— Minireview —

Endocrine-disrupting Potential of Pesticides via Nuclear Receptors and Aryl Hydrocarbon Receptor

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Nuclear receptors (NRs) and the aryl hydrocarbon receptor (AhR) form a ligand-dependent transcription factor that regulates the genes involved in key physiological functions such as cell growth and differentiation, development, homeostasis, and metabolism. These receptors are potential targets of endocrine-disrupting chemicals (EDCs). To date, many studies have shown that EDCs, such as plasticizers, pesticides, and dioxins, can function as ligands of NRs and AhR. In this review, we focus on recent studies showing that a variety of pesticides, intentionally released into the environment, have agonistic and/or antagonistic activity against NRs and AhR, and present our transactivation assay-based screening results for 200 pesticides against estrogen receptors (ERs), androgen receptor (AR), thyroid hormone receptors (TRs), pregnane X receptor (PXR), peroxisome proliferator-activated receptors (PPARs), and AhR. Our studies have shown that a number of pesticides possess ERα, ERβ, and PXR agonistic activity as well as AR antagonistic activity, whereas none of the pesticides affect the TRα_1, TRβ_1, and PPARγ-mediated signaling pathways. In addition, several of the 200 tested pesticides were found to have PPARα and AhR agonistic, and ERα and ERβ antagonistic activity. Although the activities of each of these compounds were weak compared to those of endogenous hormone or dioxins, the endocrine-disrupting potential of pesticides, particularly those which function against ERα/β, AR, and PXR, may reflect that of numerous environmental chemicals.

Key words —— endocrine-disrupting chemical, pesticide, nuclear receptor, aryl hydrocarbon receptor, transactivation assay

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are exogenous compounds that have the potential to interfere with hormonal regulation and the normal endocrine system, thereby affecting the health of animals and humans.[1,2] Many EDCs are man-made chemicals that are released into the environment; for example, phthalates[3] or bisphenol-A[4] plasticizers, organotins,[5] pesticides,[6] polychlorinated biphenyls (PCBs),[7] dioxins (polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and dioxin-like PCBs),[8] flame retardants,[9] and alkylphenols.[10] Some naturally occurring EDCs can also be found in plants or fungi, such as the so-called phytoestrogens,[11] genistein and daizein, or the mycoestrogen zearalenone.

EDCs can affect an organism's endocrine system in various ways, including the mimicking of endogenous hormones, antagonizing their action or modifying their synthesis, metabolism, and transport. A well-known mechanism of endocrine disruption is the direct interaction of chemicals, acting as receptor agonists or antagonists, with nuclear hormone receptors, thereby altering endocrine function. In particular, a number of studies have focused on the estrogen receptors (ERs), the androgen receptor (AR), and the thyroid hormone receptors (TRs), which are members of the nuclear receptor (NR) family,[12] as well as on the non-NR family aryl hydrocarbon receptor (AhR). However, recent studies have shown that several environmental chemicals affect hormone metabolism and synthesis by regulating their related enzymes, cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), or sulfotransferase, as activators of other NRs such as the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the peroxisome

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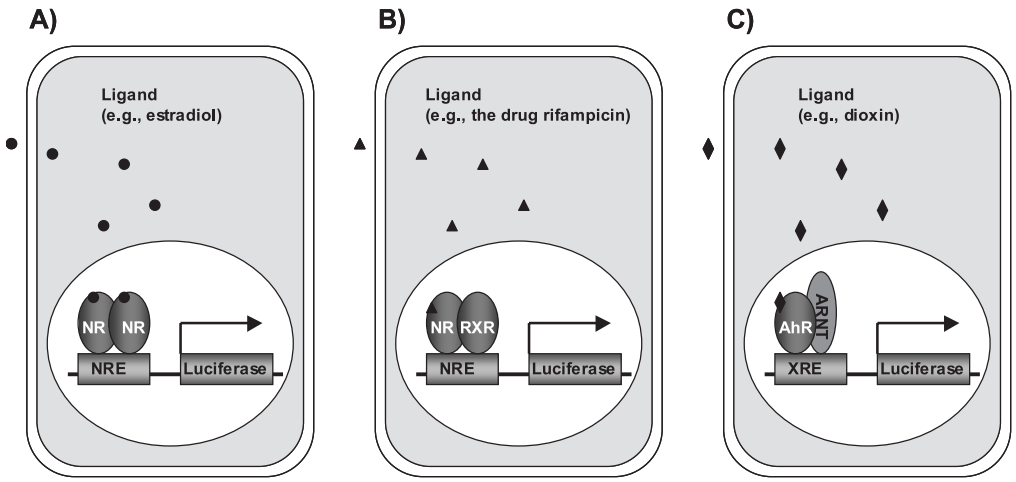

