

—Original—

## Assessing Estrogenic Activity of Pyrethroid Insecticides Using *In Vitro* Combination Assays

In Young KIM<sup>1,2)</sup>, Jae Ho SHIN<sup>1)</sup>, Hyung Sik KIM<sup>1,3)</sup>, Su Jung LEE<sup>1)</sup>,  
Il Hyun KANG<sup>1)</sup>, Tae Sung KIM<sup>1)</sup>, Hyun Ju MOON<sup>1)</sup>, Kwang Sik CHOI<sup>1)</sup>,  
Aree MOON<sup>2)</sup> and Soon Young HAN<sup>1)</sup>

<sup>1)</sup>Endocrine Toxicology Division, National Institute of Toxicological Research, Korea Food and Drug Administration, 5 Nokbun-dong, Eunpyung-ku, Seoul 122-704, <sup>2)</sup>College of Pharmacy, Duksung Women's University, 419 Ssangmun-dong, Tobong-ku, Seoul 132-714, <sup>3)</sup>Division of Molecular Toxicology, College of Pharmacy, Pusan National University, Jangjeon-dong, Geumjeong-ku, Pusan 609-735, Korea

**Abstract.** Pyrethroid insecticides are among the most commonly used classes of insecticides worldwide, but their endocrine disrupting activities remain unclear. Therefore, in the present study, we examined the estrogenic activities of pyrethroid insecticides in E-screen and competition binding assays. In addition, we measured estrogen receptor (ER) protein and pS2 mRNA levels in human breast cancer cells (MCF-7 BUS) to clarify the mechanism of their estrogenicity. Seven pyrethroid insecticides (bioallethrin, cypermethrin, deltamethrin, fenvalerate, permethrin, sumithrin, and tetramethrin) were tested because of their worldwide usage. In addition, 17 $\beta$ -estradiol was tested as a positive control. As expected, 17 $\beta$ -estradiol significantly increased MCF-7 BUS cell proliferation at concentrations of 10<sup>-11</sup> M and above. Of the pyrethroid insecticides tested, only sumithrin increased MCF-7 BUS cell proliferation in a dose-dependent manner; the maximum induction of cell proliferation was observed at a dose of 10<sup>-5</sup> M. In the anti-estrogenic activity test, bioallethrin, fenvalerate, and permethrin significantly inhibited 17 $\beta$ -estradiol-induced MCF-7 BUS cell proliferation at 10<sup>-6</sup> M, a concentration comparable to the effective dose (10<sup>-9</sup> M) of ICI 182,780, a pure ER antagonist. However, none of the pyrethroid insecticides competitively inhibited the binding of [<sup>3</sup>H]estradiol to rat uterus ERs in competition binding assays. Both 17 $\beta$ -estradiol (10<sup>-10</sup> M) and sumithrin (10<sup>-5</sup> M) decreased the levels of cytosolic ER $\alpha$  and ER $\beta$  protein expression significantly as compared with the vehicle control. In addition, 17 $\beta$ -estradiol (10<sup>-10</sup> M) increased pS2 mRNA expression markedly, and sumithrin significantly increased pS2 mRNA levels in a dose-dependent manner. The other six compounds tested in the present study did not affect ER protein levels or pS2 mRNA levels. These results suggest that certain pyrethroid insecticides may be considered to be estrogen-like chemicals that act through pathways other than direct ER binding, and may function as endocrine modulators in both wildlife and humans.

**Key words:** Pyrethroids, Human breast cancer cells (MCF-7 cell), Estrogen receptor (ER)  $\alpha$ , Estrogen receptor (ER)  $\beta$ , pS2

(J. Reprod. Dev. 50: 245-255, 2004)

---

**M**any natural and synthetic chemicals in the environment may disrupt endocrine systems

Accepted for publication: January 7, 2003  
S. Y. Han (e-mail: soonyoung@kfda.go.kr)

by mimicking or inhibiting the action of steroid hormones, thereby producing reproductive and developmental disorders in both wildlife and humans [1, 2]. There have been a number of studies

to clarify the potential adverse effects of these chemicals, referred to as endocrine disruptors (EDs). Endocrine disruption research is one of the highest priority areas in environmental toxicology, and several strategies for evaluation of the adverse effects of EDs have been developed over the past several years [3–5]. Furthermore, there is a great need for reliable testing methods that can rapidly detect a large number of probable or possible EDs. As a result, numerous methodologies for screening and testing the potential endocrine disrupting effects of chemicals have been developed [3–5]. The U.S. Environmental Protection Agency (EPA) has recommended screening strategies to detect EDS that are agonistic or antagonistic to estrogen/androgen receptors, that are steroid biosynthesis inhibitors, or that alter thyroid function both *in vitro* and *in vivo* [6, 7]. *In vitro* combination assays are powerful tools for rapidly screening large numbers of chemicals and for understanding the mechanisms of their actions. In the present study, we evaluated the estrogenicity of pyrethroid insecticides using two *in vitro* combination assays, E-screen and competitive estrogen receptor (ER) binding.

Pyrethroid insecticides are the most common pesticides currently used to control agricultural and indoor pests [8]. Synthetic pyrethroids are analogs of the natural chemical moiety pyrethrin, which is derived from the pyrethrum plant, *Chrysanthemum cinerariaefolium*. These compounds are suspected EDs [1]. Recently, some pyrethroid insecticides were evaluated for their estrogenic or anti-estrogenic activities using *in vitro* combination assays, such as cell proliferation and estrogen-sensitive pS2 expression assays [9–12]. However, the results of these studies are controversial. Some showed positive estrogenic activity in MCF-7 cell proliferation and pS2 mRNA expression assays [9, 10]. On the other hand, certain pyrethroid insecticides did not show any estrogenic or anti-estrogenic activity *in vitro* [11]. Moreover, four pyrethroid insecticides (permethrin, cypermethrin, fenvalerate, and phenothrin) did not induce estrogenic activity in an *in vitro* luciferase reporter gene assay using the metabolic activation system [12]. These results were confirmed with a uterotrophic assay in which none of the pyrethroid insecticides tested (esfenvalerate, fenvalerate, and permethrin) exhibited any estrogenic activity [13]. Although *in vitro* combination assays have

indicated that certain pyrethroid insecticides have weak estrogenic activity, their estrogenic mechanisms of action are not understood and little is known about their associations with the ERs. Measurements of ER expression levels in response to pyrethroid insecticides would provide useful information for evaluating their estrogenic or anti-estrogenic activities.

