We tested 200 pesticides, including some of their isomers and metabolites, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. The test compounds were classified into nine groups: organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. These pesticides were tested at concentrations < 10⁻³ M. Of the 200 pesticides tested, 47 and 33 showed hERα- and hERβ-mediated estrogenic activities, respectively. Among them, 29 pesticides had both hERα and hERβ agonistic activities, and the effects of the organochlorine insecticides β-benzene hexachloride (BHC) and δ-BHC and the carbamate insecticide methiocarb were predominantly hERβ rather than hERα agonistic. Weak antagonistic effects toward hERα and hERβ were shown in five and two pesticides, respectively. On the other hand, none of tested pesticides showed hAR-mediated androgenic activity, but 66 of 200 pesticides exhibited inhibitory activity against the transcriptional activity induced by 5α-dihydrotestosterone. In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlorothalonil and chloromethoxyfen, were higher than those of vinclozolin and p,p‘-dichlorodiphenyl dichloroethylene, known AR antagonists. The results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, indicating pleiotropic effects on hER and hAR. We also discussed chemical structures related to these activities. Taken together, our findings suggest that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR, and that numerous other manmade chemicals may also possess such estrogenic and antiandrogenic activities. Key words: antiandrogenic activity, Chinese hamster ovary cells, estrogenic activity, human androgen receptor, human estrogen receptor α, human estrogen receptor β, pesticide, reporter gene assay. Environ Health Perspect 112:524–531 (2004). doi:10.1289/ehp.6649 available via http://dx.doi.org/[Online 3 December 2003]

It has been well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties, which provide a potential threat to human and wildlife reproduction (Colborn 1995; Colborn et al. 1993; Jensen et al. 1995). A suggested mechanism is that these environmental contaminants alter the normal functioning of the endocrine and reproductive system by mimicking or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations (Sonnenschein and Soto 1998). A major mechanism of endocrine disruption is the action of chemicals as receptor agonists or antagonists through direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via estrogen receptor (ER) have been the focus of research for the last 20 years. Meanwhile, recent studies have shown that several chemicals may exert antiandrogenic effect by interfering with androgen receptor (AR; Sohoni and Sumpter 1998; Vinggaard et al. 1999).

Pesticides commonly used to control agricultural and indoor pests are the most likely suspects as endocrine disruptors. The ubiquitous nature of pesticide usage with minimal precautions has resulted in contamination of food, the workplace, and the environment. Recent reports showed that several pesticides exert estrogenic and antiandrogenic activities through interaction with estrogen and androgen receptors. To date, p,p‘-dichlorodiphenyl trichloroethane (DDT) (Welch et al. 1969), methoxychlor (Bulger et al. 1978; Cummings 1997), β-benzene hexachloride (BHC) (Coosen and Velsen 1989), endosulfan, toxaphene, and dieldrin (Soto et al. 1995), and fenvalerate (Garey and Wolff 1998) have been reported as estrogenic pesticides. Recently, Andersen et al. (2002) have reported that several currently used pesticides, such as methio carb, fenarimol, chlorpyrifos, deltamethrin, and tolofos methyl, possess estrogenic activity on the basis of cell proliferation assay and transactivation assay using MCF-7 human breast cancer cells. On the other hand, studies have also revealed antiandrogenic pesticides, such as vinclozolin and p,p‘-dichlorodiphenyl dichloroethylene (DDE) (Kele et al. 1994, 1995), DDT isomer and methoxychlor (Maness et al. 1998), linuron (Gray et al. 1999; Lambright et al. 2000), procymidone (Ostby et al. 1999), and fenitrothion (Tamura et al. 2001). Andersen et al. (2002) reported that dieldrin, endosulfan, methiocarb, and fenarimol possessed antiandrogenic activity on the basis of transactivation assay using Chinese hamster ovary (CHO) cells. Thus, estrogenic and antiandrogenic activities have been found in a number of pesticides, and it is conceivable that many other pesticides also have estrogenic and/or antiandrogenic activity.

