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Structural basis for androgen receptor agonists and antagonists: Interaction of SPEED 98-listed chemicals and related compounds with the androgen receptor based on an in vitro reporter gene assay and 3D-QSAR

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Abstract—The androgen receptor (AR) activity of listed chemicals, so called SPEED 98, by the Ministry of the Environment, Japan, and structurally related chemicals was characterized using MDA-kb2 human breast cancer cells stably expressing an androgen-responsive luciferase reporter gene, MMTV-luc. Since our results suggested that chemicals with diverse chemical structures were capable of disrupting the endocrine systems mediated by AR, a comparative molecular field analysis (CoMFA) model was developed to analyze the structural requirements necessary to disrupt AR function. A significant CoMFA model with $r^2 = 0.825$ and $q^2 = 0.332$ was developed for AR antagonist activity of 35 pure antagonists excluding procymidone. On the other hand, a good CoMFA model with $r^2 = 0.983$ and $q^2 = 0.555$ was obtained for antagonist activity of 13 chemicals with both agonist and antagonist activities. The steric and electrostatic properties were sufficient to describe the structural requirements for AR antagonist activity. In addition, the structural difference of AR agonists and antagonists was explained based on CoMFA results and the AR-LBD crystal structure. As several ERα agonists such as diethylstilbestrol (DES) acted as AR antagonists, the surface area of the AR ligand-binding domain (LBD) was compared with that of the ERα-LBD based on their reported crystal structures to analyze how those ligands interact with LBDs. The surface area of AR-LBD was shown to be smaller than that of ERα-LBD and therefore compounds with both estrogenic and antiandrogenic activities can fit well into the ERα-LBD but may protrude from the AR-LBD. It is likely that this subtle difference of the surface areas of the LBDs determines whether an ERα agonist acts as an AR antagonist or an agonist.

1. Introduction

The underlying mechanisms of environmental compounds that disrupt normal endocrine function 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 the estrogen receptor (ER) or androgen receptor (AR) to interfere with the ligand-dependent transcriptional function (receptor-mediated disruptors).[1] The other is the inhibition of the biosynthesis or metabolism of endogenous ligands to indirectly modulate endocrine function (non-receptor-mediated disruptors).

The Ministry of Environment in Japan (former Japan Environment Agency) has made a priority list of compounds, so called SPEED 98,[2] to preferentially examine whether they act as endocrine disruptors, and has been conducting a large-scale project to scientifically address the endocrine disruptor issues since 1998. The project includes environmental monitoring to determine the

Abbreviations: AR, androgen receptor; ER, estrogen receptor; LBD, ligand-binding domain; DHT, dihydrotestosterone; PR, progesterone receptor; GR, glucocorticoid receptor; QSAR, quantitative structure–activity relationships; SAR, structure–activity relationships; CoMFA, comparative molecular field analysis; 3D-QSAR, three-dimensional quantitative structure–activity relationship.

Keywords: CoMFA; SAR; AR; MDA-kb2; DHT; Endocrine disruptor; Estrogen receptor.

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concentrations of suspected endocrine disrupting contaminants, epidemiological surveys of the general Japanese population to examine the relationship between exposure to certain suspected endocrine disruptors 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 the 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 that stably express an androgen-responsive luciferase reporter gene, MMTV-luc. This cell line was already well characterized by other research groups.[3–7] 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.

Based on the crystal structure of the AR ligand-binding domain (LBD)-R1881 (an agonist) complex, in the transcriptionally active form of AR, the ligand-binding pocket is enclosed by the carboxy-terminal helix 12 (H12).[8] It was reported that 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 and ER.[9] Both AF1 and AF2 activities are suppressed in the absence of a ligand and after ligand binding the AF2-binding surface is completed by repositioning of H12.[9,10] It is known that bulky substituents of ER antagonists interfere with the conformational change of H12 to impair coactivator binding to ER.[10] Even if the conformational changes occurring upon antagonist binding are unknown for AR, AR also has AF2 in its LBD[11] and the positioning of H12 is considered to play an important role in the formation of the coactivator binding surface of AR.

Quantitative structure–activity relationship (QSAR) models and qualitative SAR approaches have met with some measure of success in identifying and depicting structural features that contribute to the ability of a chemical to interact with the androgen receptor.[12–16] More recent publications revealed a common pattern of steric and electronic features involved in receptor binding affinity using 3D-QSAR models such as a comparative molecular field analysis (CoMFA) model[17] based on the results of rat AR competitive binding assay of a large number of chemicals.[18–21] Since AR functions as the most important ligand depending on the transcription factor in the regulation of AR target gene expression,[22] a competitive binding assay cannot distinguish whether a ligand acts as an agonist and/or an antagonist. The 3D-QSAR models provided by the competitive binding assay may be useful to predict the endocrine activity of environmental chemicals. From the pharmacological viewpoint, however, it is difficult to adapt such models to the design of biorational pharmacophore because the models use both androgenic and antiandrogenic functional activities. The results based on a reporter gene assay in this study point to the structural elements necessary for AR antagonist and/or agonist activity. The 3D-QSAR models provided by AR reporter gene assays afford a consistent description of steric and electrostatic fields in the ligand framework forming interactions with appropriate amino acid residues within the binding pocket of the AR.