The aims of the present study were to assess the estrogenic or anti-estrogenic activities of seven pyrethroid insecticides (bioallethrin, cypermethrin, deltamethrin, fenvalerate, permethrin, sumithrin, and tetramethrin) in the E-screen and competitive ER binding assays. In addition, ER $\alpha$  and ER $\beta$  expression and pS2 mRNA levels were measured in MCF-7 BUS cells to clarify the estrogenic mechanism of action of the pyrethroid insecticides.

## Materials and Methods

### *Chemicals and reagents*

17 $\beta$ -Estradiol, dimethylsulfoxide (DMSO), and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ICI 182,780 was purchased from Tocris Chemical Co. (Bristol, UK). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and 0.25% (w/v) trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Non-fat dry milk was purchased from Bio-Rad Co. (Hercules, CA, USA). Anti-ER $\alpha$  and ER $\beta$  antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Hybond-P filters, enhanced chemiluminescence system (ECL plus), and 2,3,6,7-[<sup>3</sup>H]estradiol were purchased from Amersham Pharmacia (Buckinghamshire, UK). Bioallethrin (purity 99%), cypermethrin (purity 98%), deltamethrin (purity 99%), permethrin (purity 98%), sumithrin (purity 97%), and tetramethrin (purity 98%) were purchased from Chem Service (West Chester, PA, USA) and were dissolved in culture-grade DMSO. Fenvalerate (purity 99.9%) was purchased from Riedel-de Haën (Seelze, Germany).

### *Cell culture conditions*

Estrogen sensitive human breast cancer cells (MCF-7 BUS) were kindly provided by Dr. Soto of Tufts University. MCF-7 BUS cells were grown and maintained in DMEM supplemented with 5% (v/v)

FBS, 1% (v/v) penicillin-streptomycin, 20 mM sodium bicarbonate, and 1 mM sodium pyruvate. Cells were incubated at 37 C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

#### *E-Screen assay*

This assay was carried out using a modification of Soto's E-screen assay [14]. Briefly, subconfluent MCF-7 BUS cells grown in 5% FBS-supplemented DMEM in 25-cm<sup>2</sup> cell culture flasks (Corning 430639; Corning Co., NY, USA) were washed with 5 ml of phosphate buffered saline (PBS; pH 7.4) and trypsinized. Cells were resuspended in 5% FBS-supplemented DMEM and plated onto 96-well plates (Falcon 353072; Falcon Co., NJ, USA). The cell density at this stage was 1500 cells per well, and the culture plates were maintained at 37 C under 5% CO<sub>2</sub> in air. After 24 h, the medium was removed and replaced with experimental medium containing the test chemicals. The experimental medium consisted of phenol red-free DMEM supplemented with FBS that was treated with 5% charcoal-dextran (CD-FBS) to minimize its estrogenic activity. The culture was continued for six days, and then the SRB assay was conducted to measure cell proliferation. The optical density was measured at 570 nm (reference 630 nm) using a microplate reader (ELx808; DI bio-tek, Winooski, VT, USA). The readings from six culture wells were averaged and expressed as the fold-induction of control cultures.

#### *Competitive ER binding assay*

The assay was performed as described previously by Kim *et al.* [15] with only slight modifications [16]. Briefly, the competitive binding assay was conducted using cytosol isolated from Sprague-Dawley rat (15-week-old) uteri. Sprague-Dawley Crl:CD female rats were obtained from the Korea FDA Laboratory Animal Resources (Seoul, Korea), they were handled in an accredited Korea FDA animal facility in accordance with the AAALAC International Animal Care Policy (Accredited Unit, Korea Food and Drug Administration: Unit Number, 000936) and were maintained in a specified pathogen-free (SPF) state. Rats were housed three animals per cage in polycarbonate cages under controlled environmental conditions, including a temperature 23 ± 2 C, a relative humidity of 50 ± 10%, and a 12-h light-dark cycle. Uterus cytosol was prepared

from female rats approximately 10 days after ovariectomy (OVX). The rats were sacrificed by cervical dislocation, and the uteri were excised, trimmed of excessive fat, and weighed. One gram of tissue was placed in 10 ml of ice-cold buffer solution containing 10 mM Tris-HCl, pH 7.4, 1.5 mM ethylene diamine tetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), and 10% (v/v) glycerol (TEDG buffer). The uterine tissues were homogenized at 4 C with a polytron homogenizer (Brinkman Instruments, Westbury, NY, USA) using 5-s bursts and centrifuged at 105,000 × g for 60 min at 4 C. After centrifugation, supernatants were decanted into 15-ml conical tubes, protein concentrations were adjusted to approximately 2 mg/ml in phosphate buffer (pH 7.4), and samples were stored at -70 C until used in assays.

Aliquots (50 μl) of rat uterus cytosol were incubated with 10 μl of selected concentrations of competitor and 10 μl of [<sup>3</sup>H]estradiol (2 × 10<sup>-9</sup> M final assay concentration) in duplicate tubes. Each reaction mixture was brought to a final volume of 430 μl with 50 mM Tris buffer (pH 7.4). Reaction mixture tubes were placed in a drum roller and incubated at 4 C for 20 h. After incubation, free hormone was removed by incubation with 5% charcoal-0.5% dextran at 4 C for 30 min. The mixtures were then centrifuged at 800 × g, and the supernatants were decanted into glass vials containing scintillation cocktail. Radioactivity was measured in a liquid scintillation counter (Wallac 1450; Wallac, Milton Keynes, UK). The relative binding affinities of competitors were calculated as the ratio of the concentration of unlabelled 17β-estradiol to that of the competitive material required to inhibit specific [<sup>3</sup>H]estradiol binding at 50% of the 17β-estradiol value.

#### *Estrogen receptor (ERα and ERβ) expression*

MCF-7 BUS cells were plated at a density of 2.5 × 10<sup>5</sup> cells per 10-cm<sup>2</sup> culture dish (Corning 430167; Corning Co., NY, USA) and incubated for 24 h. The medium was then changed to phenol red-free DMEM supplemented with 5% CD-FBS, and the cells were incubated for 48 h. Then, the cells were treated with sumithrin and incubated for a further 48 h. The cells were washed twice with cold PBS, and 250 μl of lysis buffer containing 0.5% (v/v) Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM ethylene bis (oxyethylenetriolo) tetra-acetic acid (EGTA), 1 mM

DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub> and a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) were added. The protein concentration of the cell extract was determined in triplicate according to the method of Bradford, using crystalline BSA (Sigma, St. Louis, MO, USA) as the protein standard. Equal amounts of total protein were separated by 10% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-rad, Hercules, CA, USA). The membranes were blocked with 5% (w/v) non-fat dry milk in PBS containing 0.1% (v/v) Tween-20 (PBST). After a brief wash with PBST, the levels of ER $\alpha$  and ER $\beta$  were determined using anti-ER $\alpha$  and anti-ER $\beta$  antibody, respectively, and horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz). Immunoreactive bands were visualized using the Enhanced Chemiluminescence System (ECL plus; Amersham pharmacia). Relative band intensities were determined using an Image Analyzer (Uvidoc; Uvitec, Cambridge, UK).