Transactivation or reporter gene assay, which is a powerful tool for testing receptor agonists and antagonists among chemicals, has been established as a method for evaluating the receptor activity of chemicals. However, many compounds are independently evaluated for ER or AR activity by different reporter gene assays. This may lead to confusion in the evaluation of their potential as endocrine-disrupting chemicals. In addition, the recent cloning of a gene for a second estrogen receptor, ERβ, by Kuiper et al. (1996) led to the discovery that ERβ and the classic ERα differ in their ligand binding ability and transactivation properties (Kuiper et al. 1997; McNerney et al. 1998). Therefore, a screening system involving both ER subtypes (α and β) is required to completely evaluate the endocrine disruption potential of environmental estrogens. Previously, we reported highly sensitive reporter gene assays using CHO cells for detecting ERβ and AR agonists/antagonists from chemicals, and demonstrated that the diphenyl ether-type herbicide chlorothalonil (CPN) and its amino derivatives (CPN-amino) possessed both antiandrogenic and estrogenic activities (Kojima et al. 2003). In the present study, we screened a total of 200 pesticides using our reporter gene assay systems for detecting two ER subtypes, ERα and ERβ, and AR activities. These pesticides, including several well-known estrogenic and antiandrogenic pesticides such as DDT and vinclozolin, were selected according to the frequency of their use in Japan and other countries, both currently and in the past. They are classified...
into nine groups according to similarities in their chemical structure and are discussed on the basis of the relationships between chemical structure and activity via hormone receptors. In this article, we provide evidence that a variety of pesticides have estrogenic and/or antian-
drogenic potential via ER and/or AR and that their activities are related to chemical structure.

**Materials and Methods**

**Chemicals.** 17ß-Estradiol (E2; > 97% pure), 5α-dihydrotestosterone (DHT; 95% pure), and tamoxifen citrate (98% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 200 pesticides tested in the present study are listed in Table 1. These pesticides were purchased from Wako, Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), AccuStandard Inc. (New Haven, CT, USA), and Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan) and had a purity of 95–100%. Dimethylsulfoxide (DMSO) was also purchased as a vehicle from Wako.

E2 and DHT were stored as a 1 mM stock solution in DMSO at −20°C. Pesticides were dissolved in DMSO to a final concentration of 10 mM, except for asulam, paraquat, diquat, iminocaricide, glyphosate, and carbendazim, which were directly dissolved in the medium, and all pesticides were diluted to the desired concentrations in phenol red−free Dulbecco’s modified Eagle medium plus nutrient mixture Ham’s F-12 (DMEM/F-12) immediately before use. The final solvent concentration in the culture medium did not exceed 0.1%, and this concentration did not affect cell yields.

**Cell line and cell culture conditions.** CHO-K1 cells were obtained from the Dainippon Pharmaceutical Co. (Osaka, Japan). Penicillin−streptomycin solution (antibiotics) at 37°C in an atmosphere of 5% CO2/95% air under saturating humidity and passed every week by trypsinization with 0.25% trypsin/0.02% ethylenediamine tetaacetic acid (EDTA) disodium salt solution (Life Technologies, Paisley, UK).

**Construction of plasmids.** The human ERα (hERα) and AR (hAR) expression vectors (pcDNA5Rts and pZeSV2AR) were constructed as previously described (Kojima et al. 2003). The hERβ expression vector was newly constructed as follows: The ERβ cDNA was cloned by reverse transcriptase–polymerase chain reaction from human placental RNA (Clontech, Palo Alto, CA, USA). The sequence

