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Inhibitory effects of azole-type fungicides on 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. However, it remains unclear whether environmental chemicals, including pesticides, have agonistic and/or antagonistic activity against ROR α/γ . In this study, we investigated the ROR α/γ activity of several azole-type fungicides, and the effects of these fungicides on the gene expression of interleukin (IL)-17, which mediates the function of Th17 cells. In the ROR-reporter gene assays, five azole-type fungicides (imibenconazole, triflumizole, hexaconazole, tetraconazole and imazalil) suppressed ROR α - and/or ROR γ -mediated transcriptional activity as did benzenesulphonamide T0901317, a ROR inverse agonist and a liver X receptor (LXR) agonist. In particular, imibenconazole, triflumizole and hexaconazole showed ROR γ inverse agonistic activity at concentrations of 10^{-6} M. However, unlike T0901317, these fungicides failed to show any LXR α/β agonistic activity. Next, five azole-type fungicides, showing ROR inverse agonist activity, were tested on IL-17 mRNA expression in mouse T lymphoma EL4 cells treated with phorbol myristate acetate and ionomycin. The quantitative RT-PCR analysis revealed that these fungicides suppressed the expression of IL-17 mRNA without effecting ROR α and ROR γ mRNA levels. In addition, the inhibitory effect of imibenconazole as well as that of T0901317 was absorbed in ROR α/γ -knocked down EL4 cells. Taken together, these results suggest that some azole-type fungicides inhibit IL-17 production via ROR α/γ . This also

provides the first evidence that environmental chemicals can act as modulators of *IL-17* expression in immune cells.

Key Words: fungicide; interleukin 17; mouse, reporter gene assay; retinoic acid receptor-related orphan receptor.

Introduction

T helper (Th) cells with diverse effector functions differentiate from naïve CD4⁺ T cells upon stimulation by antigens in the presence of various cytokines produced by cells of the innate immune system. Th17 cells, the T helper cells that produce interleukin 17 (IL-17) and other pro-inflammatory cytokines, were recently identified as a novel Th subset and play important roles in host defense against bacterial and fungal infections, and in a wide variety of autoimmune disease, including psoriasis and rheumatoid arthritis (Harrington *et al.*, 2005; Park *et al.*, 2005; Weaver *et al.*, 2007; Stockinger and Veldhoen, 2007). In Th17 cells, the transcription of IL-17 is mediated by Th17-specific transcriptional regulators of the retinoic acid receptor-related orphan receptors, ROR α and ROR γ (Ivanov *et al.*, 2006; Yang *et al.*, 2008).

ROR α/γ are members of the nuclear hormone receptor superfamily, which contains a signature type II zinc finger DNA binding motif and a hydrophobic ligand binding pocket (Jetten *et al.*, 2001). To date, three subtypes, designated ROR α , - β , and - γ (NR1F1-3 or RORA-C), 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 named 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. The RORs bind as monomers to ROR response elements (ROREs) in the promoter regulatory region of the target genes (Giguere *et al.*, 1994; Medvedev *et al.*, 1996). Recent studies have also demonstrated that RORs function as ligand-dependent transcriptional factors. For

example, in some cholesterol metabolites, 7 α -hydroxycholesterol was reported to be a ROR α and ROR γ inverse agonist **that inhibits the basal transcription mediated by ROR α and ROR γ , respectively** (Wang *et al.*, 2010a), while cholesterol sulfate was reported to function as an ROR α agonist (Kallen *et al.*, 2004). In addition, Kumar *et al.* (2010) provided novel evidence that N-(2,2,2-trifluoroethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzene sulfonamide (T0901317), known to be a potent agonist of liver X receptor (LXR, NR1H1-3), was reported to be an inverse agonists of ROR α and ROR γ . In fact, other previous studies had shown that this compound leads to a decreased severity of experimental autoimmune encephalomyelitis (EAE), which is dependent on Th17 cell function (Hindinger *et al.*, 2006; Xu *et al.*, 2009). Recent novel studies have also provided evidences that a synthetic chemical SR1001, digoxin derivatives, and ursolic acid act as ROR inverse agonists and ameliorate EAE disease in mice (Solt *et al.*, 2011; Huh *et al.*, 2011; Xu *et al.*, 2011). As Th17 cells play an important role in both the immune response to invading pathogens and autoimmunity (Harrington *et al.*, 2005; Park *et al.*, 2005), the identification of ROR modulators, such as ROR agonists and inverse agonists, would be of great value with regard to immunotoxicity against the innate immune system and drug-therapy for autoimmune diseases. However, although exposure to environmental agents can compromise a number of immunological functions, the effects of environmental chemicals against RORs remain unclear.

Pesticides are used globally to control agricultural and indoor pests, and are

intentionally released into the environment in large quantities. As pesticides possess a wide variety of chemical structures, they may represent the most likely candidates for ligands against nuclear receptors (Kojima *et al.*, 2010). Transactivation or reporter gene assays, a powerful tool for testing receptor agonists and antagonists among chemicals, have been established as an *in vitro* method for evaluating the receptor activity of chemicals. We previously developed novel highly sensitive reporter gene assays using Chinese hamster ovary (CHO) cells for detecting human estrogen receptor (ER) α , ER β and androgen receptor (AR) activities among numerous chemicals (Kojima *et al.*, 2003; 2004), and demonstrated that a number of environmental chemicals, including pesticides, possess ER α - and ER β -mediated estrogenic activities and/or AR-mediated antiandrogenic activity (Kojima *et al.*, 2004; 2009; Takeuchi *et al.*, 2005; 2009; 2011).

We have now performed a pesticide screen of around 200 compounds using CHO cell-based ROR α / γ reporter gene assays, and identified several pesticides possessing ROR α / γ activity. In the present study, we show that five azole-type fungicides have ROR α and/or ROR γ inverse agonistic activity. In addition, we examined whether these fungicides affect IL-17 gene expression in mouse T lymphoma EL4 cells, and found that these fungicides suppress IL-17 mRNA expression. Here, we provide the first evidence that environmental chemicals might affect IL-17 gene transcriptional activity via ROR α and/or ROR γ .