#### *pS2 mRNA expression*

MCF-7 BUS cells were plated at a density of  $2.5 \times 10^5$  cells per 10-cm dish and incubated for 24 h. The medium was changed to phenol red-free DMEM supplemented with 5% charcoal-dextran FBS, and the cells were incubated for 48 h. Then, the cell cultures were each treated with a test compound and incubated for 48 h. All cells were harvested at the same time. Total RNA was purified with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequences of the primers for pS2 were 5'-GGCCACCATGGAGAACAAGG-3' and 5'-CCCCACGAACGGTGTCTCGAA-3', and those for 1A (used as an intrinsic control) were 5'-GATATAGCATTCCCACGAATA-3' and 5'-GGGCTTTTGCTCATGTGTCAT-3'. The reaction conditions for pS2 were 95 C for 5 min followed by 20 cycles of 95 C for 1 min, 50 C for 1 min, and 72 C for 1 min 30 s with a final template extension at 72 C for 15 min. Those for 1A were 95 C for 5 min followed by 20 cycles of 95 C for 1 min, 50 C for 1 min, and 72 C for 1 min 30 s with a final template extension at 72 C for 15 min. After the reactions were complete, the PCR products were separated in 1.5% (w/v) agarose gels containing 0.1% (v/v) ethidium bromide. The net intensities of the bands corresponding to pS2 and 1A mRNA were

analyzed with Kodak digital identification image analysis software (Eastman Kodak Co., Rochester, NY, USA). The pS2 mRNA level was determined by normalizing the pS2 net intensity to the 1A mRNA net intensity.

#### *Statistical analysis*

All data are expressed as means  $\pm$  SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Dunnett's test; p values of < 0.01 were considered statistically significant.

## Results

#### *E-Screen assay*

The proliferation of MCF-7 BUS cells was estimated after the cells had been treated for six days with one of seven different pyrethroid insecticides or with 17 $\beta$ -estradiol as a physiologically positive control. The chemical structures of the tested compounds are shown in Fig. 1. None of the compounds tested were cytotoxic to MCF-7 BUS cells, even at the highest concentrations used. As expected, 17 $\beta$ -estradiol induced cell proliferation in a dose-dependent manner. The maximum proliferative activity of 17 $\beta$ -estradiol was observed at a concentration of  $10^{-10}$  M, and the proliferation rate was 4.8 folds higher than that of the control culture (Fig. 2A). In the absence of 17 $\beta$ -estradiol, MCF-7 BUS cells proliferated slightly when incubated for six days. Of the seven pyrethroid insecticides, only sumithrin induced a dose-dependent increase in MCF-7 BUS cell proliferation, with significant effects at concentrations of  $10^{-7}$  M to  $10^{-5}$  M. The maximum proliferative effect was 70% of that of 17 $\beta$ -estradiol (Fig. 2B). Bioallethrin, cypermethrin, deltamethrin, fenvalerate, permethrin, and tetramethrin did not induce MCF-7 BUS cell proliferation at any concentration (data not shown). To evaluate their anti-estrogenic properties, cells were treated with  $10^{-6}$  M of each pyrethroid insecticide and  $10^{-9}$  M of ICI 182,780. Our results showed that ICI 182,780 ( $10^{-9}$  M), a pure ER antagonist, was sufficient to inhibit 17 $\beta$ -estradiol-induced MCF-7 BUS cell proliferation. Likewise, bioallethrin, fenvalerate and permethrin also inhibited MCF-7 BUS cell proliferation induced by 17 $\beta$ -estradiol, but other pyrethroid insecticides did not affect 17 $\beta$ -estradiol-induced cell proliferation

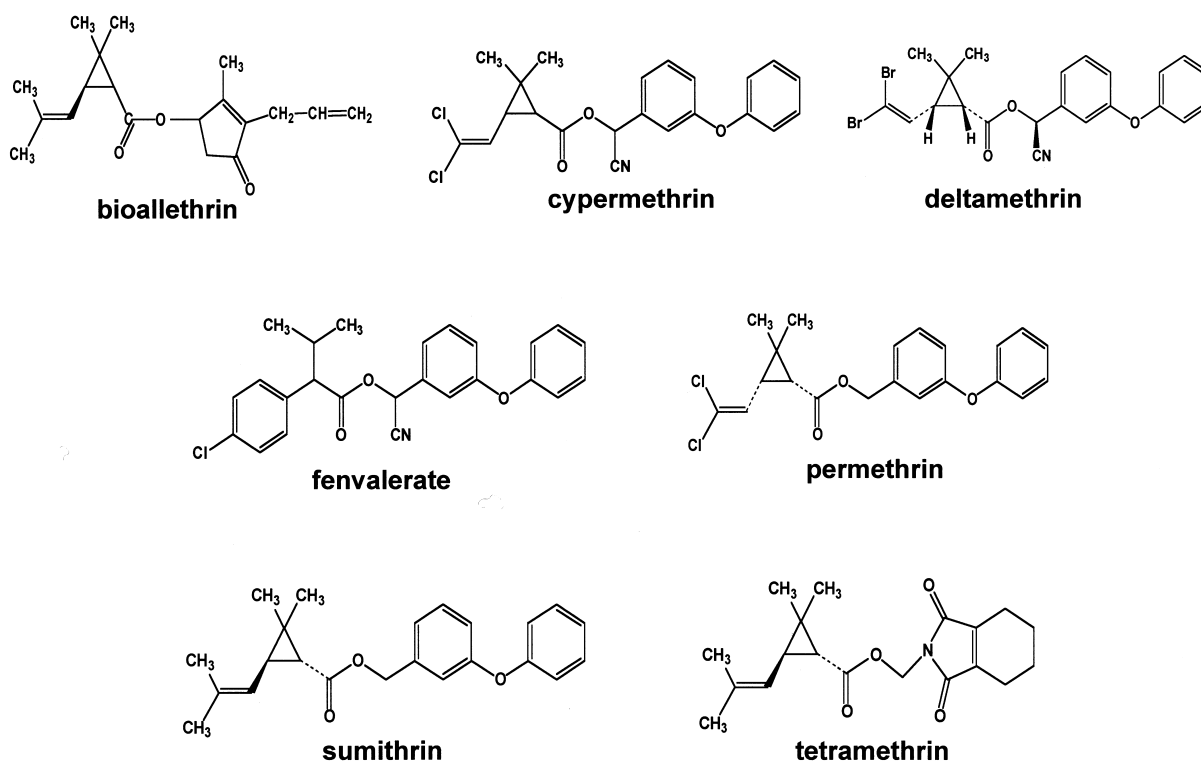


Fig. 1. Chemical structures of the pyrethroid insecticides tested.