Fig. 1. *In vitro* Cell-based Transactivation Assays of Nuclear Receptors and AhR

A) CHO cells were transiently transfected with an NR expression plasmid as well as reporter plasmid coded with an NR response element (NRE). Upon ligand binding, NR forms a homodimer with another NR, binds to NRE on the DNA, and induces transcription of the luciferase gene. B) COS-7 or CV-1 cells were transiently transfected with an NR expression plasmid as well as a reporter plasmid coded with NRE. Upon ligand binding, NR forms a heterodimer with the retinoid X receptor (RXR), binds to NRE, and induces transcription of the luciferase gene. C) Hepa1c1c7 cells were stably transfected with a reporter plasmid coded with xenobiotic response element (XRE). Upon ligand binding, AhR forms a complex with AhR nuclear translocator (ARNT), binds to XRE on the DNA, and induces transcription of the luciferase gene.

proliferator-activated receptors (PPARs).[13, 14)]

Among the many EDCs, pesticides are used globally to control agricultural and indoor pests, and are intentionally released into the environment in large quantities. In addition, pesticides possess a wide variety of chemical structures and may represent the most likely candidates for ligands against NRs and AhR. In this minireview, we will briefly provide recent evidence, including our data obtained using *in vitro* transactivation assays (Fig. 1), regarding the potential effects of various pesticides via NRs and AhR, and predict the endocrine-disrupting potential of numerous environmental chemicals on the basis of the receptor-mediated effects of pesticides.

EFFECTS OF PESTICIDES VIA ERs, AR, AND TRs

There are a number of reports on the nuclear hormone receptor agonistic and antagonistic activities of pesticides based on various transactivation assays using mammalian and yeast cells. For example, *p*,*p*′-dichlorodiphenyl trichloroethane (DDT), methoxychlor, β-benzene hexachloride (BHC), endosulfan, toxaphene, dieldrin, and fenvalerate have been reported to have estrogenic activity via ER.[15–20)] In addition, several pesticides or their metabolites, such as vinclozolin, procymidone, linuron, fenitrothion, fenthion, and *p*,*p*′-dichlorodiphenyl dichloroethylene (DDE), have been reported to have antiandrogenic activity via AR.[21–27)] Andersen *et al.*[28)] reported that several currently used pesticides, such as methiocarb, fenarimol, chloropyrifos, deltamethrin, and tolclofos-methyl, possess estrogenic activity, whereas dieldrin, endosulfan, methiocarb, and fenarimol possess antiandrogenic activity. 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 through these hormone receptors. However, many compounds were independently evaluated for ER or AR activity using different assay systems. This may lead to some limitations to the accurate evaluation of their potential as EDCs.

In order to clarify this problem, we tested 200 pesticides, including several of their isomers and metabolites, for agonism and antagonism to two human ER subtypes, ERα and ERβ, and a human AR by highly sensitive and specific transactivation assays using Chinese hamster ovary (CHO) cells (Fig. 1A).[29, 30)] The test pesticides were selected according to the frequency of their use in Japan and other countries, both currently and in the past, and were classified into nine groups according to their chemical structure: 29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides,

Table 1. Transactivation Assay-based Screening of Agonists (ag) and/or Antagonists (ant) against NRs and AhR among 200 Pesticides