Of particular interest are the structural elements in the interactions of ligands with both AR and ER ligand-binding domains (LBD) based on the detailed crystal structures and homology models of AR and ER LBD with their agonist or antagonist.[23–28] This approach also provides insight into steroidal ligand-binding specificity as well as information on the interaction of steroidal and non-steroidal ligands with specific residues within both AR- and ER-LBD. The results of this study will be beneficial not only for the prediction of endocrine-disrupting effects of environmental chemicals but also the rational drug design of hormone-dependent cancers, reproductive tract disorders, and so on.

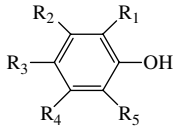
2. Results and discussion

2.1. Androgen reporter gene analysis

The IC_{50} values of compounds evaluated by an in vitro reporter gene assay consisting of MDA-kb2 human breast cancer cells are shown in Tables 1–8 and Figure 1.

2.1.1. Response of natural and synthetic estrogens and progesterone. Of the known estrogenic compounds tested in this study, only 17β-estradiol (E2) demonstrated a sufficient increase of luciferase activity over concentrations ranging from 1×10^{-8} to 3×10^{-5} M (Fig. 2a), although this activity was 10^{-3}- to 10^{-4}-fold weaker than that observed with dihydrotestosterone (DHT). AR antagonist activity was not observed at any concentration of E2 in the presence of 0.2 nM DHT (data not shown). These data were consistent with previously reported data.[4,5] To determine whether the activity induced by E2 was AR-mediated activity, a well-known

Table 1. Effect of phenols on DHT-induced AR activity in MDA-kb2 human breast cancer cells



Compound	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)
n-Pentyl phenol	H	H	*n*-C_5H_{11}	H	H	35.8
i-Pentyl phenol	H	H	*i*-C_5H_{11}	H	H	16.1
t-Pentyl phenol	H	H	*t*-C_5H_{11}	H	H	16.6
t-Octyl phenol	H	H	*t*-C_5H_{11}	H	H	5.1
Nonyl phenol (mixtures)	H	H	C_9H_{19}	H	H	8.8
2,4-Dichlorophenol	Cl	H	Cl	H	H	751.6[a]
Pentachlorophenol	Cl	Cl	Cl	Cl	Cl	nd[b]

[a] Estimated value extrapolated from the calibration curve.
[b] Not detected at the concentration of 1×10^{-4} M.

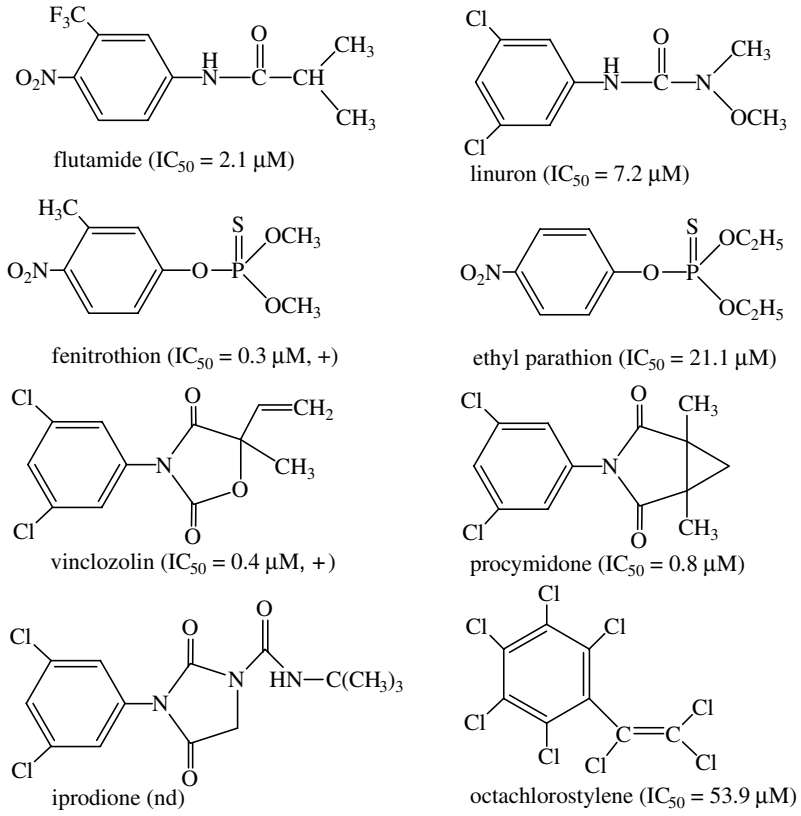


Figure 1. Effect of miscellaneous compounds on AR activity. Number in parentheses indicates the IC_{50} value and AR agonist activity as +.