(Fig. 3).

#### ER competitive-binding assay

Cytosol prepared from the OVX rat uteri was incubated with [ $^3\text{H}$ ]estradiol and increasing concentrations of radio-inert  $17\beta$ -estradiol. As expected,  $17\beta$ -estradiol effectively competed with [ $^3\text{H}$ ]estradiol for binding to ER at the concentrations tested and was found to have an  $\text{IC}_{50}$  of  $5 \times 10^{-10}$  M (Fig. 4). In contrast, sumithrin did not show any binding to ER (Fig. 4). In addition, other pyrethroid insecticides examined did not compete with [ $^3\text{H}$ ]estradiol for binding to ER at any concentration (Data not shown).

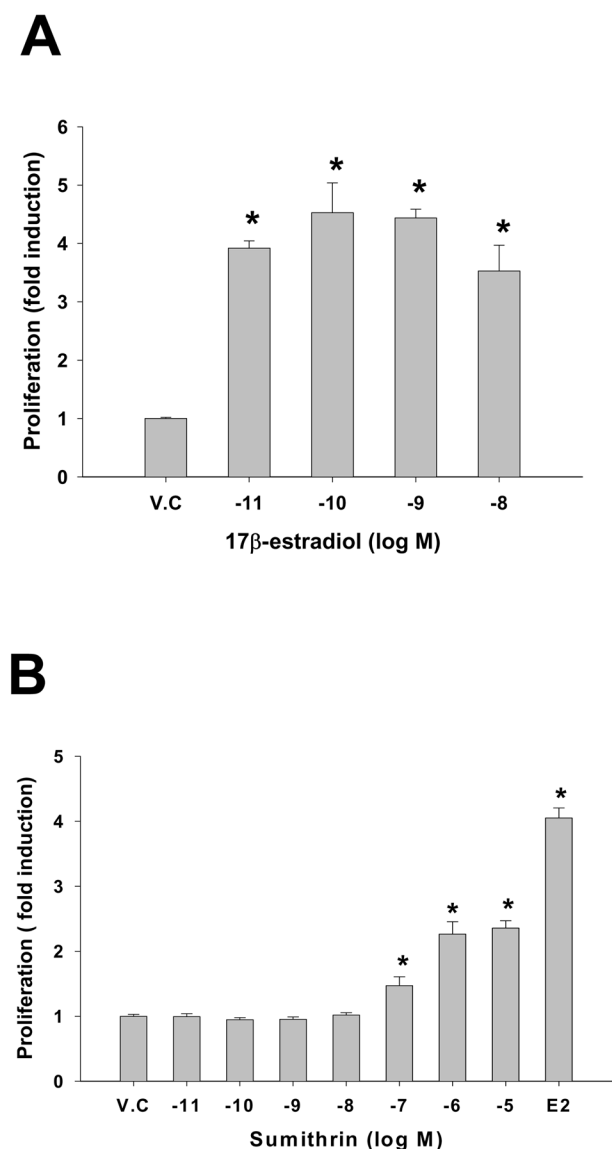
#### Expression of ER $\alpha$ and ER $\beta$

ER $\alpha$  and ER $\beta$  protein levels were measured in MCF-7 BUS cells following treatment with  $17\beta$ -estradiol and sumithrin for 48 h. The background levels of ER $\alpha$  and ER $\beta$  proteins in MCF-7 BUS cells treated with vehicle were regarded as 100%.  $17\beta$ -Estradiol ( $10^{-10}$  M) decreased the ER $\alpha$  protein levels significantly compared with those of the control

culture (53% of control). Similarly, ER $\alpha$  protein levels were decreased significantly by sumithrin at concentrations of  $10^{-7}$ ,  $10^{-6}$  and  $10^{-5}$  M (76, 73 and 64% of control, respectively) (Fig. 5). The ER $\beta$  protein level was decreased by  $17\beta$ -estradiol at  $10^{-10}$  M and significantly by sumithrin at  $10^{-7}$ ,  $10^{-6}$  and  $10^{-5}$  M (82, 56 and 48% of control, respectively) (Fig. 6).

#### Expression of pS2 mRNA

To understand the estrogenic properties of sumithrin, we measured pS2 mRNA levels in MCF-7 BUS cells by RT-PCR analysis. Induction of the pS2 gene is widely used as a biomarker for the estrogenic activity of test compounds because it is induced strongly by estrogens or estrogen-like chemicals [17]. As expected,  $17\beta$ -estradiol ( $10^{-10}$  M) induced a increase in pS2 mRNA level compared with the control culture (Fig. 7). Similarly, sumithrin (from  $10^{-7}$  to  $10^{-5}$  M) also induced a slight increase in pS2 mRNA level. The highest level of induction was observed at  $10^{-5}$  M sumithrin (137% of control).



**Fig. 2.** The effects of  $17\beta$ -estradiol and sumithrin on proliferation of MCF-7 BUS cells. Cells in DMEM supplemented with 5% (v/v) charcoal dextran-treated FBS serum were exposed to test chemicals for six days. Cell proliferation was determined by SRB assay. Values are means  $\pm$  S.D. of duplicates from three independent experiments. Asterisks indicate results significantly different from vehicle control ( $P < 0.01$ ).