Pesticide name	hERα[30]		hERβ[30]		hAR[30]		hPXR[31]	mPPARα[32]	mAhR[33]
	ag	ant	ag	ant	ag	ant	ag	ag	ag
Organochlorines (29)									
Aldrin	+	−	−	−	−	−	+	−	−
α-BHC	−	−	+	−	−	−	+	−	−
β-BHC	++	−	++	−	−	−	+	−	−
γ-BHC	−	−	+	−	−	−	+	−	−
δ-BHC	+	−	+	−	−	−	+	−	−
Captan	−	−	−	−	−	−	−	−	−
cis-Chlordane	+	−	+	−	−	+	+	−	−
trans-Chlordane	+	−	+	−	−	+	+	−	−
Chlorobenzilate	+	−	−	−	−	+	+	−	−
Chloropropylate	+	−	+	−	−	++	+	−	−
Chlorothalonil	−	−	−	−	−	−	−	−	−
o,*p*′-DDT	+ + +	−	++	−	−	++	+	−	−
p,*p*′-DDT	+	−	+	−	−	++	+	−	−
p,*p*′-DDE	+	−	+	−	−	++	+	−	−
p,*p*′-DDD	+	−	+	−	−	+	+	−	−
Dichlobenil	−	−	−	−	−	−	−	−	−
Dicofol	+	−	+	−	−	+	+	−	−
Dieldrin	+	−	−	−	−	+	+	−	−
α-Endosulfan	++	−	+	−	−	+	+	−	−
β-Endosulfan	+	−	+	−	−	+	+	−	−
Endosulfan sulfate	+	−	+	−	−	−	+	−	−
Endrin	+	−	−	−	−	−	+	−	−
Folpet	−	−	−	−	−	−	−	−	−
Fthalide	−	−	−	−	−	−	−	−	−
Heptachlor	−	−	+	−	−	−	+	−	−
Heptachlor epoxide	+	−	+	−	−	++	+	−	−
Methoxychlor	++	−	−	+	−	+	+	−	−
Pentachlorophenol	−	−	−	−	−	−	−	−	−
Quintozene	−	−	−	−	−	−	+	−	−
Diphenyl ethers (11)									
Acifluorfen	−	−	−	−	−	−	−	−	−
Acifluorfen-methyl	−	−	−	−	−	+	+	−	+
Bifenox	−	−	−	−	−	+	+	−	+
Chlomethoxyfen	−	−	−	−	−	+ + +	+	−	−
Chlornitrofen	+	−	−	−	−	+ + +	+	−	−
Chlornitrofen-amino	++	−	++	−	−	++	−	−	−
Chloroxurone	−	−	−	−	−	−	−	−	−
Dichlofop-methyl	−	−	−	−	−	−	−	+	−
Fluazifop-butyl	−	−	−	−	−	−	−	−	−
Nitrofen	−	−	−	−	−	++	+	−	−
Oxyfluorfen	−	−	−	−	−	++	+	−	−
Organophosphorus (56)									
Acephate	−	−	−	−	−	−	−	−	−
Anilofos	−	−	−	−	−	+	+	−	−
Bromophos-ethyl	+	−	+	−	−	+	+	−	−
Bromophos-methyl	+	−	+	−	−	−	−	−	−
Butamifos	++	−	−	−	−	+	++	−	−
Chlorpyrifos	+	−	−	−	−	−	+	−	+
Chlorpyrifos-methyl	−	−	−	−	−	−	−	−	−
Cyanofenphos	+	−	+	−	−	−	+	−	−
Cyanophos	−	−	−	−	−	+	−	−	−
Diazinon	−	−	−	−	−	−	−	−	−

Table 1. Continued.

Pesticide name	hERα[30]		hERβ[30]		hAR[30]		hPXR[31]	mPPARα[32]	mAhR[33]
	ag	ant	ag	ant	ag	ant	ag	ag	ag
Dichlorfenthion	+	−	+	−	−	+	+	−	−
Dichlorvos	−	−	−	−	−	−	−	−	−
Dimethoate	−	−	−	−	−	−	−	−	−
Dioxabenzofos	−	−	−	−	−	−	−	−	−
Disulfoton	−	−	−	−	−	−	−	−	−
EPN[a]	+	−	+	−	−	+	+	−	−
Edifenphos	−	−	−	−	−	−	−	−	−
Ethion	+	−	+	−	−	+	++	−	−
Ethoprophos	−	−	−	−	−	−	−	−	−
Fenamiphos	−	−	−	−	−	−	+	−	−
Fenchlorphos	−	−	−	−	−	−	−	−	−
Fenitrothion	−	−	−	−	−	++	−	−	−
Fenitrothion oxon	−	−	−	−	−	+	−	−	−
Fensulfothion	−	−	−	−	−	−	−	−	−
Fenthion	−	−	−	−	−	+	−	−	−
Glyphosate	−	−	−	−	−	−	−	−	−
Iprobenfos	−	−	−	−	−	−	+	−	−
Isofenphos	+	−	−	−	−	+	++	−	−
Isoxathion	+	−	−	−	−	−	+	−	+
Leptophos	+	−	+	−	−	+	+	−	−
Malathion	−	−	−	−	−	−	−	−	−
Mecarbam	−	−	−	−	−	−	+	−	−
Methamidophos	−	−	−	−	−	−	−	−	−
Methidathion	−	−	−	−	−	−	−	−	−
Methyl-parathion	−	−	−	−	−	+	−	−	−
Monocrotophos	−	−	−	−	−	−	−	−	−
Parathion	−	−	−	−	−	+	+	−	−
Phenthoate	+	−	−	−	−	−	+	−	−
Phorate	−	−	−	−	−	−	−	−	−
Phosalone	−	−	−	−	−	+	+	−	−
Phosmet	−	−	−	−	−	−	−	−	−
Piperophos	−	−	−	−	−	+	++	−	−
Pirimiphos-methyl	+	−	−	−	−	−	−	−	−
Profenofos	−	−	−	−	−	−	−	−	−
Propaphos	−	−	−	−	−	−	+	−	−
Prothiofos	+	−	+	−	−	+	+	−	−
Prothiofos oxon	−	−	−	−	−	−	−	−	−
Pyridaphenthion	−	−	−	−	−	−	+	−	−
Quinalphos	+	−	+	−	−	+	+	−	+
Terbufos	−	−	−	−	−	−	+	−	−
Tetrachlorvinphos	−	−	−	−	−	−	+	−	−
Thiometon	−	−	−	−	−	−	−	−	−
Tolclofos-methyl	+	−	+	−	−	+	+	−	−
Tolclofos-methyl oxon	−	−	−	−	−	−	−	−	−
Trichlorfon	−	−	−	−	−	−	−	−	−
Vamidothion	−	−	−	−	−	−	−	−	−
Pyrethroids (12)									
Cyfluthrin	+	−	−	−	−	+	+	−	−
Cyhalothrin	−	+	−	−	−	−	++	−	−
Cypermethrin	+	−	−	−	−	−	+	−	−
Deltamethrin	−	+	−	−	−	−	+	−	−
Etofenprox	−	−	−	−	−	+	+	−	−
Fenvalerate	+	−	−	−	−	+	+	−	−