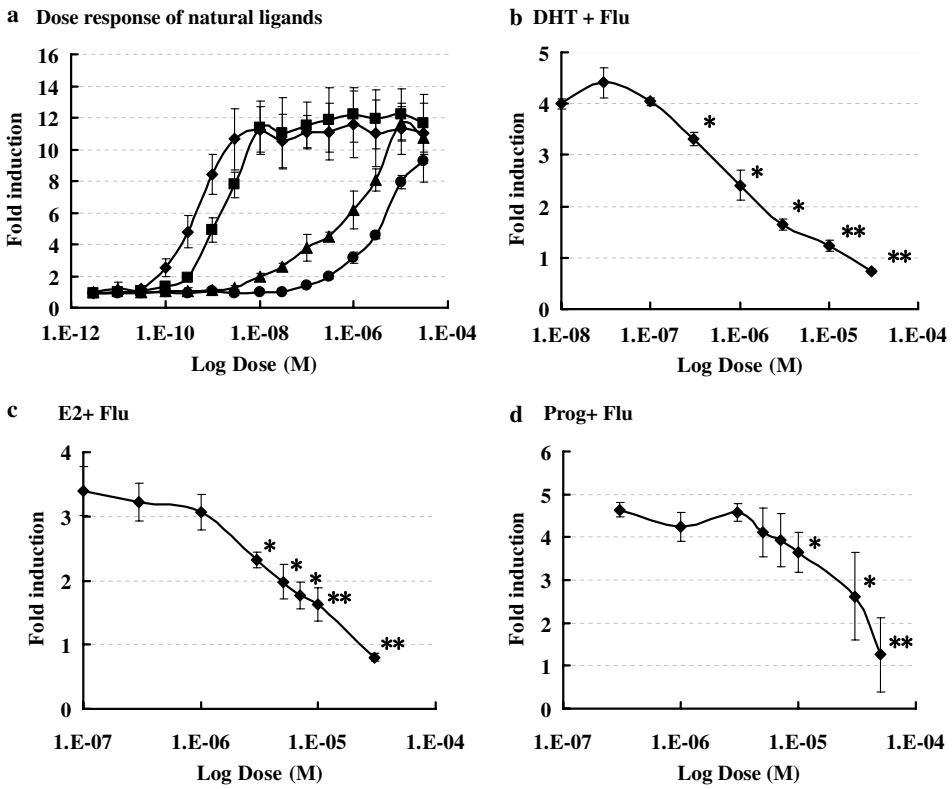
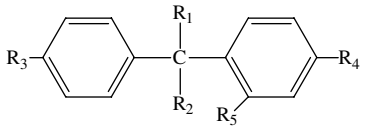
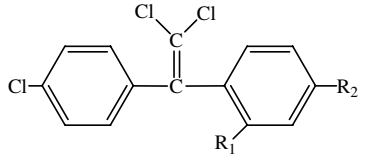


Figure 2. Effect of steroids, dihydrotestosterone, DHT (♦); testosterone (■); estradiol, E2 (▲); and progesterone, Prog (●) on luciferase activity in MDA-kb2 cells (a) and antagonism by flutamide, Flu in the presence of 0.2 nM of DHT (b), 100 nM of E2 (c), and 1 μM of Prog (d), respectively. Data are presented as the mean fold induction compared to vehicle controls of at least three independent assays (4 wells per replicate) ± standard deviation of the mean. *, Significant difference ($p < 0.05$) as compared to activation by 0.2 nM DHT, 100 nM E2, and 1 μM Prog, respectively. **, Cytotoxicity.

Table 2. Effect of halogenated compounds like DDT and bisphenol A on DHT-induced AR activity in MDA-kb2 human breast cancer cells



Compound	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)
p,*p*′-DDT	H	CCl_3	Cl	Cl	H	17.9
o,*p*′-DDT	H	CCl_3	Cl	H	Cl	22.2
p,*p*′-DDD	H	CCl_2	Cl	Cl	H	20.4
o,*p*′-DDD	H	CCl_2	Cl	H	Cl	17.0
Methoxychlor	H	CCl_3	OCH_3	OCH_3	H	52.9
Dicofol	OH	CCl_3	Cl	Cl	H	11.9
Chlorobenzilate	OH	$C(O)OC_2H_5$	Cl	Cl	H	26.8
Chloropropylate	OH	C(O)O–*i*-C_3H_7	Cl	Cl	H	33.7
Phenisobromolate	OH	C(O)O–*i*-C_3H_7	Br	Br	H	22.7
Fenarimol	OH	3-Pyrimidine	Cl	H	Cl	23.3
Bisphenol A	CH_3	CH_3	OH	OH	H	5.7