## Discussion

We tested the estrogenic potencies of pyrethroid insecticides in the E-screen assay. With the exception of sumithrin, none of the pyrethroid insecticides examined induced proliferation of MCF-7 BUS cells in our assay system. Sumithrin

showed weak estrogenic activity based on a maximum MCF-7 BUS cell proliferation that was equivalent to 70% of that of  $17\beta$ -estradiol. These results are in partial agreement with those of previous reports in which pyrethroid insecticides did not show any estrogenic activity in MCF-7 cell proliferation assay [11], and that certain pyrethroid insecticides (fenvalerate, *d*-phenothrin, permethrin, and cypermethrin) did not exhibit estrogenicity in a reporter gene assay with or without liver microsomes or S9 mix [12]. In contrast, Go *et al.* [10] found that fenvalerate, *d*-allethrin, and sumithrin, at concentrations of  $10^{-5}$  M, induced MCF-7 cell proliferation at a level comparable to the effect of  $17\beta$ -estradiol. More recently, fenvalerate, cypermethrin, deltamethrin, and permethrin were shown to exhibit partial agonistic effects, and the estrogenic effect was blocked by the addition of ICI 182,780 in the E-screen assay [18]. In the present study, only sumithrin ( $10^{-5}$  M) stimulated MCF-7 BUS cell proliferation significantly, but sumithrin did not inhibit  $17\beta$ -estradiol-induced MCF-7 BUS cell proliferation. Bioallethrin, fenvalerate and permethrin significantly inhibited  $17\beta$ -estradiol-induced MCF-7 BUS cell proliferation comparable to that of  $10^{-9}$  M of ICI 182,780. These results are similar to those of Chen *et al.* [18]. Based on these results, we propose that certain pyrethroid insecticides may act as estrogen agonists or antagonists through either an ER-mediated pathway or an alternative signaling pathway in MCF-7 BUS cells.

The E-screen assay is widely used as a primary screening method to detect a large numbers of estrogenic compounds [14, 19]. Although the E-screen assay is a simple and highly reproducible method of testing the estrogenicity of chemicals, false positive and false negative responses have been found depending on the exact method used [20, 21]. In fact, there is variability in E-screen assay methods, and the lack of standardized protocols for the E-screen assay makes inter- or intra-laboratory validation tests necessary. On the other hand, the variable results might be due to the origin of the MCF-7 cell lines, the passage number, or the serum batch used [19, 20, 22, 23]. For these reasons, in the present study,  $17\beta$ -estradiol was also measured concurrently to validate our experimental protocol.  $17\beta$ -Estradiol at a low concentration significantly stimulated MCF-7 BUS cell proliferation, and the proliferation rate was 4.8 folds higher than that of

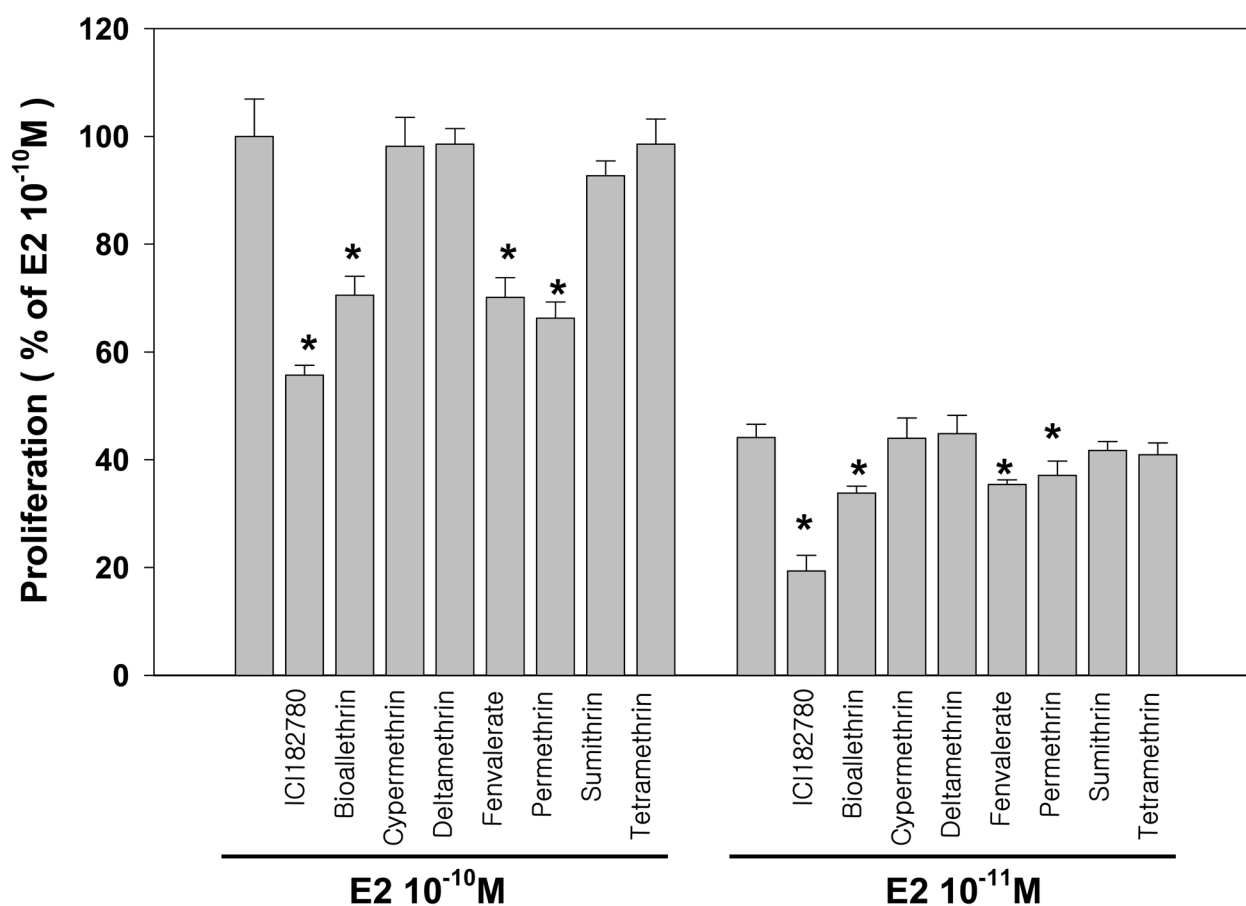


Fig. 3. Anti-estrogenic effects of pyrethroid insecticides on proliferation of MCF-7 BUS cells. Cells in DMEM supplemented with 5% (v/v) charcoal dextran-treated FBS serum were exposed to test chemicals ( $10^{-6}$  M) or ICI 182,780 ( $10^{-9}$  M) with  $17\beta$ -estradiol for six days. Cell proliferation was determined by SRB assay. Values are means  $\pm$  S.D. of duplicates from three independent experiments. Asterisks indicate results significantly different from vehicle control ( $P < 0.01$ ).

the vehicle control culture. Our data indicate that the E-screen assay using MCF-7 BUS cells is highly sensitive to  $17\beta$ -estradiol.

