Introduction

The identification of environmental chemicals capable of binding to hormone receptors such as estrogen receptors (ERs) and androgen receptors (ARs), and interfering with their normal physiological functions, has heightened concern for adverse effects across species in the animal kingdom.1–7) Such environmental chemicals are known as endocrine disruptors. The Organization for Economic Co-operation and Development (OECD) defines endocrine disrupters as follows: an endocrine disrupter is an exogenous substance that causes adverse effects in an intact organism, or its progeny, consequent to changes in endocrine function. The OECD conceptual framework for the testing and assessment of endocrine disrupting chemicals is shown in Table 1. Only compounds causing adverse effects in level 5 study can be called endocrine disruptors. Others are known as endocrine active chemicals or substances. By pursuing the underlying mechanisms of endocrine active chemicals, their mechanisms are generally thought to be divided into two main categories. One is the direct interaction of a chemical with target steroid hormone receptors, such as ERs or ARs, to interfere with the ligand-dependent transcriptional function (receptor-mediated disruptors).8) The other is inhibition of the biosynthesis or

Endocrine disruptors that disrupt the transcription mediated by androgen receptor

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The Ministry of Environment in Japan (formerly the Japan Environment Agency) has made a priority list of compounds, SPEED 98, to preferentially examine whether they act as endocrine active chemicals and conducted a project to scientifically address endocrine disruptor issues. Out of around 100 compounds listed in SPEED 98 and related compounds, thirty-six acted as pure androgen receptor (AR) antagonists, whereas 13 showed both AR agonist and antagonist activities based on an in vitro reporter gene assay. The structural difference between AR agonists and antagonists was explained by Comparative Molecular Field Analysis (CoMFA). The precise structural requirements for AR agonists and/or antagonists are described as follows: in the case of AR agonist, the distance between two functional groups with H-bonding ability corresponding to 3-keto and 17β-OH groups should be near 10 Å to maintain a favorable H-bond position while the length axis of antagonists should be less than or more than 10 Å so as not to make an H-bond by interaction with Asn705 and Thr877 in the steroid D ring anchoring pocket, preventing the correct positioning of helix 3 (H3) and helix 12 (H12). This hypothetical general rule is named “Near 10 Å polar Interaction Rule”. © Pesticide Science Society of Japan

Keywords: androgen, endocrine disruptor, nuclear receptor, environmental hormone, CoMFA, MDA-kb2.
metabolism of endogenous ligands to indirectly modulate endocrine function (non-receptor-mediated disruptors). Since, year after year, thousands of new chemicals are discharged into the environment by industrial and domestic activities, it is becoming more important to verify whether and to what extent these chemicals disrupt the animal endocrine system. Therefore, researchers have focused on the development of more rapid and efficient in vitro assay methods to screen their activities of these discharged chemicals. The representative in vitro screening methods providing mechanistic data at OECD level 2 are shown in Table 2. Among the reported in vitro assays, the reporter gene assay is one of the most promising assay methods to prove the potential activity of target chemicals because, in particular, the majority of known endocrine active chemicals binds to their corresponding hormone receptors. The characterization of known reporter gene assays is summarized in Table 3. Although the yeast system is a convenient and good for their primary assay, it has a couple of unoverlooked disadvantages as follows: 1) permeability of chemicals through the cell wall and 2) difference of transactivating factors, such as recruited transcription factors. In conclusion, a human cell line is preferred to screen the activity of chemicals because its own intact signal transduction systems mediated by own intact receptors can be used. The reporter gene assay system was validated and reviewed by Körner W. et al.9) The Ministry of Environment in Japan (formerly the Japan Environment Agency) has made a priority list of compounds, SPEED 98, to preferentially examine whether they act as endocrine active chemicals and has been conducting a project to scientifically address endocrine disruptor issues since 1998.10) The project includes environmental monitoring to determine the concentrations of suspected endocrine active chemicals, epidemiological surveys of the general Japanese population to examine the relationship between exposure to certain suspected endocrine active chemicals and the occurrence of congenital malformations such as cryptorchism, and a series of in vitro and in vivo bioassays using established cell lines and experimental animals. We have been involved in the project and have assessed around 100 chemicals listed in SPEED 98 and related compounds for potential AR-mediated activities based on an in vitro reporter gene assay using MDA-kb2 human breast cancer cells with an endogenous human AR that stably express an androgen-responsive luciferase reporter gene, MMTV-luc in the presence of androgens.11–14) The principle of this assay is summarized in Fig. 1 and the experimental procedures were described in detail previously.12,14) Briefly, AR in cytoplasm moves to the nucleus to bind to the AR response element in target DNA as a ligand-inducible transcription factor after AR with a ligand making homodimer. This binding allows the recruitment of RNA polymerase II and other transcription factors. This mini-review overviews the structural requirements capable of disrupting AR function in addition to potential AR-agonist and antagonist activities based on our previously published papers.14,15)