Compound	R_1	R_2	IC_{50} (μM)
p,*p*′-DDE	Cl	H	6.7
o,*p*′-DDE	H	Cl	20.2

Table 3. IC_{50} values of chlorinated compounds like dieldrin on DHT-induced AR activities in MDA-kb2 human breast cancer cells

Compound	IC_{50} (μM)
Dieldrin	22.8
Endrin	63.9
Aldrin	71.4
Heptachlor	nd
trans-Nonachlor	nd
Chlorden	nd
trans-Chlorden	nd
cis-Chlorden	nd
Oxychlorden	nd
Toxaphene	463.9[a]
Chlordecon	nd[b]
Milex	nd
β-Hexachlorocyclohexane	nd
γ-Hexachlorocyclohexane	57.1
Hexachlorobenzene	nd
trans-heptachlorepoxide	42.0
cis- heptachlorepoxide	nd

[a] Estimated value extrapolated from the calibration curve.
[b] Not detected at the concentration of 1×10^{-4} M.

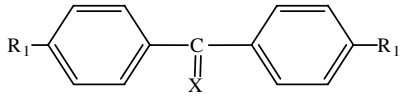
Table 4. Effect of polyaromatic hydrocarbons on DHT-induced AR activity in MDA-kb2 human breast cancer cells

Compound	Number of ring	IC_{50} (μM)	AR agonist activity
Benzo[*a*]pyrene	5	20.2	+
Peryrene	5	113.0[a]	+
Chrysene	4	3683.5[a]	+
Pyrene	4	18.6	–
Phenanthrene	3	12.7	+
Anthracene	3	36.4	–
Naphtharene	2	nd[b]	nd

[a] Estimated value extrapolated from the calibration curve.
[b] Not detected at the concentration of 1×10^{-4} M.

antiandrogen, flutamide instead of hydroxyflutamide, was co-treated with 100 nM E2 because hydroxyflutamide acts as a weak AR agonist at high concentrations in the absence of the natural ligand.[28] Flutamide antagonized luciferase activation induced by E2 in a concentration-dependent manner with an IC_{50} value of 9.1 μM (Fig. 2c). In contrast with E2, estrone (E1) and ethinyl estradiol (EE) had obvious AR antagonist activity in the presence of 0.2 nM DHT, with IC_{50} values of 1.9 and 0.7 μM, respectively. In addition, E1 and EE showed a weak increase of luciferase activity, the fold induction values of which were 2.5 and 2.0 at the concentration of 10 μM, respectively (data not shown). However, both estriol and diethylstilbestrol (DES) acted only as pure AR antagonists in a concentration-dependent manner and their IC_{50} values were 6.2 and 7 μM in the presence of 0.2 nM DHT, respectively. Taken together, ligands with hydroxyl groups such as E1, E2, EE, estriol, and DES had lower AR binding affinities than ligands with stronger H-bond acceptors like the 3-keto group of DHT. Although Hong et al.[18] showed that these steroids and DES are capable of binding to androgen receptor by AR competitive binding assay, it was difficult to distinguish whether the ligands act as agonists and/or antagonists based on such a competitive binding assay. This is the first report of how estrogenic compounds affect AR-mediated actions.

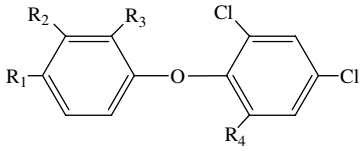
Table 5. Effect of benzophenons on DHT-induced AR activity in MDA-kb2 human breast cancer cells



Compound	X	R_1	IC_{50} (μM)	AR agonist activity
Benzophenone	O	H	109.6[a]	–
4,4′-Dichlorobenzophenone	O	Cl	2357.7[a]	+
4,4′-Dihydroxybenzophenone	O	OH	63.7	+
1,1′-Diphenylethene	CH_2	H	223.8[a]	–

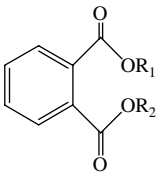
[a] Estimated value extrapolated from the calibration curve.

Table 6. Effect of diphenyl ethers on DHT-induced AR activity in MDA-kb2 human breast cancer cells



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)	AR agonist activity
Nitrofen	NO_2	H	H	H	2.7	+
Chlornitrofen	NO_2	H	H	Cl	1.9	+++
Chlomethoxynil	NO_2	CH_3O	H	H	0.6	++
Biphenox	NO_2	$C(O)OCH_3$	H	H	36.8	–
Trichlosan	Cl	H	Cl	H	7.5	–

Table 7. Effect of phthalate esters on DHT-induced AR activity in MDA-kb2 human breast cancer cells



Compound	R_1	R_2	IC_{50} (μM)
Dimethyl phthalate	CH_3	CH_3	104.2[a]
Diethyl phthalate	C_2H_5	C_2H_5	82.2
Di-*n*-propyl phthalate	*n*-C_3H_7	*n*-C_3H_7	709.4[a]
Butylbenzyl phthalate	*n*-C_4H_9	$CH_2C_6H_5$	89.2
Dibenzyl phthalate	$CH_2C_6H_5$	$CH_2C_6H_5$	54.9

[a] Estimated value extrapolated from the calibration curve.