An *in vitro* competitive ER binding assay was conducted to determine the ability of pyrethroid insecticides to bind to rat uterus ER. In contrast to the E-screen assay, the competitive ER binding assay cannot distinguish between estrogen agonistic and antagonistic activities and cannot detect pro-estrogens. In the present study, none of the pyrethroid insecticides examined interacted with the rat cytosolic ER. Similarly, Saito *et al.* [11] reported that certain pyrethroid insecticides did not show any binding to the hER $\alpha$  in competitive ligand-binding assays. In contrast, Chen *et al.* [18] found that certain pyrethroid insecticides (permethrin, fenvalerate, cypermethrin, and

deltamethrin) at high concentrations inhibited the binding of [ $^3$ H]estradiol to rat uterus cytosolic ERs in a competitive binding study. We suggest that signaling pathways, other than ERs, are involved in mediating pyrethroid-induced or -inhibited MCF-7 BUS cell proliferation and that pyrethroid insecticides may alter the conformation of ER without binding to it. Although linear relationships have been determined between the MCF-7 cell proliferation assay and the competitive ER binding assay for many chemicals [24], certain compounds may exhibit estrogenic or anti-estrogenic activities via other signaling pathways [25]. Based on these observations we suggest that the estrogenic (sumithrin) or anti-estrogenic (bioallethrin, permethrin and fenvalerate) properties of pyrethroid insecticides are associated

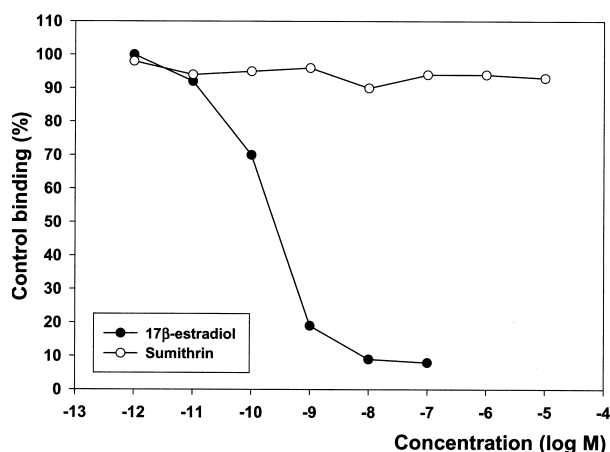


Fig. 4. Competitive binding of 17 $\beta$ -estradiol and sumithrin to estrogen receptors isolated from the uterus of ovariectomized female Sprague-Dawley rats.

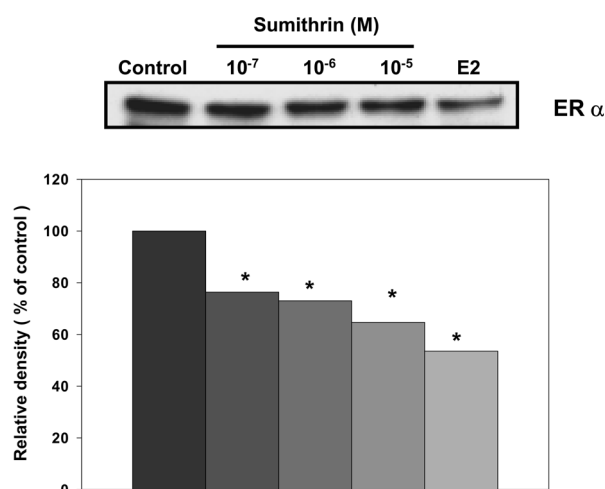


Fig. 5. Western blot analysis of ER $\alpha$  protein in MCF-7 BUS cells treated with 17 $\beta$ -estradiol or sumithrin. Cells in DMEM supplemented with 5% (v/v) charcoal dextran-treated FBS serum were exposed to 17 $\beta$ -estradiol ( $10^{-10}$  M) or sumithrin ( $10^{-7}$  to  $10^{-5}$  M) for 48 h.

with indirect ER-mediated mechanisms.

Estrogen-responsive ER protein levels were measured to understand the precise estrogenic mechanism of action of sumithrin because it showed a positive response in the E-screen assay. In general, ER has a critical role in the biological effects of estrogens and anti-estrogens. Environmental estrogenic chemicals can alter the expression of estrogen-regulated genes and can initiate the transcription of these genes by

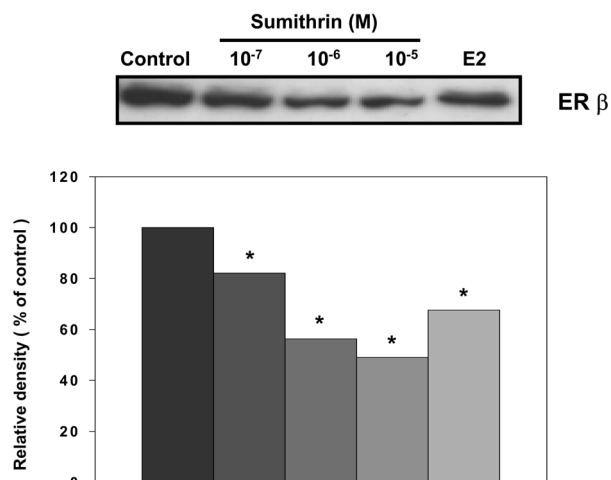


Fig. 6. Western blot analysis of ER $\beta$  protein in MCF-7 BUS cells treated with 17 $\beta$ -estradiol or sumithrin. Cells in DMEM supplemented with 5% (v/v) charcoal dextran-treated FBS serum were exposed to 17 $\beta$ -estradiol ( $10^{-10}$  M) or sumithrin ( $10^{-7}$  to  $10^{-5}$  M) for 48 h.