### Table 2. Screening methods of endocrine disruptors at level 2

<table>
<thead>
<tr>
<th>Method</th>
<th>Notes</th>
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<tbody>
<tr>
<td>ER, AR, TR; receptor binding affinity</td>
<td>a)</td>
</tr>
<tr>
<td>High throughput prescreens</td>
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<tr>
<td>Transcriptional activation</td>
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<td>Thyroid function</td>
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<td>Aromatase and steroid genesis in vitro</td>
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<tr>
<td>Aryl hydrocarbon receptor recognition/binding</td>
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<td>QSARs=in silico assay</td>
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<tr>
<td>Fish hepatocyte VTG assay</td>
<td></td>
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<tr>
<td>Others (as appropriate)</td>
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</table>

a) ER, estrogen receptor; AR, androgen receptor; TR, thyroid receptor.

### Table 3. Characterization of reporter gene assay

<table>
<thead>
<tr>
<th>Items</th>
<th>Yeast two hybrid assay</th>
<th>Human cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor</td>
<td>plasmid</td>
<td>plasmid</td>
</tr>
<tr>
<td>Reporter gene</td>
<td>plasmid (transit)</td>
<td>plasmid (transit)</td>
</tr>
<tr>
<td>Activators</td>
<td>plasmid (transit)</td>
<td>constitutive</td>
</tr>
<tr>
<td>Transport barrier</td>
<td>cell wall</td>
<td>cell membrane</td>
</tr>
<tr>
<td>HepG2</td>
<td></td>
<td>constitutive</td>
</tr>
<tr>
<td>MDA453 kb2</td>
<td></td>
<td>plasmid (stable)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>constitutive</td>
</tr>
</tbody>
</table>
affinities than one with a stronger H-bond acceptor like the 3-keto group of dihydrotestosterone (DHT). Progesterone also acted as a pure AR agonist. Since a well-known ER agonist, DES, acted as a pure AR antagonist, the surface area of the AR ligand binding domain (LBD) was compared with that of the ERα-LBD based on their reported crystal structures\(^{16,17}\) to analyze how ligands interact with LBDs. The surface area of AR-LBD was shown to be smaller than that of ERα-LBD; therefore, compounds with both estrogenic and antiandrogenic activities like DES can fit well into the ERα-LBD but may protrude from the AR-LBD. It is likely that this subtle difference in the surface areas of LBDs determines whether an ERα agonist acts as an AR antagonist or an agonist. In phenols (industrial material), the fact that the order of AR an-
agonist activity was 4-tert-octylphenol > 4-nonylphenol (mixtures) > 4-iso- and tert-pentylphenol > 4-n-pentylphenol, which was 7-fold less potent than 4-tert-octylphenol, indicates that steric hindrance at the para position of phenols increases pure AR antagonist activity. In all 12 tested halogenated compounds, including DDT (insecticide), they showed AR antagonist activity and had fairly similar IC$_{50}$ values except for methoxychlor and $p,p'$-DDE. The result that

Fig. 2. List of tested chemicals. Calculated IC$_{50}$ means extrapolation values obtained by a calibration curve. IC$_{50}$ values were obtained from the previously reported manuscript.¹⁴)
bisphenol A (plasticizer) with hydroxyl groups substituted for chlorine atoms, had higher AR antagonist activity than chlorinated compounds implies that AR antagonist activity significantly depends on the H-bond forming capacity of the substituents. Moreover, the fact that fenarimol (fungicide) and α-tri-substituted acetates, which have an sp³ carbon atom between two benzene rings, displayed identical AR antagonist activity to DDT means that no steric hindrance exists around the sp³ carbon atom for interaction with AR. In ethylene derivatives having an sp² carbon atom between two benzene rings, only p,p'-DDE showed both AR agonist and antagonist activities (Fig. 2). This suggests that the steric effect derived from the sp² carbon atom reduces the degree of flexibility of the compounds and thereby increases the binding affinity of p,p'-DDE to the AR-LBD. Dieldrin (insecticide), along with endrin, aldrin, toxaphene, r-hexachlorocyclohexane, and trans-heptachlorepoxide, showed AR antagonist activity; however, the steric isomer of trans-heptachlorepoxide, cis-heptachlorepoxide and the isomer of r-hexachlorocyclohexane, β-hexachlorocyclohexane, had no activity. Those different activities between stereo isomers will give important clues to solve the interaction mechanism between AR-LBD and ligand in the future. In diphenyl ethers (herbicide), 2,4,6-trichlorophenyl-4'-nitrophenyl ether (chloronitrofen) acted not only as the most effective AR antagonist among the used diphenyl ethers, but also as an agonist (Fig. 3). This U-shaped dose-response effect was also observed by both 2,4-dichlorophenyl-4'-nitrophenyl ether (nitrofen) and 2,4-dichlorophenyl-3'-methoxy-4'-nitrophenyl ether (chloromethoxylin). However, 2,4-dichlorophenyl-3'-methylcarbonyl-4'-nitrophenyl ether (biphenox) acts only as an AR antagonist and its activity is approximately 61-fold less active than that observed in chloromethoxylin. Substitution of a methoxy group (chloromethoxylin) for a hydrogen atom (nitrofen) at the meta position increased AR antagonist potency. On the other hand, substitution to a bulkier group at the meta position, like methylenecarboxylate (biphenox), significantly decreased AR antagonist activity, implying that this meta position as well as the para position plays an key role in their interaction with AR. In miscellaneous compounds, linulon (herbicide) had the same IC₅₀ values of a known antiandrogen, flutamide (anticancer agent). Among dicarboxyl imide derivatives (fungicide), vinclozolin and procymidone are highly active AR antagonists. Although vinclozolin has potent AR agonist activity at higher concentration, procymidone acts only as a pure AR antagonist. The AR agonist activity of vinclozolin might come from the parent compound and not from its metabolites because of the suggested low esterase activity of MDA-kb2 cell lines. In contrast with these compounds, iprodione had no detectable AR agonist or antagonist activity based on this assay. Fenitrothion (organophosphate insecticide) had 70-fold higher AR antagonist activity than ethyl