Progesterone increased luciferase activity in the absence of DHT (Fig. 2a) and did not inhibit luciferase activity induced by DHT. When progesterone at a concentration of 1 μM, which induces a fold induction value of 4.6, was assayed concurrently with a series of concentrations of flutamide, an AR antagonist, flutamide decreased the fold induction value dose-dependently (Fig. 2d), showing that the IC_{50} value, 68.3 μM, was about 35 times higher than that observed in DHT (IC_{50} = 2.1 μM). Wilson et al.[5] previously concluded that progesterone is a glucocorticoid receptor (GR) agonist based on their result that the luciferase activity induced by progesterone was not inhibited by co-treatment with 1 μM of hydroxyflutamide, which interacts with AR. However, our results indicate that progesterone at least acts as an AR agonist because flutamide has no effect on GR activity. This inconsistency comes from the difference between the two experimental procedures. In our experiments, the dose–response curve of flutamide was determined in the presence of progesterone, which induced sufficient luciferase activity, while the dose–response curve of progesterone was determined in the presence of hydroxyflutamide in their experiment. Since the treated concentration of hydroxyflutamide may be insufficient to abolish the AR agonistic activity of progesterone, no difference in the dose–response curve of progesterone between in the presence and absence of hydroxyflutamide was observed. To avoid such misreading of the result, we strongly recommend that the dose–response curve of flutamide be depicted in the presence of the agonist at a concentration capable of inducing suitable fold induction. Flutamide is also preferred as an AR antagonist rather than hydroxyflutamide for both AR antagonist and agonist activities.

2.1.2. Response of phenols. Antiandrogenic activities of 4-*tert*-octylphenol and bisphenol A were prominent among the phenols tested (Tables 1 and 2). Their IC_{50} values were consistent with results obtained by Paris et al.[29] The order of AR antagonist activity of the tested phenol compounds 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 (Table 1). The observed results

Table 8. Experimental and CoMFA-calculated pIC_{50}

Compound	pIC_{50}		Residual[c]
	Actual[a]	Calculated[b]	
(a) Compounds with both AR agonist and antagonist activities			
4,4′-Dichlorobenzophenone	2.63	2.87	−0.24
4,4′-Dihydroxybenzophenone	4.20	3.92	0.28
Benzo[*a*]pyrene	4.70	4.82	−0.12
Perylene	3.95	3.97	−0.02
Phenanthrene	4.90	4.74	0.16
Estrone	5.74	5.89	−0.15
Ethinyl estradiol	6.17	6.20	−0.03
Nitrofene	5.57	5.38	0.19
Chlornitrofen	5.71	5.66	0.05
Chlomethoxynil	6.22	6.12	0.10
p,*p*′-DDE	5.17	5.25	−0.08
Vinclozolin	6.38	6.44	−0.06
Fenitrothion	6.50	6.58	−0.08
(b) Compounds with pure AR antagonist activity			
2,4-Dichlorophenol	3.12	3.86	−0.74
n-Pentyl phenol	4.45	4.63	−0.18
t-Pentyl phenol	4.78	4.63	0.15
i-Pentyl phenol	4.79	4.73	0.06
t-Octyl phenol	5.29	5.05	0.24
Bisphenol A	5.25	5.10	0.15
p,*p*′-DDT	4.75	4.71	0.04
p,*p*′-DDD	4.69	4.71	−0.02
Methoxychlor(MXC)	4.28	4.13	0.15
Dicofol	4.92	4.69	0.23
Chlorobenzilate	4.57	4.58	−0.01
Phenisobromolate	4.64	4.96	−0.32
Chloropropylate	4.47	4.37	0.10
1,1′-Diphenylethene	3.65	3.79	−0.14
Benzophenone	3.96	3.71	0.25
γ-Hexachlorocyclohexane	4.24	4.00	0.24
Aldrin	4.15	4.48	−0.33
Endrin	4.64	4.56	0.08
Dieldrin	4.64	4.55	0.09
trans-Heptachlor epoxide	4.38	4.54	−0.16
Octachlorostylene	4.27	4.27	0.00
Dimethyl phthalate	3.98	3.87	0.11
Diethyl phthalate	4.09	3.82	0.27
Di-*n*-propyl phthalate	3.15	3.56	−0.41
Butylbenzyl phthalate	4.27	4.27	0.00
Dibenzyl phthalate	4.26	4.39	−0.13
Anthracene	4.44	4.21	0.23
Pyrene	4.73	4.67	0.06
Biphenox	4.43	4.33	0.10
Triclosan	5.13	4.92	0.21
Estriol	5.21	5.44	−0.23
DES	5.16	5.19	−0.03
Ethyl parathion	4.68	4.96	−0.28
Flutamide	5.68	5.85	−0.17
Linuron	5.14	4.77	0.37
Procymidone	6.08	—	—

[a] pIC_{50} based on experimental data.
[b] pIC_{50} calculated by Eqs. (3) and (2) in (a) and (b) respectively.
[c] Residual value from [pIC_{50} (actual) − pIC_{50} (calculated)].

indicate that steric hindrance at the *para* position of phenols increased AR antagonist activity. None of the tested chemicals displayed agonist activity when tested alone even at the highest concentration (1×10^{-4} M).