| Group, compound | Group, compound | Group, compound |
|----------------|----------------|----------------|
| 1. Organochlorines (n = 29) | Isofenphos | Mepronil |
| Aldrin | Isoxathion | Metolaxyl |
| α-BHC | Leptophos | Metolachlor |
| β-BHC | Malathion | Pretialachlor |
| γ-BHC | Mecarbam | Propyzamide |
| b-BHC | Methamidophos | Thychlchlor |
| Captan | Methidathion | Thychlchlor |
| cis-Chlordane | Methyl-parathion | Thychlchlor |
| trans-Chlordane | Monocrotophos | Thychlchlor |
| Chlorobenzilate | Parathion | Thychlchlor |
| Chloropropionate | Phenthioate | Thychlchlor |
| Chlorothalonil | Phorate | Thychlchlor |
| p,p’-DDT | Phosalone | Thychlchlor |
| p,p’-DDE | Phosmet | Thychlchlor |
| p,p’-DDE | Piperophos | α-DHC |
| Dichlorobenzene | Pyrimidinophos-methyl | β-DHC |
| Dichlorobenzen | Profenofos | d-BHC |
| Dieldrin | Propophos | m-BHC |
| α-Endosulfan | Prothiofos | p-BHC |
| β-Endosulfan | Prothiofos oxon | o-BHC |
| Endosulfan sulfate | Pyridaphenthion | endrin |
| Endrin | Quinalphos | γ-BHC |
| Folpet | Pencycuron | α-BHC |
| Fthalide | Piperophos | β-BHC |
| Heptachlor | Prothiofos | p,p’-DDE |
| Heptachlor epoxide | Prothiofos oxon | p,p’-DDD |
| Methoxychlor | Pyridaphenthion | p,p’-DDD |
| Pentachlorophenol | Quinalphos | p,p’-DDD |
| Quintozene | Pimelicidinophos | p,p’-DDD |
| 2. Diphenyl ethers (n = 11) | Cyfluthrin | p,p’-DDD |
| Acifluorfen | Cyhalothrin | p,p’-DDD |
| Acifluorfen-methyl | Cypermethrin | α,α-DHC |
| Bifenox | Deltamethrin | a,BHC |
| Chlormethylox | Etopenprox | a,BHC |
| Chlorothalonil | Fenvalerate | a,BHC |
| Chlorobenzilate | Flocytrinate | a,BHC |
| Chlorpyrifos-methyl | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |
| Chlorpyrifos | Flucytrinate | a,BHC |

Table 1. The 200 pesticides tested in the reporter gene assays for hERα, hERβ, and hAR.
of the cloned hERβ cDNA was verified and was inserted into the mammalian expression vector pcDNA3.1Zeo(-) (Invitrogen, San Diego, CA, USA), creating pcDNAERβ.

The estrogen-responsive element (ERE)—containing reporter plasmid pG3l3-ERE and the androgen-responsive element (ARE)—containing reporter plasmid pIND-ARE were constructed as described previously (Kojima et al. 2003). pRL-SV40 containing the Renilla luciferase gene was purchased from Promega (Madison, WI, USA) and used as an internal control for transfection efficiency.

**Reporter gene assays for hERα, hERβ, and hAR.** The host CHO-K1 cells were plated in 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) at a density of 8,400 cells/well in phenol red–free DMEM/F-12 containing 5% CD-FBS (complete medium) 1 day before transfection. For detection of hERα or hERβ activity, cells were transfected with 5 ng pcDNAERα or 5 ng pcDNAERβ, 50 ng pG3l3-ERE, and 5 ng pRL-SV40 per well using the transfection reagent FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). For detection of hAR activity, cells were transfected with 2.5 ng pZeoSV2AR, 50 ng pGL3-tkERE, and 5 ng pRL-SV40 per well.

After the transfection, cells were washed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 µL/well) provided with the Dual-Luciferase Reporter Assay kit (Promega). We measured the firefly luciferase activity with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) before measuring *Renilla* luciferase activity in one reaction tube with 5-µL aliquots of cell lysates using the Dual-Luciferase Reporter Assay kit, following the manufacturer’s instructions. The firefly luciferase activity was normalized based on the *Renilla* luciferase activity of the cotransfected pRL-SV40. The values shown are mean ± SD from at least three independent experiments.

We evaluated the results for the agonistic activities of the pesticides by relative activity, expressed as REC20 (20% relative effective concentration)—that is, the concentration of the test compound showing 20% of the activity of 10–12 M E2, 10–9 M E2, or 10–9 M DHT for ERα, ERβ, or AR, respectively. When the activity of the test compound was higher than REC20 within the concentration tested (~10–8 to 10–5 M), we judged the pesticide to be positive for activity. For the antagonistic activities of the pesticides, we expressed them as IC50 (50% inhibitory concentration), that is, the concentration of the test compound showing 50% inhibition of the activity induced by 10–12 M E2, 10–10 M E2, or 10–10 M DHT for ERα, ERβ, or AR, respectively. When the activity of the test compound was lower than the IC50 within the concentration tested, we judged the pesticide to be inhibitory for activity. To avoid cell toxicity by the pesticides, assays were performed for pesticides at concentrations ≤ 10–5 M.

**Data analysis.** We evaluated the statistical significance of differences using the Student’s t-test (two-tailed, equal variance) calculated by software (Excel; Microsoft, Redmond, WA, USA). The level of significance was p < 0.05. Data are presented as the mean and, where shown, the SD of at least three separate experiments with duplicate wells.