Table 1. Continued.

Pesticide name	hERα[30]		hERβ[30]		hAR[30]		hPXR[31]	mPPARα[32]	mAhR[33]
	ag	ant	ag	ant	ag	ant	ag	ag	ag
Flucythrinate	+	−	−	−	−	+	++	−	−
Fluvalinate	−	−	−	−	−	−	+	−	−
Permethrin	+	−	−	−	−	−	+	−	−
Pyrethrin	−	−	−	−	−	−	++	+	−
Tefluthrin	−	−	−	−	−	−	+	−	−
Tralomethrin	−	−	−	−	−	−	+	−	−
Carbamates (22)									
Bendiocarb	−	−	−	−	−	−	−	−	−
Benomyl	−	−	−	−	−	−	−	−	−
Carbaryl	−	−	−	−	−	−	−	−	−
Carbendazim	−	−	−	−	−	−	−	−	−
Carbofuran	−	−	−	−	−	−	−	−	−
Chlorpropham	−	−	−	−	−	−	+	−	+
Diethofencarb	−	−	−	−	−	−	+	−	+
Dimepiperate	−	−	−	−	−	−	+	−	−
Esprocarb	−	−	−	−	−	−	++	−	−
Ethiofencarb	−	−	−	−	−	−	−	−	−
Fenobucarb	−	−	−	−	−	−	−	−	−
Isoprocarb	−	−	−	−	−	−	−	−	−
Methiocarb	+	−	++	−	−	+	−	−	−
Methomyl	−	−	−	−	−	−	−	−	−
Molinate	−	−	−	−	−	−	−	−	−
Oxamyl	−	−	−	−	−	−	−	−	−
Phenmedipham	−	−	−	−	−	−	−	−	−
Pirimicarb	−	−	−	−	−	−	−	−	−
Pyributicarb	−	−	−	−	−	−	+ + +	−	−
Thiobencarb	−	−	−	−	−	+	++	−	−
Thiobencarb sulfon	−	−	−	−	−	−	−	−	−
Thiram	−	−	−	−	−	−	−	−	−
Acid amides (12)									
Alachlor	−	+	−	−	−	+	+	−	−
Asulam	−	−	−	−	−	−	−	−	−
Cafenstrole	−	−	−	−	−	−	−	−	−
Flutolanil	−	−	−	−	−	−	+	−	−
Mefenacet	−	−	−	−	−	+	+	−	−
Mepronil	−	−	−	−	−	−	+	−	−
Metalaxyl	−	−	−	−	−	−	+	−	−
Metolachlor	−	−	−	−	−	−	++	−	−
Pretilachlor	−	−	−	−	−	−	+ + +	−	−
Propanil	−	−	−	−	−	+	−	−	+
Propyzamide	−	−	−	−	−	−	+	−	−
Thenylchlor	+	−	+	−	−	+	++	−	−
Triazines (7)									
Anilazine	−	−	−	−	−	−	−	−	−
Atrazine	−	−	−	−	−	−	−	−	−
Metribuzin	−	−	−	−	−	−	−	−	−
Prometon	−	−	−	−	−	−	+	−	−
Prometryn	−	−	−	−	−	−	−	−	−
Simazine	−	−	−	−	−	−	−	−	−
Simetryn	−	−	−	−	−	−	−	−	−
Ureas (7)									
Bensulfuron-methyl	−	−	−	−	−	−	−	−	−
Dymuron	−	−	−	−	−	−	++	−	−

Table 1. Continued.