2.1.3. Response of halogenated compounds. The result that all 12 tested halogenated compounds like DDT showed AR antagonist activity and had fairly similar IC_{50} values except for methoxychlor and *p*,*p*′-DDE (Table 2). This result is consistent with the AR binding affinities by Hong et al.[18] Although methoxychlor, in which methoxy groups substituted for chlorine atoms in DDT, was the weakest AR antagonist among halogenated compounds, bisphenol A, in which hydroxyl

groups were substituted for chlorine atoms, had higher AR antagonist activity than chlorinated compounds. This implies that AR antagonist activity significantly depends on the hydrogen bonding and/or electrostatic interaction capacity of the substituents. As fenarimol and α-tri-substituted acetates, which have an sp^3 carbon atom between two benzene rings, displayed identical AR antagonist activity to DDT, it is suggested that no steric hindrance exists around the sp^3 carbon atom for interaction with AR. In ethylene derivatives having an sp^2 carbon atom between two benzene rings, only *p,p′*–DDE showed both AR agonist and antagonist activities (Fig. 3a). This suggests that the steric effect derived from the sp^2 carbon atom reduces the degree of flexibility of the compounds and thereby increases the binding affinity of *p,p′*–DDE to the AR ligand binding domain (AR-LBD).

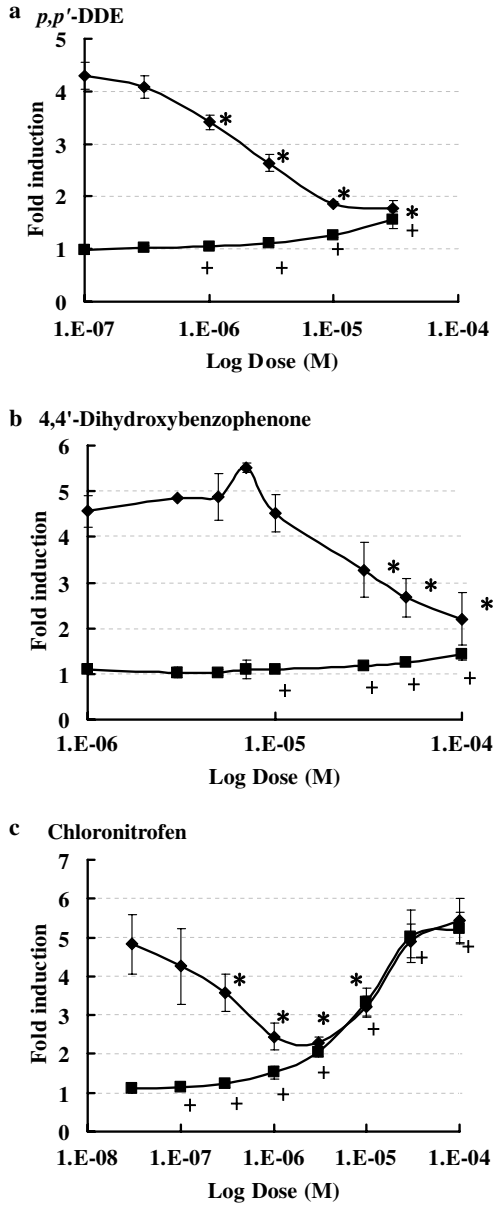


Figure 3. Responsiveness of MDA-kb2 to *p,p′*-DDE (a), 4,4′-dihydroxybenzophenone (b), and chloronitrofen (c) in the presence (♦) or absence (■) of 0.2 nM DHT. Data are presented as the mean fold induction compared to the vehicle controls of three independent assays (4 wells per replicate) ± standard deviation of the mean for *p,p′*-DDE and chloronitrofen and two independent assays (4 wells per replicate) ± standard deviation of the mean for 4,4′-dihydroxybenzophenone. * and $^{+}$, Significant difference ($p < 0.05$) as compared to activation by 0.2 nM DHT and the vehicle control, respectively.

Dieldrin along with endrin, aldrin, toxaphene, *r*-hexachlorocyclohexane, and *trans*-heptachlorepoxide showed AR antagonist activity of the 17 compounds in Table 3. Although steric isomers like dieldrin and endrin had moderate AR antagonist activity, the steric isomer of *trans*-heptachlorepoxide, *cis*-heptachlorepoxide and the isomer of *r*-hexachlorocyclohexane, β-hexachlorocyclohexane had no activity. Hexachlorobenzene also had no AR activity. This evidence from the results of stereo isomers will give important clues to solve an interaction mechanism between AR-LBD and ligand in the future.