**Results**

**Response of 17β-E2 in ERα and ERβ assays, and of 5α-DHT in AR assay.** Figure 1A shows the dose-dependent transactivation of ERα and ERβ by 17β-E2, indicating that both receptors can be activated at very low hormone concentrations. The maximal ERα activity was achieved at 10–10 M E2 or more, exhibiting approximately 10-fold that of the control solvent. The maximal ERβ activity induced was 8.5-fold that of the solvent control at 10–9 M E2 or more. Thus, E2 was more potent for ERα than for ERβ. From these dose–response curves, REC20 values of E2 for ERα and ERβ were deduced to be 2.5 × 10–11 M and 5.3 × 10–12 M, respectively.

Figure 1B shows the dose-dependent transactivation of AR by 5α-DHT. Its activity was detectable from 10–11 M DHT and reached a plateau at 10–9 M DHT. The maximum induction was 21-fold that of the control solvent. The REC20 value of DHT for AR was 3.1 × 10–11 M.

**Estrogenic effects of the pesticides.** Table 2 shows the REC20 values and relative estrogenic activities at 10–5 M of pesticides evaluated as positive for ERα agonistic activity. As shown in Table 2, 47 of the 200 pesticides were found to induce estrogenic activity in the ERα assay. A comparison of the potency of estrogenic activities among these active pesticides shows that the REC20 values of α,p′-DDT, β-BHC, methoxychlor, and α-endosulfan among the organochlorine pesticides, the CNP metabolite CNP-amino among the diphenyl ether pesticides, and butamifos among the organophosphorus pesticides were all lower than 10–6 M, indicating that they possess potent estrogenic activity.

The results of ERβ agonistic activity are presented in Table 3. Thirty-three of 200 pesticides increased the ERβ-mediated transactivation gene response. Twenty-nine of these pesticides also have estrogenic activity via ERα (Table 2). The REC20 values of β-BHC and α,p′-DDT among the organochlorine pesticides, CNP-amino among the diphenyl ether pesticides, and methiocarb among the carbamate pesticides were all lower than 10–6 M. However, butamifos, which showed potent ERα agonistic activity among the organophosphorus pesticides, was inactive in the ERβ assay. Dose–response curves of β-BHC, δ-BHC, and methiocarb for ERα and ERβ are shown in Figure 2. These pesticides stimulated ERβ more strongly than they did ERα.

**Antiestrogenic effects of the pesticides.** Of 200 test pesticides, five (cyhalothrin, delta-methrin, alachlor, pyrazoxyfen, and triflumizole) showed antiestrogenic properties in the hERα transactivation assay with 10–10 M E2. As shown in Figure 3A, 10–5 M of these pesticides significantly inhibited the estrogenic response by 10–11 M E2 as did the well-known...
ER antagonist tamoxifen (10⁻⁸ and 10⁻⁷ M). The RIC₂₀ values of cyhalothrin, deltamethrin, alachlor, pyrazoxyn, triflumizole, and tamoxifen for hERα were 9.0 × 10⁻⁶ M, 8.1 × 10⁻⁶ M, 4.5 × 10⁻⁶ M, 6.0 × 10⁻⁶ M, 9.8 × 10⁻⁶ M, and 3.2 × 10⁻⁷ M, respectively.

In the hERα transactivation assay of the 200 tested pesticides, only methoxychlor and pyrazoxyfen were antiestrogenic. As shown in Figure 3B, 10⁻⁵ M methoxychlor or pyrazoxyfen inhibited by more than 20% the estrogenic activity induced by 10⁻⁶ M E₂. In this assay, tamoxifen also showed antiestrogenic activities at concentrations of 10⁻⁸ and 10⁻⁷ M. The RIC₂₀ values of methoxychlor, pyrazoxyfen, and tamoxifen for hERα were 9.0 × 10⁻⁶ M, 7.8 × 10⁻⁶ M, and 6.0 × 10⁻⁹ M, respectively.

Androgenic effects of the pesticides. None of the pesticides tested showed androgenic transcriptional activity of more than 20% that induced by 10⁻⁹ M DHT at the tested concentrations (data not shown).