Pesticide name	hERα[30]		hERβ[30]		hAR[30]		hPXR[31]	mPPARα[32]	mAhR[33]
	ag	ant	ag	ant	ag	ant	ag	ag	ag
Diflubenzuron	−	−	−	−	−	−	−	−	−
Diuron	−	−	−	−	−	+	+	−	++
Linuron	−	−	−	−	−	+	+	−	++
Pencycuron	−	−	−	−	−	+	+	−	−
Prochloraz	−	−	−	−	−	+	+	−	++
Others (44)									
Amitraz	−	−	−	−	−	−	−	−	−
Benfuresate	−	−	−	−	−	−	+	−	−
Bentazone	−	−	−	−	−	−	−	−	−
Benzoximate	−	−	−	−	−	−	−	−	−
Biphenyl	−	−	−	−	−	−	−	−	−
Bitertanol	−	−	−	−	−	+	+	−	−
Bromopropylate	+	−	+	−	−	++	++	−	−
Chinomethionat	−	−	−	−	−	−	−	−	−
Chloridazon	−	−	−	−	−	−	−	−	−
Dazomet	−	−	−	−	−	−	−	−	−
Diquat	−	−	−	−	−	−	−	−	−
Ethoxyquin	−	−	−	−	−	+	+	−	−
Fenarimol	+	−	+	−	−	+	+	−	−
Ferimzone	−	−	−	−	−	−	+	−	−
Fluazinam	−	−	−	−	−	−	−	−	−
Imazalil	−	−	−	−	−	+	+	+	−
Imidacloprid	−	−	−	−	−	−	−	−	−
Iminoctadine	−	−	−	−	−	−	−	−	−
Indanofan	−	−	−	−	−	−	++	−	−
Ioxynil	−	−	−	−	−	−	−	−	−
Iprodione	−	−	−	−	−	−	−	−	−
Isoprothiolane	−	−	−	−	−	−	+	−	−
Lenacil	−	−	−	−	−	−	−	−	−
MCPA[b]	−	−	−	−	−	−	−	−	−
2,4-D[c]	−	−	−	−	−	−	+	−	−
Paraquat	−	−	−	−	−	−	−	−	−
Pendimethalin	+	−	+	−	−	+	+	−	−
2-Phenylphenol	−	−	−	−	−	+	+	−	−
Probenazole	−	−	−	−	−	−	−	−	−
Procymidone	−	−	−	−	−	++	+	−	−
Propiconazole	−	−	−	−	−	+	+	−	−
Pyrazolynate	−	−	−	−	−	−	−	−	−
Pyrazoxyfen	−	+	−	+	−	+	+	−	−
Pyroquilon	−	−	−	−	−	−	−	−	−
Sethoxydim	−	−	−	−	−	−	−	−	−
Thiabendazole	−	−	−	−	−	−	−	−	−
Thiocyclam	−	−	−	−	−	−	−	−	−
Thiophanate-methyl	−	−	−	−	−	−	−	−	−
Triadimefon	−	−	−	−	−	−	++	−	−
Tricyclazole	−	−	−	−	−	−	−	−	−
Triflumizole	−	+	−	−	−	+	+	−	−
Trifluralin	−	−	−	−	−	−	+	−	−
Triforine	−	−	−	−	−	−	+	−	−
Vinclozolin	−	−	−	−	−	++	−	−	−

a) *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate. *b*) 4-Chloro-*o*-toloxyacetic acid. *c*) 2,4-Dichlorophenoxyacetic acid.

Symbols: + + +, REC_{20} or $RIC_{20} \leq 1 \times 10^{-7}$ M; ++, 1×10^{-7} M < REC_{20} or $RIC_{20} \leq 1 \times 10^{-6}$ M; +, 1×10^{-6} M < REC_{20} or $RIC_{20} \leq 1 \times 10^{-5}$ M; −, negative.