Materials and Methods

Chemicals. Hexaconazole (>99% pure) and imazalil (>98% pure) were purchased from Wako Pure Chemical Industries Ltd. (Wako; Osaka, Japan). Imibenconazole (>99% pure) and triflumizole (>99% pure) were purchased from Kanto Chemical Company Ltd. (Tokyo, Japan). Tetraconazole (97.5% pure) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). A synthetic ROR inverse agonist and LXR agonist, T0901317 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ionomycin was purchased from LKT Laboratories (St. Paul, MN, USA). We used dimethylsulfoxide (DMSO, Wako) as a vehicle, and all compounds were dissolved in DMSO at a concentration of 3×10^{-2} M.

Cell line and cell culture materials. We obtained Chinese hamster ovary (CHO-K1) cells, COS-7 simian kidney cells, and EL4 cells 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 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 solution and penicillin-streptomycin (antibiotics) solution 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. COS-7 cells were maintained in DMEM containing 10% FBS and antibiotics. EL4 cells were maintained in RPMI-1640 medium containing 10% FBS and antibiotics.

Plasmids. We used the expression plasmids pCMV10-mROR α 4 and pCMV10.ROR γ 1 encoding the full-length receptor protein and the reporter plasmid pGL4.27(RORE)5-Luc (pRORE-Luc) for determining ROR α/γ activity (Takeda, Y., unpublished). We used the expression plasmids pTARGET-hLXR α and pTARGET-hLXR β and fusion protein expression plasmids hLXR-N/pBIND and hLXR-C/pACT for determining LXR α/β activity (Kobayashi *et al.*, 2010). We purchased the internal control plasmid pCMV- β Gal from Clontech (Palo Alto, CA, USA).

Transfection of plasmids to cells and reporter gene assays for ROR α and ROR γ . We plated the host CHO-K1 cells in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of 8,400 cells per well in DMEM/F-12 containing 5% CD-FBS (complete medium) 1 day before transfection. For detection of ROR α or ROR γ activity, cells were transfected with 12 ng pCMV-mROR α or pCMV-mROR γ , 48 ng pRORE-Luc, and 12 ng pCMV β -Gal per well using FuGENE[®]6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA). After a 3-hr transfection period, cells were dosed with various concentrations of the test compounds or with 0.1% DMSO (vehicle control) in

complete medium. After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 μ l/well; Promega, Madison, WI, USA). We measured *firefly* luciferase activity with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) in one reaction tube with a 5- μ l aliquot of the cell lysate using the Luciferase Assay System (Promega), according to the manufacturer's instructions. We measured β -galactosidase activity to check the cytotoxicity of the test compounds against transcriptional activity using a fluorescence method as described previously (Takeuchi *et al.*, 2005; 2006). The luciferase activity was normalized against the β -galactosidase activity for each treatment. Results are expressed as mean $\pm$ SD from at least three independent experiments performed in triplicate.

To estimate the potency of the receptor-inverse agonistic activity of the compounds tested, the concentration of the compound exhibiting a response equal to 20% that of the vehicle control response was evaluated from a dose-response curve of the luminescence intensity, and expressed as the 20% relative inhibitory concentration (RIC₂₀).

Reporter gene assays for LXR α and LXR β . The LXR α and LXR β assays were performed using the same procedure as that for the ROR α/γ assays. Namely, COS-7 cells were transfected with 50 ng of either pTAGET-hLXR α or pTARGET-hLXR β , 50 ng hLXR-N/pBIND, 50 ng hLXR-C/pACT, and 10ng pCMV- β Gal per well using the FuGene6 transfection reagent. The LXR agonist, T0901317, was utilized as a positive

control in these LXR α/β assays.

EL4 cells experiments, isolation of RNA and reverse transcription. We plated the host EL4 cells 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, EL4 cells were treated with vehicle or both PMA (5 ng/ml) and ionomycin (1 μ M) for induction of IL-17. Simultaneously, the cells were treated with vehicle, 1, 3, 10 or 30 μ M of the fungicides or 0.1-10 μ M T0901317 as a ROR inverse agonist (positive control), and incubated at 37°C for 6 h. The EL4 cells were removed, rinsed with isotonic saline, and homogenized in TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA). Total RNA was isolated from the EL4 cells using TRIzol Reagent (1 mL/100 mg of tissue) following the manufacturer's instructions. The complementary DNA (cDNA) was synthesized using 5 μ g of total RNA by Revertra Ace reverse transcriptase (TOYOBO, Osaka, Japan). The cDNAs were then stored at -20°C until analysis.

Reverse transcription polymerase chain reaction (RT-PCR). The following primers were synthesized: for IL-17A, 5'-TCCAGAAGGCCCTCAGACTA-3' and 5'-AGCATCTTCTCGACCCTGAA-3'; and for β -actin, 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3'. PCR amplification was conducted on cDNA (20 μ L) synthesized from 0.25 μ g of the total RNA of EL4 cells treated with T0901317 or a vehicle using Taq DNA polymerase

(Promega, USA) for 22 to 35 cycles. The PCR cycle consisted of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR product was size-fractionated on 1.5% agarose gel and visualized by ethidium bromide staining. The expected sizes of the amplified cDNAs were 239 bp and 165 bp for IL-17A and β -actin, respectively.

Real-time quantitative PCR for IL-17, ROR α , ROR γ , and β -actin. We measured IL-17, ROR α , ROR γ , 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 for detection were designed by Applied Biosystems: for IL-17A, 5'-FAM d(CTTCATCTGTGTCTCTGATGCTGTT) NFQ-3'; for ROR α , 5'-FAM d(AAGGCTGCAAGGGCTTTTTCAGGAG) NFQ-3'; for ROR γ , 5'-FAM d(GCTACCAGAGGAAGTCAATGTGGGA) NFQ-3'; and for β -actin, 5'-FAM d(ACTGAGCTGCGTTTTACACCCTTTC) NFQ-3'. The amounts of IL-17, ROR α , and ROR γ mRNAs were normalized to that of β -actin mRNA.

Establishment of ROR α / γ -knockdown EL4 cells using RNA-mediated interference. EL4 cells were maintained in RPMI 1640 medium containing 10% FBS. A ROR α and ROR γ -knockdown EL4 cell clone (EL4/shRorac) was established by electroporation with the pGPU6/GFP/Neo vector (Shanghai GenePharma, Shanghai, China), bearing

short hairpin RNA (shRNA) targeting Rora (5'-GGATCAAACCCGAACCCATAT-3') and Rorc (5'-GCTACCAGAGGAAGTCAATGT-3'), and subsequent selection in G418-containing medium. Similarly, a control shRNA (nonsilencing; 5'-TTCTCCGAACGTGTCACGT-3')-transfected EL4 cell line (EL4/shNC) was also established.