Antiandrogenic effects of the pesticides. We tested 200 pesticides for their inhibitory effect on the androgenic activity induced by DHT (10⁻¹⁰ M). The RIC₂₀ values and relative luciferase activities (RLA) of 66 pesticides evaluated as having an inhibitory effect are summarized in Table 4. In particular, 13 pesticides (α, p′DDE, p,DDE, p,p′DDT, chlorophenyl, CNP, chlorothion, nitrofen, CNP-ami, oxyfluorfen, fenitrothion, vinclozolin, procyomidine, and bromopropophylate) showed a potent antiandrogenic effect with RIC₂₀ < 10⁻⁶ M. Among these active pesticides, the RIC₂₀ of two diphenyl ether-type herbicides, CNP and chlorothion, were 4.3 × 10⁻⁸ M and 6.8 × 10⁻⁸ M, respectively, which is distinctly more potent than known AR antagonists such as vinclozolin and p,p′-DDE (1.6 × 10⁻⁷ M and 6.5 × 10⁻⁷ M, respectively). In addition, weak antiandrogenic effects with RIC₂₀ > 10⁻⁶ M were found in 53 pesticides: 10 organochlorines, 2 diphenyl ethers, 18 organophosphorus pesticides, 4 pyrethroids, 2 carbamates, 3 acid amides, 5 ureas, and 9 others.

Both ER agonists and AR antagonists in the pesticides. Table 5 summarizes the 34 pesticides exhibiting dual activities as ER agonists and AR antagonists. Among these pesticides, organochlorine and organophosphorus pesticides were predominant. Above all, α, p′-DDE and CNP-ami were the most potent pesticides, having both estrogenic and antiandrogenic activities. Cytotoxicity was not observed for any of the tested compounds at the selected dose range (data not shown).

Discussion
To our knowledge, there are two reports on the screening of the endocrine-disrupting effects of a large number of chemicals: on estrogenic activity of 514 chemicals using a yeast two-hybrid assay (Nishihara et al. 2000) and on binding ability of 188 chemicals to rat ER using a competitive binding assay (Blair et al. 2000). However, in these studies 118 pesticides of 514 chemicals and 20 pesticides of 188 chemicals showed little estrogenic activity or little binding ability to ER, respectively. In addition, there is no report on screening of ERβ-mediated estrogenic activity from a large number of chemicals. We previously developed highly sensitive and specific reporter gene assays for ERα and AR (Kojima et al. 2003), and in the present study we established the ERβ assay by constructing the hERβ expression plasmid pcDNAhERβ in our screening of 200 pesticides for their estrogenicity via hERα/β and androgenicity via hAR. As a result, we found estrogenic activity for hERα in 47 pesticides and for hERβ in 33 pesticides, and antiandrogenic activity for hERα and hERβ in five and two pesticides, respectively. In the AR assay, although none of the tested pesticides showed AR agonistic activity, 66 of the 200 pesticides surprisingly showed antiandrogenic activities. Thus, a number of pesticides were newly found to possess ER agonistic and/or AR antagonistic activities in addition to the pesticides already reported to be estrogenic and antiandrogenic. This suggests that our reporter gene assays are highly sensitive and specific.

We classified 200 pesticides into nine groups according to their chemical structure:
organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, uracils, and others. Organochlorine-type pesticides should be of the most concern among the nine groups of pesticides suggested as candidates to be endocrine disruptors, because of their global distribution by widespread use and bioaccumulation through the ecosystem by high lipophilic property (Kutz et al. 1991; Simonich and Hites 1995). Several of these compounds (DDT, methoxychlor, BHC, endosulfan, and dieldrin) have been reported to possess estrogenic activity by studies with animals and cells (Bulger et al. 1978; Coosen and Velsen 1989; Soto et al. 1995; Welch et al. 1969). We have also demonstrated that o,p′-DDT, β-BHC, methoxychlor, α-endosulfan, and cis-(trans)-chlordane exert transcriptional reporter activity via ERα and/or ERβ by our assays. In addition, we newly identified estrogenic organochlorine pesticides such as dicofol, chloropropylate, and chlorobenzilate, whose chemical structures resemble those of DDT and its isomer (Figure 4). Among BHC isomers, β-BHC is most prevalent in the fatty tissues because of its greater stability, lipophilicity, and accumulation potential (Dejonckheere et al. 1978). In the present study, β-BHC and δ-BHC exerted potent estrogenic activity especially via ERβ (Figure 2) but showed no effect in the androgen assay. Moreover, we also found 14 antiandrogenic pesticides among the organochlorine pesticides. The antiandrogenic properties of DDT isomers, methoxychlor, dieldrin, and endosulfan have already been reported (Andersen et al. 2002; Kelce et al. 1995; Maness et al. 1998), but in the present study several other pesticides were newly defined as AR antagonists. Although many organochlorine pesticides have weak hormonal activity, their lipophilic nature and long half-lives allow them to accumulate in the fatty tissues of the body, increasing their concentration and bioavailability.