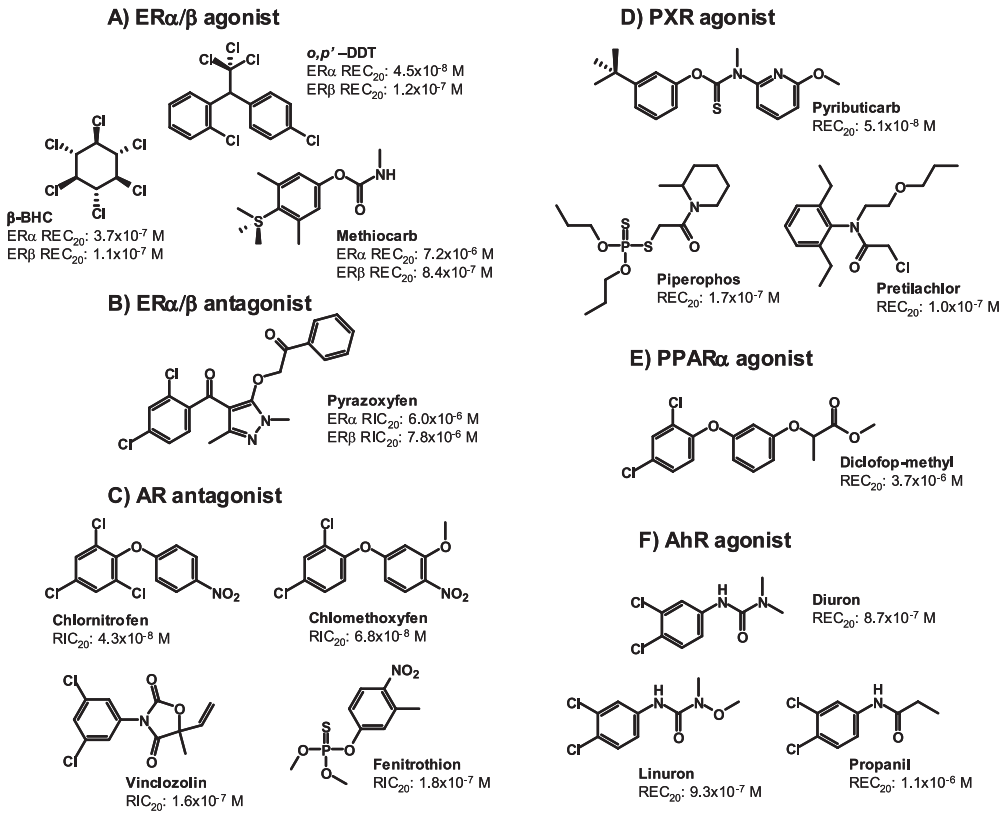

Fig. 2. Chemical Structures of Representative Pesticides Showing Agonistic or Antagonistic Activity against NRs and AhR

REC_{20} (20% relative effective concentration) is represented as the concentration of the test compound showing 20% of maximum agonistic activity of a typical agonist. RIC_{20} (20% relative inhibitory concentration) is represented as the concentration of the test compound showing 20% inhibition of the agonistic activity induced by an endogenous hormone.

12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 7 ureas, and 44 others. As shown in Table 1, of the 200 pesticides tested, 47 and 33 showed human (h)ERα- and hERβ-mediated estrogenic activities, respectively.[30] Among them, 29 pesticides had both hERα and hERβ agonistic activities. In this study, o,p'-DDT and β-BHC (Fig. 2A) were shown to have more potent estrogenic activity via both receptors than any other tested pesticide. In addition, the effects of β-BHC, δ-BHC, and methiocarb (Fig. 2A) were predominantly hERβ rather than hERα agonistic. Weak antagonistic effects toward hERα and hERβ were shown in five and two pesticides, respectively. Among these pesticides, pyrazoxyfen (Fig. 2B) showed antiestrogenic activities via both hERα and hERβ. On the other hand, none of the tested pesticides showed any hAR-mediated androgenic activity, whereas 66 of the 200 pesticides exhibited inhibitory effects against 5α-dihydrotestosterone-induced transcriptional activity (Table 1).[30] In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlornitrofen and chlomethoxyfen, were higher than those of vinclozolin, fenitrothion, and p,p'-DDE, well-known AR antagonists (Fig. 2C). In addition, the results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, supporting the notion of pleiotropic effects on hER and hAR suggested by Sohoni and Sumpter.[34]

Compared with ERs and AR, very little is known regarding the ability of pesticides to bind to the TRs. Although we also examined the agonistic and antagonistic activity of the 200 pesticides against human TRα_1 and TRβ_1 by transactivation assays using CHO cells (Fig. 1A), we couldn't find any TR activity in these pesticides (unpublished data). The ligand-binding pockets of TRs have been shown to be internal and a tight fit for the thyroid hormone.[35] Therefore, candidate chemicals that could potentially bind to the TRs may be those with a structure closely resembling the thyroid hormone; *e.g.*, some hydroxylated PCBs[36] and polybrominated flame retardants.[9,37] On the other hand, it was reported that a number of environmental chemicals, including pesticides, could alter thyroid function by acting as thyroid disruptors through

altering the synthesis, transport or metabolism of the thyroid hormone, rather than through interaction with the TR.[38,39]

EFFECTS OF PESTICIDES VIA PXR, CAR, AND PPARs

Both PXR and CAR are important regulators of several steroid and xenobiotic detoxification enzymes (*e.g.*, CYP3A, CYP2B, and UGT1A) as well as transporters in the liver and intestines,[40,41] and their signaling pathways can alter the bioavailability of endogenous steroid and thyroid hormones.[13,39,42] PXR, like most NRs, activates transcription upon ligand binding. On the other hand, the first step of CAR activation by drugs and xenobiotics is the induction of cytosolic CAR nuclear translocation, which is considered to have two mechanisms: one is dependent on the direct binding of a ligand such as 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) for mouse CAR,[43] and the other relies on the translocation of CAR into the nucleus through a dephosphorylation cascade by chemicals such as phenobarbital (PB),[44] thereby inducing target genes. Activation of PXR and CAR by xenobiotics can increase the level of endocrine-disrupting metabolites while at the same time altering the local bioavailability of endogenous androgens, estrogens and thyroid hormones. This provides a route through which EDCs can alter steroid and thyroid hormone receptor activity without directly binding to their receptors.