Data analysis. We used an 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.

Results

Effects of azole-type fungicides on transcriptional activity in the ROR α and ROR γ assays.

Figure 1 shows the chemical structures of T0901317 and five azole-type fungicides tested in this study. We examined the effects of these compounds on ROR α - and ROR γ -mediated transcriptional activity using CHO cell-based reporter gene assays. As a result, T0901317 and five azole-type fungicides didn't show any ROR α/γ agonistic activity. However, we found that these compounds have ROR α and/or ROR γ inverse agonistic activity, which inhibits their constitutive activation. Figures 2A and B show the inhibitory effects of five azole-type fungicides together with T0901317 on transcriptional activity via ROR α and ROR γ , respectively. In the ROR α assay, although imazalil and tetraconazole did not show almost any inverse agonistic activity, imibenconazole, triflumizole and hexaconazole were found to be weak ROR α inverse agonists similar to T0901317 (Fig. 2A). However, in the ROR γ assay, these five fungicides and T0901317 showed inverse agonistic activity in a dose-dependent manner from 1 to 30 μ M (Fig. 2B). The order of relative potency for ROR γ inverse agonistic activity were T0901317 > Imibenconazole > triflumizole, hexaconazole > tetraconazole, Imazalil. Based on their β -galactosidase activity, none of the compounds tested in this study showed any cytotoxic effects, except for the highest dose (30 μ M) of T0901317 and imibenconazole. From these dose-response curves, we estimated the RIC₂₀ values of five azole-type fungicides and T0901317 for ROR α and ROR γ , and summarized in

Table 1.

Effects of azole-type fungicides in the LXR α / β transactivation assays.

As T0901317 is a potent LXR agonist, we investigated whether the five azole-type fungicides also possess LXR agonistic activity similar to T0901317. Figures 3A and B show LXR α - and LXR β -mediated transactivation by the fungicides and T0901317. Although T0901317, as expected, induced LXR α and LXR β activation at low concentrations of 10^{-7} M order, none of the five fungicides showed any LXR α or LXR β agonistic activity even at a high dose of 1×10^{-5} M.

Effects of T0901317 on in vitro expression of IL-17A mRNA in mouse T lymphoma EL4 cells.

Since induction of IL-17A via ROR is a specific positive marker in Th17 cell proliferation pathway, we examined whether azole-type fungicides affect IL-17A level in EL4 cells. Figure 4 shows the inhibitory effect of T0901317 on IL-17A gene expression in PMA/ionomycin-stimulated EL4 cells. Although neither T0901317 nor the vehicle induced the expression of IL-17A gene in non-stimulated EL4 cells, T0901317 inhibited IL-17A mRNA expression in a dose-dependent manner in PMA/ionomycin-stimulated EL4 cells (Fig. 4A). Real-time PCR analysis revealed that T0901317 significantly inhibited IL-17A mRNA expression at concentrations of 0.1, 1, and 10 μ M, although it did not affect ROR α and ROR γ mRNA expressions (Fig. 4B).

Effects of five azole-type fungicides on in vitro expressions of IL-17A, ROR α , and ROR γ mRNA in EL4 cells.

Based on the real-time PCR analysis in PMA/ionomycin-stimulated EL4 cells, we investigated the effects of the five azole-type fungicides on *IL-17A*, *ROR α* , and *ROR γ* expressions. As shown in Fig. 5, we found that five azole-type fungicides possessed inhibitory effects of PMA/ionomycin-stimulated *IL-17A* mRNA expression. In particular, imibenconazole (10 μ M) strongly suppressed *IL-17A* mRNA expression similar to T0901317 (Fig. 5B), and triflumizole and hexaconazole significantly inhibited it at a concentration of 10 μ M (Figs. 5D and E). In addition, treatment with the highest doses (30 μ M) of imazalil and tetraconazole significantly suppressed *IL-17* mRNAs compared with the vehicle (Figs. 5A and C). On the other hand, these fungicides failed to affect the expression levels of *ROR α* and of *ROR γ* mRNAs (Fig. 5A-E).

Effect of Imibenconazole and T0901317 on IL-17A mRNA expression in the ROR α / γ knockdown EL4 cells.

We established EL4/shRorac and EL4/shNC cells as endogenous *ROR α* and *ROR γ* double-knockdown EL4 and control EL4 cell lines, respectively, using shRNA technology. As shown in Figs. 6A and B, *ROR α* and *ROR γ* 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. Figure 6C shows the effects of

imibenconazole and T0901317 (each 10 μ M) on IL-17A mRNA levels in EL4/shRorac and EL4/shNC cells treated with PMA/ionomycin. The IL-17A mRNA expression in EL4/shRorac cells was found to potently decrease in comparison with that in EL4/shNC cells. Although imibenconazole and T0901317 showed inhibitory effects on IL-17A mRNA expression in EL4/shNC cells, these compounds also suppressed IL-17 mRNA expression in EL4/shRorac cells.

Discussion

Unlike steroid hormone receptors, apo-ROR α and - γ are constitutively active and retain the ability to interact with a range of coactivator NR box peptides in the absence of binding to any ligand (Jetten *et al.*, 2001). In fact, RORs are orphan receptors whose endogenous ligands are still unclear. However, recent studies have reported that the binding of cholesterol metabolites (e.g., 7-oxygenated sterols) to ROR α/γ reduces the level of this constitutive interaction, resulting in the repression of the transcriptional output of the receptors (Wang *et al.*, 2010a). In addition, Kumar *et al.* (2010) provided evidence that the synthetic chemical T0901317, which is also a potent LXR agonist, directly binds to ROR α and ROR γ with high affinity (K_i = 132 and 51 nM, respectively), and represses ROR α/γ -dependent transactivation of RORE reporter genes. **Therefore, these cholesterol metabolites and T0901317 are classified as ROR inverse agonists that inhibit ROR basal transcriptional activity in the absence of agonist, and are distinct from ROR antagonists that themselves do not effect the receptor equilibrium but are capable of competitive antagonism of both agonists and inverse agonists (Klein *et al.*, 1996).** In this study using CHO-K1 cell-based reporter gene assays, we found that T0901317 decreased ROR α - and ROR γ -dependent luciferase induction at a RIC₂₀ of 12 μ M and 0.97 μ M, respectively (Table 1), thereby supporting the results obtained by Kumar *et al.* (2010). More recently, they have reported that synthetic compounds derived from the benzenesulfonamide scaffold, SR1078 and SR1001, act as a ROR α/γ agonist and ROR α/γ inverse agonist,

respectively (Wang *et al.*, 2010b; Solt *et al.*, 2011). This suggested the possibility that small molecule compounds, such as environmental chemicals, may also act as ligands of ROR.