Diphenyl ether pesticides are no longer used, but a few decades ago this type of chemical was extensively used as an herbicide due to its low cost and toxicity. We have reported that a diphenyl ether–type pesticide, CNP, and its amino derivative (CNP-amino) act as both ERα agonists and AR antagonists in vitro (Kojima et al. 2003). In the present study, we additionally found that CNP-amino, but not CNP, possessed an ERβ agonistic effect, and that except for fluazifop-butyl, which demonstrated the weak ERα agonistic activity, no other diphenyl ether–type pesticides showed estrogenic activity. This suggests that CNP-amino itself, rather than the structure of diphenyl ether, may contribute to the transactivation of ERs. Nevertheless, the greatest concern of diphenyl ether pesticides as endocrine-disrupting agents should reside in their potent AR antagonist activity. Recently, Tomura et al. (2001) demonstrated that a diphenyl ether herbicide, nitrofen, was antiandrogenic by transactivation assay and three-dimensional image analysis using COS-7 simian renal carcinoma cells. In the present study, seven of 11 diphenyl ether pesticides, including CNP, CNP-amino, and nitrofen showed antiandrogenic activity, and in

Figure 2. Dose–response curves of (A) β-BHC, (B) δ-BHC, and (C) methiocarb on hERα- and hERβ-mediated estrogenic activities. CHO cells were transiently cotransfected with pCDNAERα or pCDNAERβ, pGL3-tkERE, and pRL-SV40 as described in “Materials and Methods.” Cells were incubated with various concentrations of (A) β-BHC, (B) δ-BHC, or (C) methiocarb. Values represent the mean ± SD of three independent experiments and are presented as percentage induction, with 100% activity defined as the activity achieved with 10−10 M and 10−9 M E2 for ERα and ERβ, respectively.

Figure 3. Antagonistic effects of pesticides in the hERα and hERβ transactivation assays. (A) CHO cells, transiently cotransfected with pCDNAERα, pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10−5 M of cyhalothrin, deltamethrin, alachlor, pyrazoxyfen, or triflumizole in the presence of 10−11 M E2. Effect of ER antagonist tamoxifen (~10−9 to 10−7 M) was also measured as a positive control. Values represent the mean ± SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10−11 M E2. (B) CHO cells, transiently cotransfected with pCDNAERβ, pGL3-tkERE, and pRL-SV40, were incubated with the vehicle control (0.1% DMSO) or 10−5 M of methoxychlor or pyrazoxyfen in the presence of 10−10 M E2. Effect of ER antagonist tamoxifen (~10−4 to 10−3 M) was also measured as a positive control. Values represent the mean ± SD of three independent experiments and are presented as percent induction, with 100% activity defined as the activity achieved with 10−10 M E2.

*Significantly different (p < 0.05) from vehicle control (=100%).
particular, the antiandrogenic activities of CNP and chlormethoxyfen were more potent than those of known AR antagonists such as vinclozolin and p,p'-DDE. Chlormethoxyfen is structurally similar to CNP and nitrofen as well as the antiandrogen drug flutamide and the organophosphorus insecticide fenitrothion, all of which commonly contain nitrobenzene in the molecular structure (Figure 4). This may be the key point in screening AR antagonists from the multitude of chemicals (Kojima et al. 2003).