![Chemical Structure](image)

**Fig. 3.** Responsiveness of MDA-kb2 to chloronitrofen in the presence (●) or absence (■) of 0.2 nM DHT. Data are presented as the mean fold induction compared to the vehicle controls of three independent assays (4 wells per replicate) ± standard deviation of the mean for chloronitrofen. * and + show significant difference (p<0.05) as compared to the activation by 0.2 nM DHT and the vehicle control, respectively.
parathion and has AR agonist activity at higher concentration. Amitrol (herbicide), metiram (fungicide), aldicarb (insecticide), 1,2-dibromo-3-chloropropane (industrial material), 4-nitrotoluene (industrial material) had no AR activity at any treated concentrations.

### Difference between AR Agonists and Antagonists

The structural diversity of these chemicals has heightened interest in the structural requirements necessary to disrupt AR function and has motivated the development of models and strategies for predicting potential AR activity based on chemical structures. The obtained antagonist activities were analyzed by using a three-dimensional quantitative structure-activity relationship (3D-QSAR) technique, Comparative Molecular Field Analysis (CoMFA), and the structural requirements for the activity in each group were compared. The steric and electrostatic properties were sufficient to describe the structural requirements for AR antagonist activity. In addition, the structural difference between AR agonists and antagonists was explained based on CoMFA results and the AR-LBD crystal structure. To clarify whether chemicals act as an AR agonist and/or antagonist and how they increase their activity, the precise structural requirements for AR agonists and/or antagonists are described as follows:

1) In general, ligands should have a strong H-bond acceptor, such as nitro groups, at the position corresponding to the 3-keto group of DHT to interact with AR-LBD. Moreover, a meta-substituent such as methyl or trifluoromethyl groups, which can induce interaction with the hydrophobic pocket surrounded by Gln711, Met745 and Leu707 in AR-LBD, plays a prominent role in increasing AR binding activity.

2) Agonists should not stick out from the ligand binding cavity of AR and have an H-bond acceptor or donor group at the position corresponding to the 17β-OH group of DHT to accurately position Asn705, Arg752, and Thr877. The distance between two functional groups with H-bonding ability corresponding to 3-keto and 17β-OH groups should be near 10 Å to maintain a favorable H-bond position (Fig. 4a).

3) The length axis of antagonists should be less than or more than 10 Å so as not to make an H-bond by interaction with Asn705 and Thr877 in the steroid D ring anchoring pocket, preventing the correct positioning of helix 3 and helix 12 (H12) (Fig. 4b).

This hypothetical general rule is named “Near 10 Å polar Interaction Rule” (Fig. 4). Based on this rule, the U-shaped phenomena observed by chloronitrofen and fenitrothion is also explained without any contradiction. Although transcriptional activation functions are present in the amino-terminal domain (activation function 1: AF1) and the LBD (activation function 2: AF2) of many nuclear receptors including AR, both AF1 and AF2 activities are suppressed in the absence of a ligand. However, after ligand binding to the nuclear receptors, the AF2 binding surface is completed by repositioning of H12. After agonist binding to AR, the ligand-binding pocket is enclosed by the carboxy-terminal helix H12 to be a transcriptionally active form of AR (at high concentration in the case of a U-shape), while antagonists interfere with the conformational change of H12 to impair coactivator binding to AR (at low concentration in the case of a U-shape). The positioning of H12 in AR-LBD is considered to play an important role in the formation of the coactivator binding surface of AR. This subtle difference in structural requirements determines whether a ligand acts as an AR antagonist or an agonist as well as an ER agonist.

### Conclusion

In conclusion, while *in vitro* study is suitable for rapid screening and mechanistic understanding as to whether chemicals
act as endocrine active chemicals, animal studies are also necessary to confirm whether they influence endocrine functions by modulating transcription within target organs through the interaction with nuclear receptors in vivo. Therefore, scientists should consider the results provided by not only in vitro study but also in vivo study.

References