We investigated the potential agonistic and inverse agonistic activity of about 200 pesticides against ROR α and ROR γ using CHO-K1 cell-based reporter gene assays. As a result, we have found that five azole-type fungicides inhibited constitutive activation of ROR α and ROR γ in a dose-dependent manner similar to T0901317 (Fig. 2). In particular, imibenconazole, triflumizole and hexaconazole were found to inhibit basal ROR γ -mediated transactivation at concentrations of 10^{-6} M order. According to a recent study by Martin *et al.* (2010), hexaconazole has been listed as a potent ROR inhibitor together with other 13 pesticides, supporting our results. As shown in Fig. 1, five fungicides acting as ROR inverse agonists have structures similar to each other and resembling T0901317, although the ROR α/γ activities of these fungicides were weaker than those of T0901317.

It is topical that, in recent immunological studies, a novel Th subset, termed Th17, which preferentially produces IL-17, has been identified as a distinct Th lineage mediating tissue inflammation (McGeachy and Cua, 2008). Th17 cells are defined by their secretion of the pro-inflammatory cytokine IL-17, which mediates the recruitment of inflammatory cells and the induction of pro-inflammatory mediators by endothelial cells (Ouyang *et al.*, 2008). Therefore, Th17 cells and their effector cytokines mediate the host defense mechanisms to various infections, especially extracellular bacteria

infections, and are involved in the pathogenesis of many autoimmune diseases (Ouyang *et al.*, 2008; Stockinger and Veldhoen, 2007). Several mechanism studies have revealed that ROR α and ROR γ , and particularly the ROR γ t isoform, act as Th17 lineage-specific master transcription factors (Ivanov *et al.*, 2006; Yang *et al.*, 2008). For example, Yang *et al.* (2008) demonstrated that ROR α and ROR γ co-expression synergistically leads to greater IL-17 production in Th17 cells, whereas double deficiencies in ROR α and ROR γ globally impair IL-17 production and completely protect mice against EAE. Thus, it is important to investigate whether chemicals having ROR activity affect Th17 differentiation, and to identify ROR agonists and antagonists. In this study, we examined the effects of ROR inverse agonistic fungicides on IL-17 gene expression in mouse T lymphoma EL4 cells treated with PMA/ionomycin. Interestingly, as with T0901317, five azole-type fungicides (imibenconazole, triflumizole, hexaconazole, tetraconazole and imazalil) were found to inhibit IL-17 gene expression in a dose-dependent manner (Figs. 5A-E). This result suggests that ROR inverse agonistic activities in reporter gene assays well corresponded to the potency of IL-17 inhibitory effects in EL4 cells.

A synthetic compound, T0901317, is well known as a potent LXR agonist. T0901317 has been reported to suppress Th17 function in a mouse autoimmune model of EAE (Xu *et al.*, 2009), supporting our results regarding the inhibition of IL-17 expression by T0901317. However, Cui *et al.* (2011) have recently reported that the LXR agonistic activity of T0901317 is involved in the repression of IL-17 production,

and leads to EAE prevention. Therefore, we examined whether these fungicides show LXR agonistic activity, and confirmed that they did not act as LXR agonists (Fig. 3). This suggests that the five fungicides affect IL-17 gene expression via ROR, not LXR, unlike T0901317, a potent LXR agonist. Thus, our findings indicate that environmental chemicals, such as pesticides, may act as regulatory molecules of Th17 cell function as ROR inverse agonists, leading to potential effects on the immune system in whole organisms.

The EL4 cells constitutively express ROR α (encoded by *Rora*), ROR γ (*Rorc*) and IL-17A (*Il17a*) (Ichiyama *et al.*, 2008). Next, we established the ROR α/γ knockdown EL4 (EL4/shRorac) cells using shRNA technology. As a result, we found that IL-17A mRNA expression was clearly suppressed in EL4/shRorac cells when they were treated with PMA/ionomycin. This suggests that ROR α/γ is a critical regulator of IL-17 gene expression in EL4 cells, as pointed out by other studies (Ichiyama *et al.*, 2008; Solt *et al.*, 2011). More importantly, although treatment of EL4/shNC cells with imibenconazole and T0901317 potently suppressed IL-17A mRNA expression, treatment of EL4/shRorc cells with those also resulted in decrease. This may suggest that the remained ROR α/γ are involved in suppression of IL-17A mRNA expression by imibenconazole and T0901317.

Azole fungicides are widely used in agriculture and in human mycosis (Zarn *et al.*, 2003; Giavini and Menegola, 2010). Their antifungal activity is based on their ability to inhibit fungal CYP51 (lanosterol 14 α -demethylase), a key enzyme in the formation of

the fungal wall. In addition, several azoles are also used in antiestrogen therapy such as in breast cancer treatment, as they inhibit CYP19 (aromatase), a key enzyme in the estrogen biosynthesis (Zarn *et al.*, 2003). Although five azole-type fungicides showed ROR inverse agonistic activity, triazole or imidazole structures, which play an important role in antifungal activity, might be not necessary for ROR activity as other azole fungicides (bitertanol, triadimefon, propiconazole, and prochloraz) were inactive (data not shown). Thus, further studies on the structural property of azole-type fungicides are required for elucidating their ROR inverse agonistic activity.

The present study also indicates the structural design of potent and selective ROR ligands with potential application in the treatment of metabolic and immune disorders (Kumar *et al.*, 2010; Solt *et al.*, 2010). The development for ROR inhibitors represents a promising therapeutic strategy in the treatment of Th17-mediated diseases. Thus, ROR α/γ inverse agonists, such as imibenconazole, might be useful as lead compounds for drug development in autoimmune diseases involving Th17 cells. On the other hand, inhibiting ROR α/γ might adversely affect the beneficial functions of Th17 cells in fighting pathogens. Although the physiological concentrations reached during exposure to these fungicides might be lower than the concentrations by which those affect ROR activity, unexpected additive exposure to numerous environmental chemicals should be concerned about their suppressive effect of the immune response, rather than their therapeutic merit for autoimmune disease.