Organophosphorus pesticides are widely used in both agriculture and pest control. We found that a number of organophosphorus-type pesticides possess estrogenic and/or antiandrogenic activities. To date, it has been reported that tolclofos-methyl and quinalphos have estrogenic activities via ERα and methyl parathion have antiandrogenic activities via AR (Sohoni et al. 2001; Tamura et al. 2001). We also found that tolclofos-methyl and quinalphos have estrogenic activities via ERE and ERF, and that fenitrothion, parathion, and methyl parathion have antiandrogenic activities via AR. Moreover, here we provide new evidence that butamifos, prothiofos, lepthos, cyanofenox, ethion, bromophosethyl, O-ethyl O,p-nitrophenyl phenylphosphonothioate (EPN), and dieldrin induce EREt-mediated transcriptional activity at concentrations lower than that of quinalphos. In addition, we found that 19 organophosphorus pesticides, including fenitrothion, parathion, and methyl parathion, possess antiandrogenic activity. Thus, the similarity in chemical structure among these pesticides may be the primary cause for their estrogenicity and/or antiandrogenicity. Interestingly, organophosphorus pesticides displaying these effects commonly contain a thiophosphoryl residue (P = S), as shown in Figure 4, whereas pesticides having an oxophosphoryl residue (P = O) such as prothiofos oxon, tolclofos-methyl oxon, acephate, and propaphos show few effects. This indicates that the estrogenic and/or antiandrogenic activities of parent compounds may disappear through oxidizing metabolism in the environment or body.

Pyrethroid and organophosphorus pesticides are the pesticides used most in Japan and other countries. Several pyrethroid pesticides (fenvalerate, permethrin, and cypermethrin) have been reported to show estrogenic activities in MCF-7 cell proliferation and transactivation assays (Chen et al. 2002; Garey and Wolff 1998; Go et al. 1999). In contrast, Saito et al. (2000) reported that fenvalerate did not have estrogenic activity in vitro at a concentration of 10−5 M. In our assays, five pyrethroid pesticides including fenvalerate were shown to increase transcriptional activity via EREt, and two pyrethroid pesticides decreased EREt activity induced by 10−11 M E2. This suggests that several pyrethroid pesticides may act as weak ER agonists or antagonists. In addition, four pyrethroid pesticides were newly found to have weak antiandrogenic activity, and three of them (fenvalerate, flucytosine, and cyfluthrin; Figure 4) displayed pleiotropic effects via both EREt and AR.

Klotz et al. (1997) reported that several carbamate insecticides (e.g., carbaryl, methomyl, and other compounds) can inhibit aromatase and hAR activities of 200 pesticides.

Table 5. Thirty-four pesticides possessing both estrogenic and antiandrogenic activities in vitro.

| Pesticide | hERα | hERβ | hAR |
|-----------|------|------|-----|
| Group 1   | ↑    | —    | —   |
| Group 2   | ↑    | ↑    | —   |
| Group 3   | ↑    | ↑    | ↑   |
| Group 4   | ↑    | ↑    | ↑   |
| Group 5   | ↑    | ↑    | —   |
| Group 6   | ↑    | ↑    | ↑   |
| Group 7   | ↑    | ↑    | —   |
| Group 8   | ↑    | —    | —   |

Abbreviations: EPN, O-ethyl O,p-nitrophenyl phenylphosphonothioate; RLA, relative luciferase activity.

*Nine compound groups are listed in Table 1. Concentration of the test compound showing 20% inhibition of the androgenic activity achieved by 10−10 M DHT. Percentage response at a concentration of 10−5 M with 100% activity defined as the activity achieved with 10−8 M DHT.
oxamyl) decreased estrogen- or progesterone-responsive reporter genes at concentrations of 10^{-7} M in breast (MCF-7) and endometrial (Ishikawa) cancer cells. However, Andersen et al. (2002) reported that methomyl induced no significant effects in proliferation and ER transactivation assays using MCF-7 cells and that methiocarb showed both estrogenic and weak antiandrogenic properties. In our study, of the 22 carbamates tested only methiocarb (see Figure 4) showed both estrogenic and antiandrogenic activities, and other carbamate pesticides showed no ER or AR activity. Thus, our results support the evidence of Andersen et al. (2002) but not that of Klotz et al. (1997).

With regard to acid amide–type pesticides, only one relevant study was available. Vonier et al. (1996) reported the interaction of the herbicide alachlor with the estrogen receptors from the oviduct of the American alligator. They showed that this pesticide competed with E2 for binding to the ER, but the binding affinity was about 3,500-fold lower than that of E2. In our assays using hERs and hAR, alachlor showed both antiestrogenic activity via ERα and antiandrogenic activity. This suggests that alachlor can interact not only with alligator ER but also with hERα and hAR. Furthermore, among 13 acid amide pesticides, we newly found thenylchlor to have both estrogenic and antiandrogenic activities, and methametac to possess antiandrogenic activity.