Recent studies have reported that many pesticides induce transcriptional activity via PXR.[45–49] For example, some organochlorine pesticides (chlordane, dieldrin, and endosulfan) were found to be hPXR agonist in transactivation assays using human hepatocarcinoma HepG2 and breast carcinoma MCF-7 cells.[45] It has been reported that 30 pesticides, including organochlorine and pyrethroids, demonstrated hPXR-mediated activity in experiments using a stable reporter cell system.[46,47] In addition, Matsubara *et al.*[48] found that several pesticides induce CYP3A4 via hPXR, with the herbicide pyributicarb, in particular, being a potent activator of the CYP3A4 gene. On the other hand, Mikamo *et al.*[49] reported that some pesticides, including methoxychlor, activated PXR in a yeast two-hybrid assay and induced CYP3A1 mRNA in the rat liver. We have recently examined the hPXR agonistic activity of 200 pesticides in a transactivation assay using COS-7 simian kidney cells (Fig. 1B). Surprisingly, 106 of the 200 tested pesticides, which is more than those showing ER or AR activities, showed hPXR-mediated transcriptional activity.[31] These 106 pesticides included not only organochlorines and pyrethroids but representatives from all groups classified (Table 1). In particular, pyributicarb, pretilachlor, and piperophos (Fig. 2D) were potent hPXR activators. Taken together, these results show that many pesticides have PXR agonistic activity, indicating that PXR can bind structurally diverse ligands due to the large size (≥ 1200 Å^3) of its ligand-binding domain relative to other NRs.[50]

The CAR ligand-binding pocket is smaller and less flexible than that of PXR and, therefore, CAR is less promiscuous than PXR.[40,41] Actually, the list of chemicals known to activate CAR is much shorter than that of known PXR activators.[40] Some studies have reported that methoxychlor[51,52] and DDT[51,53] induce CAR-mediated transcriptional activation, and more recent studies have indicated that several pesticides, including cypermethrin and chlorpyrifos, activate CAR.[54] In addition, metofluthrin has been shown to induce CYP2B1 through CAR activation.[55] However, these CAR-activating pesticides may work through an indirect pathway as well as PB, not as TCPOBOP. Our study of 200 pesticides using a hCAR-mediated transactivation assay is currently ongoing.

PPARs are ligand-dependent transcription factors and key regulators of lipid metabolism and cell differentiation. Three subtypes have been identified and designated as PPARα, PPARγ and PPARβ/δ on the basis of their differential tissue distributions and biological functions.[56] The ligands for PPARs include endogenous compounds, such as fatty acids, as well as synthetic compounds, such as fibrates (hypolipidemic drugs) and thiazolidinedions (antidiabetic drugs). With regard to endocrine disruption by PPAR activation, mono-(2-ethylhexyl) phthalate (MEHP) and thiazolidinedion troglitazone, which act as PPARα or PPARγ agonists, have been reported to inhibit the gene expression of aromatase (CYP19), the enzyme of which catalyzes the conversion of testosterone to estrogen.[14,57] These studies imply that environmental contaminants possessing PPAR agonistic activity might act through a PPARs-mediated signaling pathway to suppress estradiol levels, leading to endocrine system dysfunction. On the other hand,

there have been few studies on pesticides that might interact with these receptors. Therefore, we characterized the mouse PPARα and PPARγ agonistic activities of 200 pesticides by transactivation assays using CV-1 simian kidney cells (Fig. 1B). Only three of the 200 pesticides, diclofop-methyl, pyrethrins and imazalil, all of which have different chemical structures, showed PPARα-mediated transcriptional activity (Table 1).[32] In particular, diclofop-methyl (Fig. 2E) induced levels of CYP4A (a target gene of PPARα) mRNAs similar to those induced by WY-14643, a potent PPARα agonist, in the liver of mice intraperitoneally injected with these compounds. However, none of the 200 pesticides showed PPARγ agonistic activity (Table 1).[32] Thus, this screening result suggests that most of the tested pesticides do not interact with PPARs despite many of them showing interactions with steroid hormone receptors such as ERs and AR.