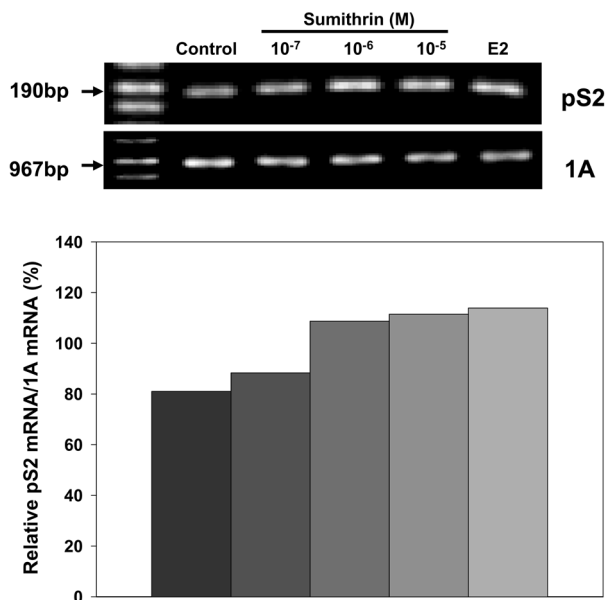


Fig. 7. The expression of pS2 mRNA in MCF-7 BUS cells detected by RT-PCR. Cells in DMEM supplemented with 5% (v/v) charcoal dextran-treated FBS were exposed to 17 $\beta$ -estradiol ( $10^{-10}$  M) or sumithrin ( $10^{-7}$  to  $10^{-5}$  M) for 48 h.

interacting with ERs [25, 26]. As shown in Figs. 5 and 6, 17 $\beta$ -estradiol ( $10^{-10}$  M) induced marked decreases in the protein levels of ER $\alpha$  and ER $\beta$ . ER $\alpha$  and ER $\beta$  are expressed differentially in



different tissues; ER expression has been widely investigated in human breast cancer cell lines [27, 28]. MCF-7 cells express both ER $\alpha$  and ER $\beta$ , but ER $\alpha$  expression is predominant [29]. Based on the results of studies showing that both ER $\alpha$  protein and mRNA levels were decreased markedly in MCF-7 cells after addition of 17 $\beta$ -estradiol [30–32], it is suggested that the reduced levels of cytosolic ERs could be the result of translocation of ERs to the nucleus. Furthermore, Diel *et al.* [30] demonstrated that the reduction of ER $\alpha$  protein level after administration of probable environmental estrogens (DDT and coumestrol) was comparable to the effect of 17 $\beta$ -estradiol, indicating that these compounds act as weak estrogen agonists in MCF-7 cells. Our results similarly revealed that sumithrin induced significant decreases in both ER $\alpha$  and ER $\beta$  protein levels. However, sumithrin did not compete directly with 17 $\beta$ -estradiol for binding to rat uterus cytosolic ERs (Fig. 3), indicating that sumithrin does not interact with the ER ligand-binding domain. Accordingly, our results suggest that sumithrin was able to act as an estrogen agonist by down-regulating ER activity, but not by binding directly to ER.

To investigate the estrogenic properties of sumithrin, pS2 mRNA expression was measured in MCF-7 BUS cells by RT-PCR analysis. The induction of pS2 is considered a direct reflection of estrogenic responses because the expression of the pS2 gene is directly associated with estrogens at the transcriptional level [33, 34]. Thus, pS2 induction is frequently used as a surrogate biomarker for detecting environmental estrogen-like chemicals [10, 18, 35]. We measured the pS2 mRNA levels as an estrogen-responsive endpoint in MCF-7 BUS cells. As shown in Figure 7, MCF-7 BUS cells cultured in phenol red-free medium did not have detectable levels of pS2 mRNA; treatment with 17 $\beta$ -estradiol increased the pS2 mRNA level slightly. Sumithrin also slightly increased the pS2 mRNA level in a dose-dependent manner. Go *et al.* [10] similarly observed that sumithrin and fenvalerate significantly induced the expression of pS2 to levels comparable to those elicited by 10<sup>-8</sup> M of 17 $\beta$ -estradiol (5.0 folds) and that the estrogenic activity of sumithrin was abolished by co-treatment with ICI 164,386. In addition, Chen *et al.* [18] reported

that some pyrethroid insecticides (fenvalerate, permethrin, and cypermethrin) induced pS2 expression in MCF-7 cells, and there was no significant effect after deltamethrin treatment. Although it is unclear how estrogen regulates pS2 expression and whether the ERs are involved in this process, 17 $\beta$ -estradiol stimulated ER expression in a manner similar to pS2 expression in MCF-7 cells [36]. Ren *et al.* [36] also reported that the weak estrogenic compound, nonylphenol, altered pS2 gene expression and ER gene expression in MCF-7 cells. Therefore, pS2 mRNA expression is an ideal model for evaluating the effects of estrogenic chemicals in human breast cancer cells [36, 37].

Although estrogen can directly stimulate MCF-7 cell proliferation, our observations indicate that the induction of pS2 expression is not always correlated with cell proliferation. We found that 17 $\beta$ -estradiol concentrations above 10<sup>-10</sup> M induced pS2 expression moderately, while the same dose induced cell proliferation significantly. Similar to 17 $\beta$ -estradiol, 10<sup>-5</sup> M sumithrin induced pS2 mRNA expression slightly (1.3 folds), but increased the cell proliferation rate significantly. Moreover, high concentrations (more than 10<sup>-5</sup> M) of pyrethroid insecticides showed slight toxicity in MCF-7 BUS cells, which may have affected the outcome of these assays.

Our results demonstrate that certain pyrethroid insecticides exhibit either estrogenic or anti-estrogenic activity via ER-independent pathways, inhibiting the expression of ERs and influencing the expression of estrogen-dependent pS2 genes. Accordingly, we propose that certain pyrethroid insecticides may interfere with the action of endogenous hormones by several mechanisms in humans.

### Acknowledgments

This study was supported by the 2002 Endocrine Disruptors Research fund of the National Institute of Toxicological Research/Korea Food & Drug Administration. We would like to thank Drs. A. M. Soto and C. Sonnenschein of Tufts University School of Medicine for kindly providing the MCF-7 BUS cells.