Urea-type pesticides are mainly used as herbicides. Bauer et al. (1998) reported evidence that propanil (DCPA), linuron, and diuron in phenyl urea herbicide have the ability to bind to AR. In addition, recent reports have shown that linuron and prochloraz to be antiandrogenic in vitro and in vivo (Lambright et al. 2000; Vinggaard et al. 2002). In our AR assay, five urea-type herbicides (DCPA, pencycuron, linuron, prochloraz, and diuron) inhibited transcriptional activity by DHT, and the antiandrogenic activities of DCPA and pencycuron were more potent than those of linuron and prochloraz, which have been shown to be antiandrogenic in vivo. On chemical structure, these pesticides have some similarities (Figure 4). These suggest that DCPA and pencycuron would also show antiandrogenic activity in vivo and should therefore be considered endocrine disruptors.

Among the triazine-type pesticides, atrazine, which is the most widely used herbicide in the United States, has been reported to be an estrogen by yeast transactivation assay (Tran et al. 1996). In the present study, seven triazine-type pesticides were tested, but none showed any ER or AR activity. Friedmann (2002) recently reported that atrazine acts as an endocrine disruptor in rat males by directly inhibiting Leydig cell testosterone production. Hayes et al. (2002) hypothesize that atrazine induces aromatase and promotes the conversion of testosterone to estrogen in Xenopus laevis. Thus, this type of pesticide may exert hormonal activity through mechanisms other than those associated with ER and AR.

| Organochlorines | Pyrethroids |
|-----------------|-------------|
| ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |
| ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |
| Diphenyl ethers | Carbamates |
| ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |
| Chlornitrofen (CNP) | Nitenpyram |
| Chlornitrofen (CNP)-amino | Methiocarb |
| Chlorpropham | Prothiofos |
| Chloropyrifos | Tolclofos-methyl |
| Chlorobenzilate | EPN |
| Chlorobenzilate | Fenvalerate |

**Figure 4.** Chemical structures of some exemplar pesticides showing estrogenic and/or antiandrogenic activity.
In the present study, 44 pesticides could not be classified into the above eight groups could be collectively grouped as “others.” The dicarboxyimide fungicides vinclozolin and procyomide are structurally similar and showed potent antianandrogen effects. This result corresponded to those of other studies (Kelce et al. 1994; Orsby et al. 1999). In addition, bromopropylate, fenamiph, and pendimethalin showed both estrogenic and antianandrogenic properties (Figure 4). Because the chemical structures of bromopropylate and chloropropylate are similar to those of DDT isomers, it was thought that the two pesticides would have similar effects. The in vitro estrogenic and antianandrogenic effects of the fungicide fenamiph have been also described by Andersen et al. (2002). The effects of fenamiph in our assays were more potent than described in that study, likely because of the difference in sensitivity of the assay systems. The herbicide pendimethalin has not been reported as having endocrine-disrupting effects, and thus we are the first to demonstrate the effects of this pesticide.

To date, no AR agonists have been found among environmental chemicals, and in this study we also failed to isolate an AR agonist from among 200 pesticides tested but identified 66 antianandrogenic pesticides. In addition, although there are many ER agonistic pesticides, we also find quite a few pesticides with antiestrogenic properties. This phenomenon is, as Sohoni and Sumpter (1998) pointed out, quite enigmatic. Furthermore, we demonstrated that a lot of pesticides possessed both estrogenic and antianandrogenic activities. Taken together, most of these chemical compounds may act as ER agonists and/or AR antagonists in the environment, a situation leading to feminization in animals.

Our experiments demonstrate that many pesticides possess in vitro estrogenic and antianadrogenic activities through ERs and/or AR. Although it appears that various pesticides exert hormonal effects at concentration-orders of magnitude higher than that required for physiologic hormones, wide exposure to large numbers of these pesticides may have additive and synergistic effects.

The first aim of this study was the comprehensive evaluation of 200 pesticides for their estrogenic and androgenic activity. In fact, we found it in a number of pesticides. This is an important point in identifying endocrine disruptors from the multitude of chemicals commonly in use. We herein propose that many compounds should be tested using the same method and under the same conditions to prevent confusion resulting from the use of different methods, and an international agreement should be reached for this purpose.

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