EFFECTS OF PESTICIDES VIA AhR

AhR is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim family.[58] Many toxicological studies have reported that the interaction of AhR ligand with this receptor is involved in a wide variety of toxic and biological effects, such as birth defects, immunotoxicity, neurotoxicity, lethality, tumor promotion, and enzyme (*e.g.*, CYP1A1/2 and CYP1B1) induction.[58] The representative ligands for AhR are dioxins, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), having a very high affinity for this receptor and, therefore, AhR is a so-called dioxin-receptor. Environmental chemicals including some pesticides have been identified as AhR agonists, a group that consists of structurally diverse chemicals that are relatively weak inducers of CYP1A1 and/or have a markedly lower affinity for AhR than TCDD.[59] Long *et al.*[60] reported that 8 of 23 pesticides currently used in Denmark revealed AhR-mediated activity in human and rat hepatoma cell lines. In addition, a carbamate-type insecticide, carbaryl,[61,62] and a urea-type herbicide, diuron,[63,64] were found to act as AhR agonists in AhR-mediated reporter gene assays using mammalian hepatoma cells or recombinant yeast cells.

Recently, we developed DR-EcoScreen, a cell line that stably expresses a AhR-responsive luciferase reporter gene construct into mouse hepatoma Hepa1c1c7 cells (Fig. 1C).[33] The reporter gene assay using this cell line (DR-assay) is highly sensitive and specific to dioxins, and can identify the AhR activity of various other chemicals. In addition, this DR-assay potentially affords an alternative to high-resolution gas chromatography/high-resolution mass spectrometry for the screening of dioxins, a task which currently requires expensive equipment and highly trained analysts. In fact, we demonstrated that this sensitive DR-assay was applicable to a simple monitoring system for the determination of dioxins in emission gas, ash and ambient air samples.[65,66] Using the DR-assay, we characterized the AhR agonistic activities of the 200 pesticides tested in the above NR assays. As a result, eleven (acifluorfen-methyl, bifenox, chlorpyrifos, isoxathion, quinalphos, chlorpropham, diethofencarb, propanil, diuron, linuron, and prochloraz) of the 200 pesticides were found to induce AhR-mediated transcriptional activity (Table 1).[33] In particular, three herbicides (diuron, linuron, and propanil) with a common chemical structure (Fig. 2F) showed more potent agonistic activity than did any other pesticides, and were also *in vivo* inducers of *CYP1A* genes in the mouse liver.

We have demonstrated that 88 of 200 pesticides have estrogenic, anti-estrogenic and/or anti-androgenic activities through ERα, ERβ and AR:[30] chlorpyrifos and isoxathion are estrogenic via ERα: acifluorfen-methyl, bifenox, propanil, diuron, linuron and prochloraz are antiandrogenic via AR: and it is interesting that quinalphos acts as both an ERα/β agonist and an AR antagonist (Table 1). Recently, it has been shown that AhR ligands both positively and negatively modulate ER and AR signaling pathways.[67,68] Notably, Ohtake *et al.*[69] have demonstrated that liganded AhR acts as an E3 ubiquitin ligase, and degradates ER and AR not only *in vitro*, but also *in vivo*. Collectively, these evidences suggest that the above pesticides with AhR agonistic activity may act, not only AhR agonists, but also as chemicals that affect ERs and AR function through direct receptor binding or multiple indirect receptor pathways via AhR.

CONCLUSIONS

The involvement of EDCs in the disruption of development, reproduction, and the immune and neural systems has been confirmed in a wide range of fish and animal species, whereas their role in humans remains controversial.[70] On the other hand,

the number of identified environmental antiandrogens keeps growing and these compounds show clear dose-additive effects, leading to concerns that a mixture of these chemicals could cause adverse effects even when each compound is present at a low concentration.[71] In the present paper, we focused on pesticides as they are the most likely candidates for EDCs. From our studies based on *in vitro* screening assays of 200 pesticides (Table 1), we showed that a variety of pesticides can affect the ERs-, AR-, PXR- and AhR-mediated signaling pathways. This implies that numerous other man-made chemicals as well as pesticides have shared these receptor activities. More recently, it has been reported that EDCs are possibly involved in the development of obesity and metabolic syndrome as a number of NRs and AhR are crucially involved in fat metabolism and glucose uptake.[13, 72, 73] Therefore, exposure to environmental chemicals, including pesticides having NR and AhR activities, not only increases reproductive problems but may also be an exacerbating factor in the development of other debilitating diseases such as metabolic syndrome. Although, to date, 48 members of the NR family have been identified in humans, about half of these are nuclear orphan receptors, the physiological roles and ligands of which are unclear. Thus, as all members of the NR family may be potential EDC targets, further studies including ligand-screening against nuclear orphan receptors are needed to fully elucidate the biological effects of a large number of environmental pollutants.

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