2.1.4. Response of polyaromatic hydrocarbons. A well-known polyaromatic hydrocarbon (PAH), benzo [a] pyrene, listed in SPEED 98, acted as both an AR agonist and antagonist, resulting in an IC_{50} value of 20.2 μM. To elucidate its activity, related compounds were tested and the results summarized in Table 4. Although AR agonist and antagonist activities were also observed by chrycene, peryrene and phenanthrene, pyrene and anthracene showed only AR antagonist activity.

While their AR antagonist activities were consistent with the results in CHO cells transiently cotransfected with AR and MMTV-LUV vectors,[30] no such AR agonist activity was detected in the CHO cell assay. This difference may come from the characteristics of the used cell line including the transfection system by recruited coactivators, although further study is necessary.

2.1.5. Response of benzophenones. Benzophenone listed in SPEED 98 acted only as an AR antagonist (IC_{50} = 109.6 μM). An ultraviolet (UV) filter, 2-hydroxy-4-methoxybenzophenone (methoxybenzophenone-3), was also an AR antagonist in the in vitro MDA-kb2 cell transcription assay.[30] Although 4, 4′-dihydroxybenzophenone had the highest AR antagonist activity among 4 related compounds with an IC_{50} value of 63.7 μM (Table 5), a slight increase in luciferase activity in the absence of DHT suggests that the compound also has weak AR agonist activity (Fig. 3b). This is consistent with the competitive binding affinity data by Hong et al.[18] The substitution of 2 hydroxy groups in 4,4′-dihydroxybenzophenone by chlorine atoms (4,4′-dichlorobenzophenone) also induced luciferase activity in the absence of DHT, while its AR antagonist activity was 37-fold less than that of the hydroxy derivative. Therefore, *para*-substituents like hydroxy groups and chlorine atoms may play an important role in the AR transcription function. The fact that 1,1-diphenylethane displayed AR antagonist activity with an IC_{50} value only 2-fold lower than that of benzophenone implies that a carbonyl group of benzophenone may not be important for interaction with AR-LBD. Moreover, the result that anthrone and anthraquinone,

conformations of which are rigid because of the bridge between two phenyl groups, had no AR agonist and/or antagonist activities suggests that flexibility in the vicinity of a carbonyl group may play a prominent role in their interaction with AR.

2.1.6. Response of diphenyl ethers. Diphenyl ether compounds have been mainly used as herbicides in paddy fields in Japan to control annual broad leaf weeds. The most typical effect provided by 2,4,6-trichlorophenyl-4′-nitrophenyl ether (chloronitrofen) was that chloronitrofen acted not only as the most effective AR antagonist among the diphenyl ethers used in this study, but also as an agonist because of its increased luciferase activity as its concentration increased in the absence of DHT (Fig. 3c). 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 (chlormethoxynil) (data not shown). However, 2,4-dichlorophenyl-3′-methylcarbonate-4′-nitrophenyl ether (biphenox) displayed only AR antagonist activity and its IC_{50} value was approximately 61-fold less active than that observed in chlomethoxynil (Table 6). Although AR antagonist but not agonist activity of chloronitrofen has been previously reported,[31] this difference may come from the difference of the used cell lines. Substitution of a methoxy group (chlormethoxynil) 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 methylcarboxylate (biphenox) significantly decreased AR antagonist activity. It is obvious that this *meta* position as well as the *para* position plays a key role in their interaction with AR.

Trichlosan, 2,4-dichlorophenyl-2′-hydroxy-4′-chlorophenyl ether, widely used as an antimicrobial agent in toothpastes, also had only AR antagonist activity with an IC_{50} value of 7.5 μM, which was as potent as linuron (Fig. 1) based on in vitro MDA-kb2 reporter gene assay. This is the first report of AR antagonist activity of this compound.

2.1.7. Response of phthalate esters. Although phthalate esters such as di-*n*-butyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) have antiandrogenic-like effects in some model systems, they do not act as AR-mediated antagonists.[32,33] However, dimethyl, diethyl, and di-*n*-propyl phthalates acted as AR antagonists with IC_{50} values of 104.2, 82.2, and 709.4 μM, respectively (Table 7). Phthalate diesters with a longer *n*-alkyl chain than four, and cyclohexyl or 2-ethylhexyl groups did not show AR activity at the treated concentrations (data not shown). The observation demonstrated that the increase in steric hindrance by long alkyl chains as well as bulkiness in the vicinity of the carbonyl group is unfavorable for AR binding affinity. However, benzyl phthalate derivatives such as butyl benzyl phthalate and dibenzyl phthalate had relatively high potent AR antagonist activity (Table 7). The fact that monomethyl phthalate had no activity at any treated concentrations (data not shown) suggested that MDA-kb2 cells may have low esterase activity and, therefore, phthalate diesters with lower alkyl and benzyl groups directly interact with AR. Consequently, the obtained results offer a potential mechanism for the effect of those phthalate derivatives with lower alkyl and benzyl groups on AR and extend the previous findings, although it remains to be solved how phthalate derivatives interact with AR. Bis-(2-ethylhexyl) adipate listed in SPEED 98 as a phthalate derivative had no AR activity (data not shown).