## References

1. **Colborn T, vom Saal FS, Soto AM.** Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 1993; 101: 378–384.
2. **Safe SH.** Endocrine disruptors and human health—is there a problem? An update. *Environ Health Perspect* 2000; 108: 487–493.
3. **Gray LE.** Tiered screening and testing strategy for xenoestrogens and antiandrogens. *Toxicol Lett* 1998; 102–103: 677–680.
4. **O'Connor JC, Cook JC, Marty MS, Davis LG, Kaplan AM, Carney EW.** Evaluation of Tier I screening approaches for detecting endocrine-active compounds (EACs). *Crit Rev Toxicol* 2002; 32: 521–549.
5. **Choi KC, Jeung EB.** The biomarker and endocrine disruptors in mammals. *J Reprod Dev* 2003; 49: 337–345.
6. **EDSTAC.** Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) final report. Washington, DC; U.S. EPA. 1998; August.
7. **Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C, Miller R, Moore J, Rolland R, Scott G, Sheehan DM, Sinks T, Tilsonj HA.** Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *Environ Health Perspect* 1996; 104: 715–740.
8. **Martin D.** Synthetic pyrethroid insecticides: Structures and properties. In: Naumann K (eds.), *Chemistry of Plant Protection*. Vol. 4. New York: Springer-Verlag Press; 1990.
9. **Garey J, Wolff MS.** Estrogenic and anti-progestagenic activities of pyrethroid insecticides. *Biochem Biophys Res Commun* 1998; 251: 855–859.
10. **Go V, Garey J, Wolff MS, Pogo BG.** Estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. *Environ Health Perspect* 1999; 107: 173–177.
11. **Saito K, Tomigahara Y, Ohe N, Isobe N, Nakatsuka I, Kaneto H.** Lack of significant estrogenic or anti-estrogenic activity of pyrethroid insecticides in three *in vitro* assays based on classic estrogen receptor alpha-mediated mechanisms. *Toxicol Sci* 2000; 57: 54–60.
12. **Sumida K, Ooe N, Nagahori H, Saito K, Isobe N, Kaneko H, Nakatsuka I.** An *in vitro* reporter gene assay method incorporating metabolic activation with human and rat S9 or liver microsomes. *Biochem Biophys Res Commun* 2001; 280: 85–91.
13. **Kunimatsu T, Yamada T, Ose K, Sunami O, Kamita T, Okuno Y, Seki T, Nakatsuka I.** Lack of (anti-) androgenic or estrogenic effects of three pyrethroids (esfenvalerate, fenvalerate, and permethrin) in the Hershberger and uterotrophic assays. *Regul Toxicol Pharmacol* 2001; 35: 227–237.
14. **Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO.** The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ Health Perspect* 1995; 103: 113–122.
15. **Kim HS, Han SY, Lee BM, Yoo SD, Park KL.** Potential estrogenic effects of bisphenol-A in both *in vitro* and *in vivo* combination assays. *J Toxicol Sci* 2001; 26: 111–118.
16. **Berthois Y, Pons M, Dussert C, Crastes de Paulet A, Martin PM.** Agonist-antagonist activity of anti-estrogens in the human breast cancer cell line MCF-7: a hypothesis for the interaction with a site distinct from the estrogen binding site. *Mol Cell Endocrinol* 1994; 99: 259–268.
17. **Nesaretnam K, Corcoran D, Dils RR, Darbre P.** 3,4,3',4'-Tetrachloro-biphenyl acts as an estrogen *in vitro* and *in vivo*. *Mol Endocrinol* 1996; 10: 923–936.
18. **Chen H, Xiao J, Hu G, Zhou J, Xiao H, Wang X.** Estrogenicity of organophosphorus and pyrethroid pesticides. *J Toxicol Environ Health A* 2002; 65: 1419–1435.
19. **Odum J, Tittensor S, Ashby J.** Limitations of the MCF-7 cell proliferation assay for detecting xenobiotic estrogens. *Toxicol In Vitro* 1998; 12: 273–278.
20. **Jones PA, Baker VA, Irwin AJE, Earl LK.** Modulation of MCF-7 cell proliferative responses by manipulation of assay conditions. *Toxicol In Vitro* 1997; 11: 769–773.
21. **Lackey BR, Gray SI, Henricks DM.** Cross-talk and considerations in endocrine disruptor research. *Med Hypotheses* 2001; 56: 644–647.
22. **Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V.** The E-screen assay: a comparison of different MCF7 cell stocks. *Environ Health Perspect* 1995; 103: 844–850.
23. **Blom A, Ekman E, Johannisson A, Norrgren L, Pesonen M.** Effects of xenoestrogenic environmental pollutants on the proliferation of a human breast cancer cell line (MCF-7). *Arch Environ Contam Toxicol* 1998; 34: 306–310.
24. **Fang H, Tong W, Perkins R, Soto AM, Prechtel NV, Sheehan DM.** Quantitative comparisons of *in vitro* assays for estrogenic activities. *Environ Health Perspect* 2000; 108: 723–729.
25. **Klotz DM, Arnold SF, McLachlan JA.** Inhibition of 17beta-estradiol and progesterone activity in human breast and endometrial cancer cells by carbamate insecticides. *Life Sci* 1997; 60: 1467–1475.
26. **Jorgensen M, Vendelbo B, Skakkebaek NE, Leffers H.** Assessing estrogenicity by quantitating

- the expression levels of endogenous estrogen-regulated genes. *Environ Health Perspect* 2000; 108: 403–412.
27. **Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA.** Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997; 82: 4258–4265.
  28. **Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA.** Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; 93: 5925–5930.
  29. **Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R.** Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. *Oncol Rep* 2000; 7: 157–167.
  30. **Diel P, Olf S, Schmidt S, Michna H.** Effects of the environmental estrogens bisphenol A, *o,p'*-DDT, *p-tert*-octylphenol and coumesterol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. *J Steroid Biochem Mol Biol* 2002; 80: 61–70.
  31. **Okubo T, Yokoyama K, Kano K, Kano I.** ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ER $\alpha$  and PR. *Food Chem Toxicol* 2001; 39: 1225–1232.
  32. **Olsen CM, Meussen-Elholm ET, Holme JA, Hongslo JK.** Brominated phenols: characterization of estrogen-like activity in the human breast cancer cell-line MCF-7. *Toxicol Lett* 2002; 129: 55–63.
  33. **Brown AM, Jeltsch JM, Roberts M, Chambon P.** Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *Proc Natl Acad Sci USA* 1984; 81: 6344–6348.
  34. **Shekhar PV, Werdell J, Basrur VS.** Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. *J Natl Cancer Inst* 1997; 89: 1774–1782.
  35. **Andersen HR, Vinggaard AM, Rasmussen TH, Gjermansen IM, Bonefeld-Jorgensen EC.** Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*. *Toxicol Appl Pharmacol* 2002; 179: 1–12.
  36. **Ren L, Marquardt MA, Lech JL.** Estrogenic effects of nonylphenol on pS2, ER, and MUC1 gene expression in human breast cancer cells-MCF-7. *Chem Biol Interact* 1997; 104: 55–64.
  37. **Kida N, Yoshimura T, Takahashi H, Nagao S, Nozawa Y, Furukawa Y, Mori K, Hayashi K.** Estrogen-inducible pS2 protein is not the key regulatory component in the proliferation of human breast cancer cells (MCF-7). *Eur J Biochem* 1993; 215: 671–676.