The ROR inverse agonistic activity obtained from our ROR assays well

corresponded with the data for IL-17A mRNA expression obtained from RT-PCR analysis. Therefore, this study also indicates that our CHO-K1 cell-based reporter gene assay is highly suitable for identifying the effects on IL-17 production, via ROR, in immune cells by a large number of chemicals. Recently, a synthetic chemical SR1078 has been reported to be an agonist of ROR α and ROR γ (Wang *et al.*, 2010b). Thus, in addition to identifying ROR inverse agonists, the study for identifying ROR agonists among numerous environmental chemicals is also required to prevent the acceleration of immune inflammation by environmental factors.

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Figure captions

Fig. 1. Chemical structures of five azole-type fungicides and T0901317 tested in this study.

Fig. 2. Effects of five azole-type fungicides and T0901317 on ROR α / γ -mediated transcriptional activity. CHO-K1 cells were transiently cotransfected with an expression plasmid for mouse ROR α (A) or ROR γ (B) as well as a reporter-responsive *firefly* luciferase plasmid and a constitutively active β -galactosidase expression plasmid. Cells were treated with increasing concentrations of the five fungicides or T0901317, a ROR α / γ inverse agonist. The *firefly* luciferase activity was normalized against β -galactosidase activity. Values represent the means $\pm$ SD of three independent experiments and are presented as percentage induction, with 100% activity defined as the constitutive activity by the vehicle control.

Fig. 3. Effects of five azole-type fungicides in hLXR α and hLXR β transactivation assays. COS-7 cells were transiently cotransfected with an expression plasmid for mouse LXR α (A) or LXR β (B) as well as two fusion protein expression plasmids and a constitutively active β -galactosidase expression plasmid. Cells were treated with increasing concentrations of the five fungicides or T0901317, a potent LXR agonist. The *firefly* luciferase activity was normalized against β -galactosidase activity. Values

represent the means $\pm$ SD of three independent experiments and are presented as the mean n-fold induction over the vehicle control.

Fig. 4. Qualitative and quantitative analysis of IL-17A mRNA expression in the EL4 cells treated with T0901314. (A) EL4 cells were treated with vehicle (no stimulation) or both PMA (5 ng/ml) and ionomycin (1 μ M) for induction of IL-17A. Simultaneously, the cells were treated with 0.1% DMSO (vehicle control), 0.1, 1, or 10 μ M of T0901317 at 37°C for 6 h. IL-17A and β -actin mRNA expressions were amplified by qualitative RT-PCR using specific primers, and amplified products (5 μ L) were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. β -actin was used as a housekeeping gene. (B) IL-17A, ROR α , and ROR γ mRNA expressions in the cells were amplified by real-time RT-PCR and normalized to the expression of a housekeeping gene β -actin. Values are expressed relative to vehicle control with PMA/ionomycin stimulation (taken as 1) and represent the mean $\pm$ SD of three experiments. ND, not detected (< 0.005). * Significant difference ($p < 0.05$; ANOVA) from vehicle control with PMA/ionomycine stimulation.

Fig. 5. Suppression of IL-17 mRNA expression by azole-type fungicides in EL4 cells treated with PMA and ionomycin. EL4 cells were treated with PMA (5 ng/ml) and ionomycin (1 μ M) for IL-17 gene induction. Simultaneously, the cells were treated with vehicle, 1, 3, 10 or 30 μ M of the fungicide, and incubated at 37°C for 6 h. IL-17A,

ROR α , and ROR γ mRNA expressions in the cells were amplified by real-time RT-PCR and normalized to the expression of a housekeeping gene β -actin. Values are expressed relative to vehicle control with PMA/ionomycin stimulation (taken as 1) and represent the mean $\pm$ SD of three experiments. * Significant difference ($p < 0.05$; ANOVA) from vehicle control with PMA/ionomycine stimulation.

Fig. 6. Comparison of inhibitory effects for IL-17A mRNA expression by imibenconazole using EL4/shRorc cells and EL4/shNC cells. ROR α (A) and ROR γ (B) mRNA expressions in EL4/shRorac cells and EL4/shNC cells, with or without PMA/ionomycine (P.I.) stimulation, were amplified by real-time RT-PCR and normalized to the expression of a housekeeping gene β -actin, respectively. (C) IL-17 mRNA expression was measured in EL4/shRorac cells and EL4/shNC cells in the presence of T0901317 (T09; 10 μ M) or imibenconazole (Imib; 10 μ M), with or without PMA/ionomycine stimulation, and normalized to the expression of a housekeeping gene β -actin. Values are expressed relative to vehicle control with PMA/ionomycin stimulation in EL4/shNC cells (taken as 1) and represent the mean $\pm$ SD of three experiments. ND, not detected (< 0.01).

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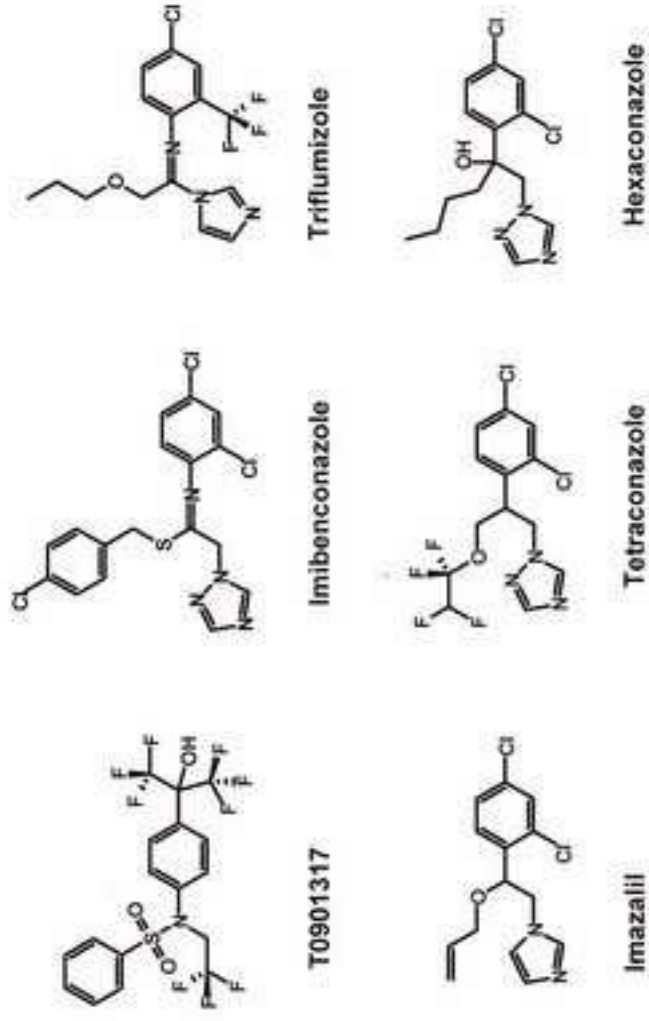


Figure 1

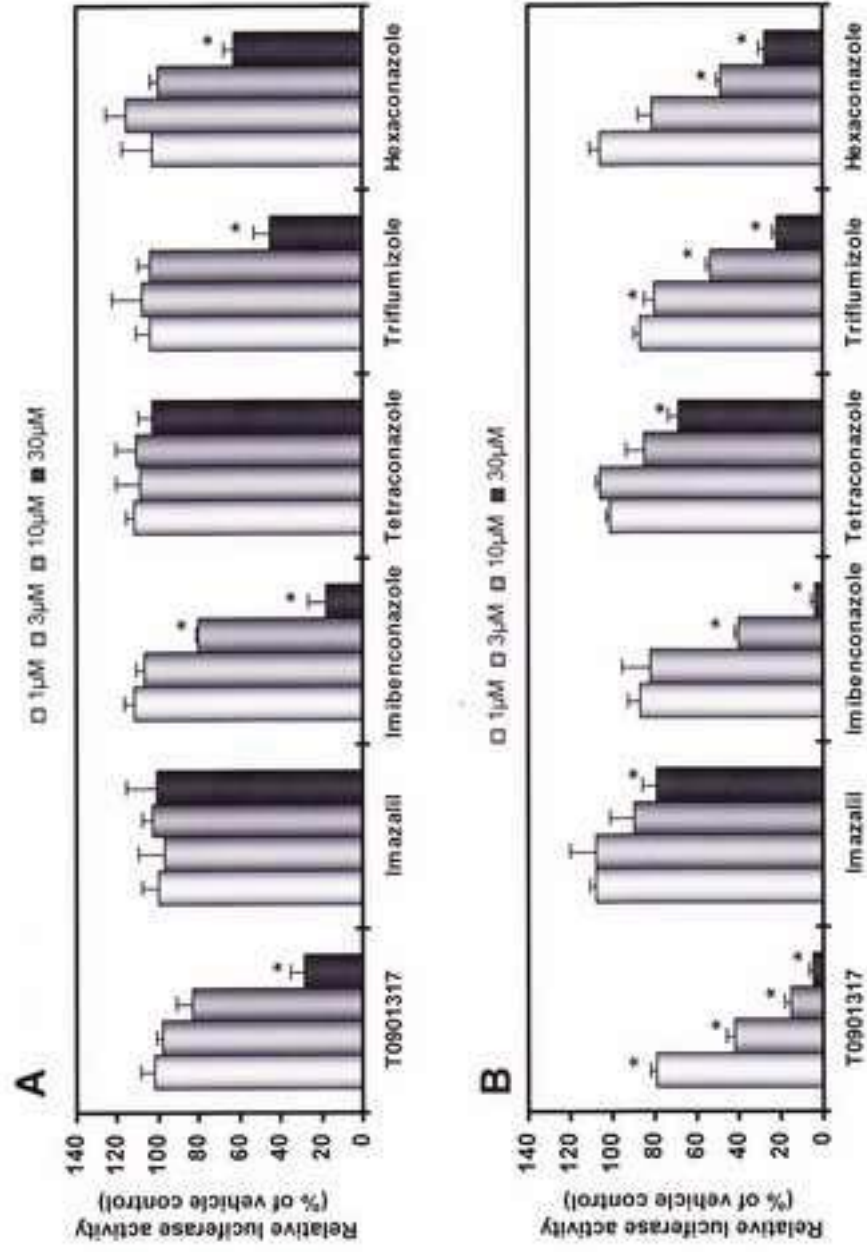


Figure 2

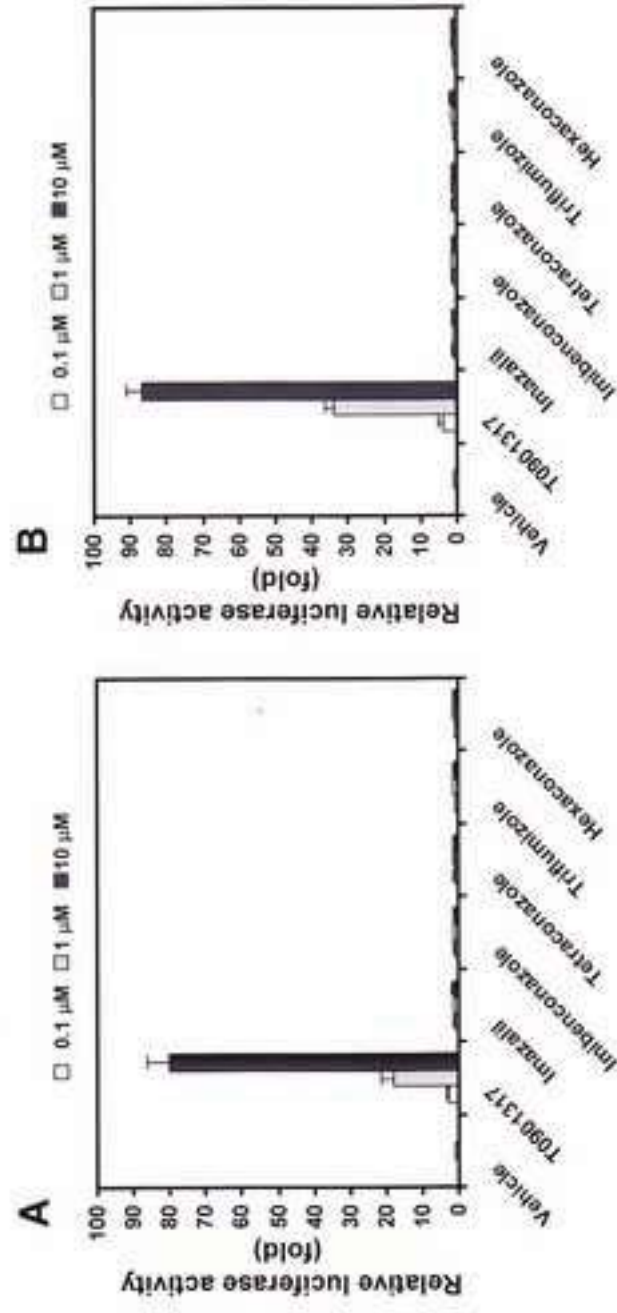


Figure 3

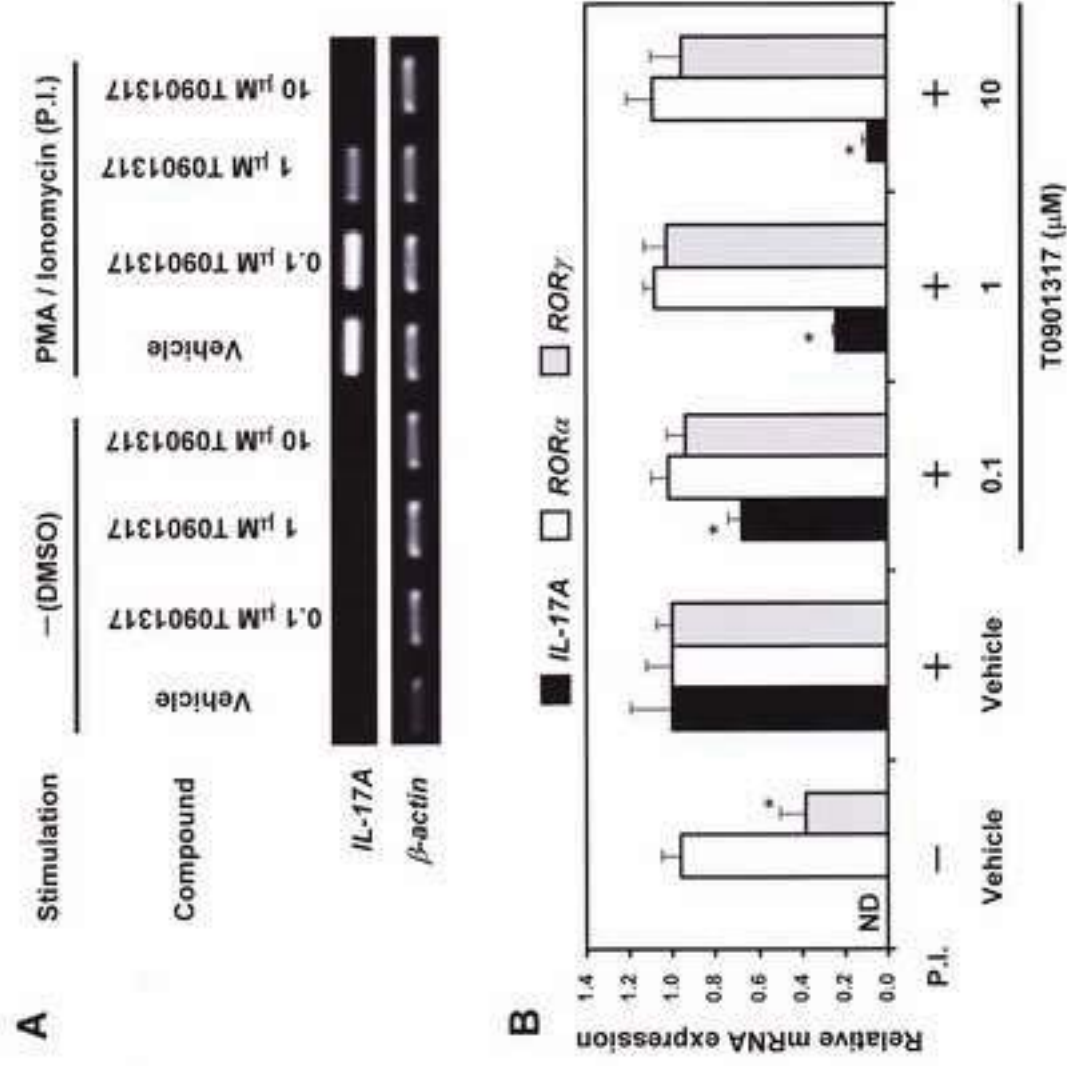


Figure 4

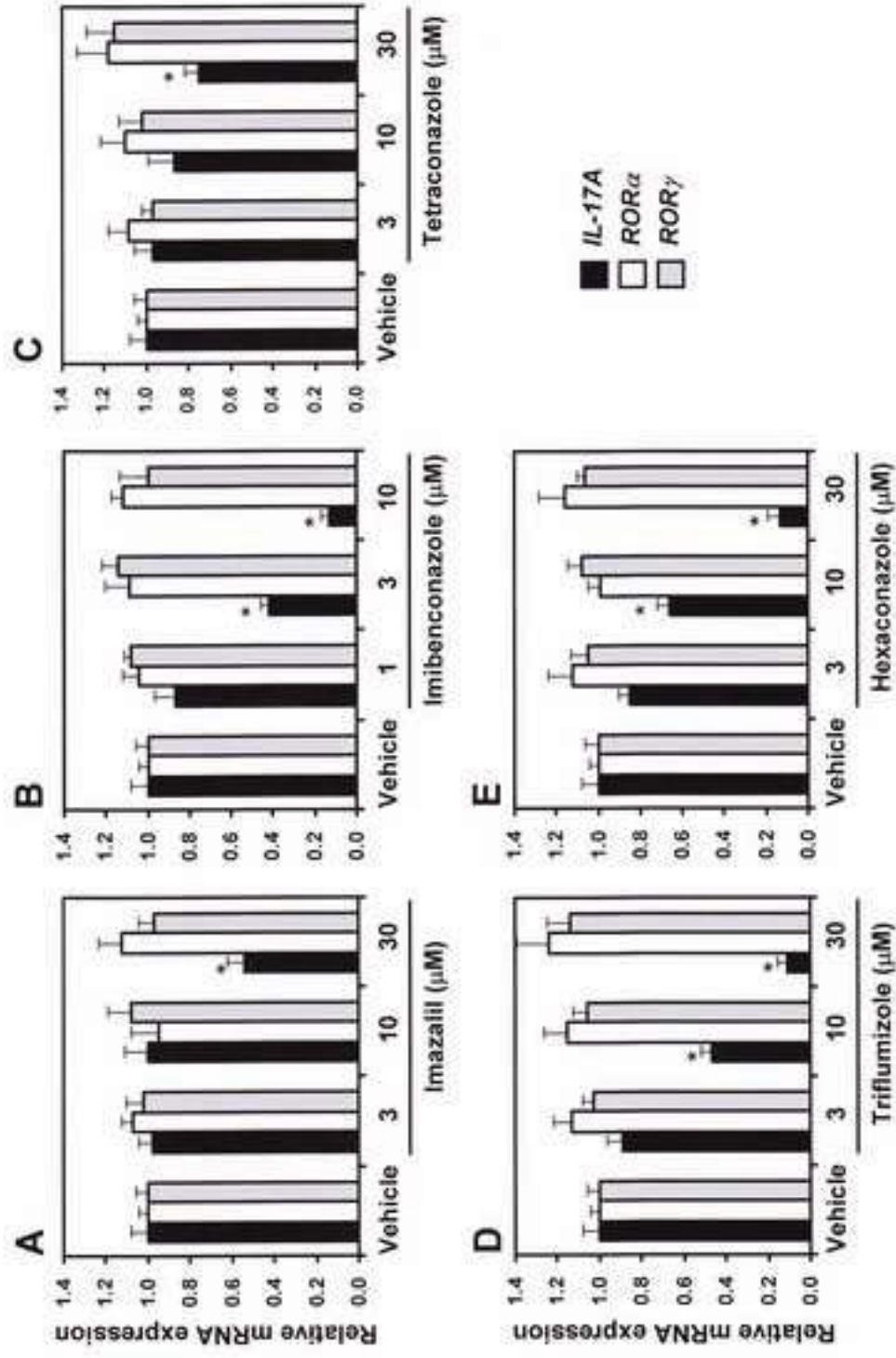


Figure 5

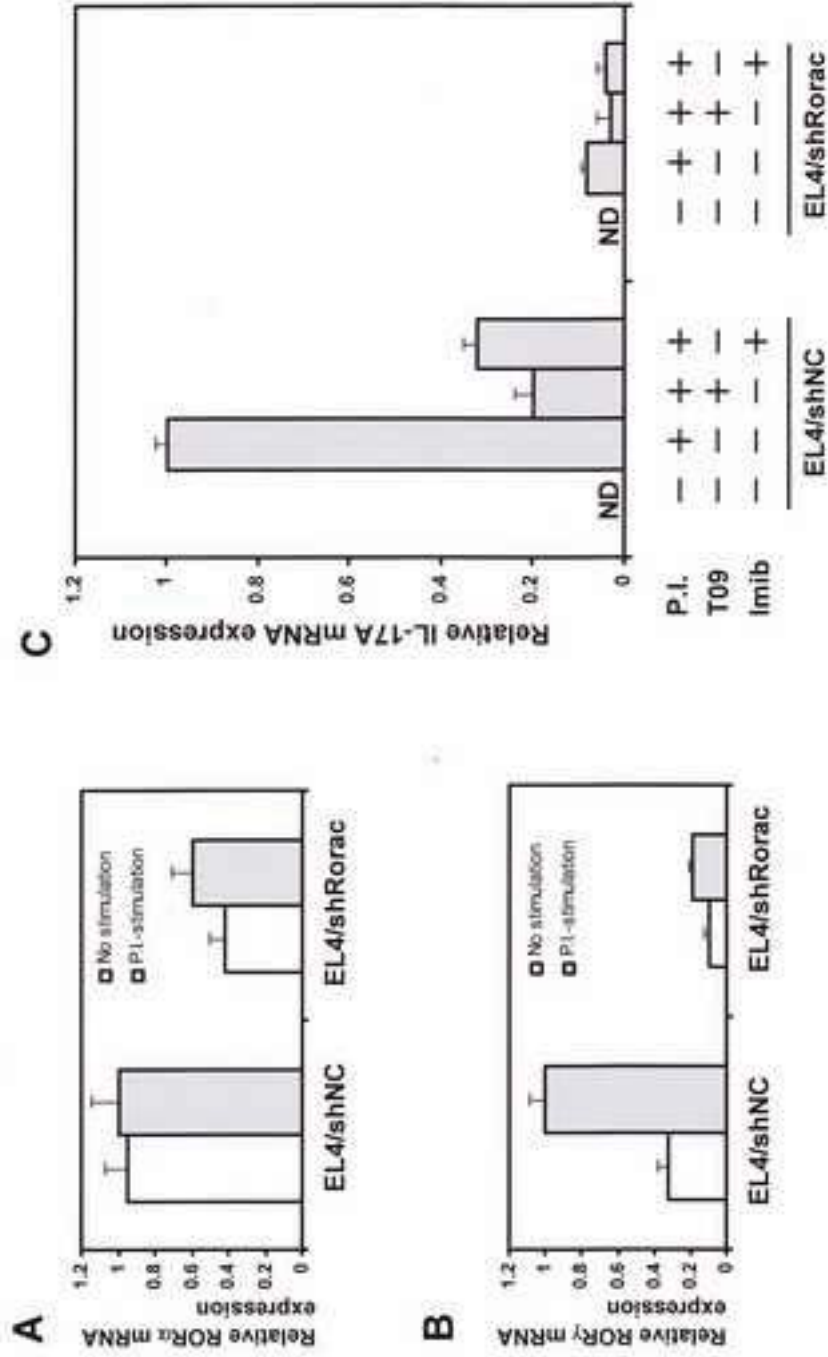


Figure 6

Table 1 RIC₂₀ for ROR α / γ inverse agonistic activities of five azole-type fungicides.

Compound	ROR α assay	ROR γ assay
	RIC ₂₀ (M)	RIC ₂₀ (M)
T0901317	1.2×10^{-5}	9.7×10^{-7}
Imazalil	$> 3.0\times10^{-5}$	2.9×10^{-5}
Imibenconazole	1.0×10^{-5}	3.3×10^{-6}
Tetraconazole	$> 3.0\times10^{-5}$	1.5×10^{-5}
Triflumizole	1.8×10^{-5}	3.0×10^{-6}
Hexaconazole	2.1×10^{-5}	3.2×10^{-6}

RIC₂₀ (20% relative inhibitory concentration) is represented as the concentration of the compound exhibiting a response equal to 20% that of the vehicle control response.