2.1.8. Response of other compounds. The effect of miscellaneous compounds on AR activity is summarized in Figure 1. The IC_{50} values of known antiandrogens, flutamide and linuron, were 2.1 and 7.2 μM, respectively. These values were consistent with those obtained by other studies.[3,5] Among dicarboxyl imide derivatives widely used as agricultural fungicides throughout the world, vinclozolin and procymidone are highly active AR antagonists with IC_{50} values of 0.4 and 0.8 μM, respectively. While vinclozolin had potent AR agonist activity above 3 μM, procymidone only acted as a pure AR antagonist at any treated concentrations. AR agonist activity of vinclozolin might come from the parent compound and not from its metabolites such as M1 and M2 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 at any treated concentrations. The organophosphate fenitrothion had 70-fold higher AR antagonist activity than ethyl parathion. Octachlorostylene, known as an industrial material, had AR antagonist activity with an IC_{50} value of 53.9 μM. None of the other compounds listed in SPEED 98, amitrol (herbicide), metiram (fungicide), aldicarb (insecticide), 1,2-dibromo-3-chloropropane (industrial material), and 4-nitrotoluene (industrial material), had AR activity at any concentration ranging from 10^{-8} to 10^{-4} M. Organotins such as triphenyl tin chloride, diphenyl tin dichloride, tributyltin chloride, and dibutyltin dichloride showed no AR activity under the experimental conditions used in this study because of their cytotoxicity observed by microscope. In particular, triphenyl tin chloride showed serious cytotoxicity even at the concentration of 0.3 μM.

2.2. CoMFA

A recent publication revealed a common pattern of steric and electronic features involved in receptor binding affinity using the 3 D-QSAR model based on a large number of chemicals determined with an AR competitive binding assay using recombinant rat AR-LBD.[18–21] Although the information derived from such a 3D-QSAR model is useful to predict whether chemicals bind to AR, it is difficult to distinguish whether the ligand acts as an agonist and/or an antagonist. Therefore, we used an in vitro reporter gene assay to clarify the nature of compounds and provided a comprehensive model based on the data.

In this study, we did not include chiral isomers (*o*,*p*′-DDT, *o*,*p*′-DDD, *o*,*p*′-DDE, and fenarimol) and

mixtures (nonyl phenol and toxaphene) because the data generated by such mixtures cannot be applied to 3D-QSAR. Chrysene was also excluded from the analyses for the following reasons: since chrysene had very weak AR antagonistic and agonistic activity at the highest treated concentrations it was difficult to obtain a reliable IC_{50} value by extrapolation (Table 4). The antagonist activity of other compounds with both activities was not affected by their agonist activity because they showed a sufficient U-shaped dose–response curve to calculate a reliable IC_{50} value. Therefore, CoMFA was carried out for pIC_{50} (log $(1/IC_{50})$) values of 36 pure antagonists, 13 agonists /antagonists, and 49 combined compounds, respectively (Table 8, Eqs. 1–4). The statistical results of the obtained CoMFA Eqs. 1–4 are summarized in Table 9. Although the initial CoMFA model developed for 36 pure antagonists gave no significant regression results (Eq. 1), the exclusion of procymidone from the 36 compounds did exert a significant influence on the CoMFA regression results to obtain Eq. 2 ($r^2 = 0.825$ and $q^2 = 0.332$). Meanwhile, a CoMFA model with $r^2 = 0.911$ and $q^2 = 0.407$ (three components) was developed with the inclusion of vinclozolin, which has both antagonist and agonist activities, but a similar chemical structure and IC_{50} value to procymidone, without a steric/electrostatic weighting ratio. These results suggest that their chemical structures are significantly unique in the model because they have no H-bond acceptor at the *para* position of the phenyl ring corresponding to the 3-keto group of DHT. Therefore, the antagonist activity of procymidone was predicted as much lower than its actual value. With the inclusion of procymidone, the analysis of the combined set of 49 compounds was shown as Eq. 4. Figure 4 shows the overlay of the structure of flutamide with major electrostatic and steric potential contour maps drawn according to Eq. 2, The red areas in Figure 4 indicate regions where negative electrostatic interactions with the receptor binding site increase activity, whereas the blue areas show the reverse case. The green areas in Figure 4 indicate regions where submolecular bulk is well accommodated with an increase in AR binding activity on AR-LBD, whereas the yellow areas indicate regions where submolecular bulk is unfavorable for activity.

In contrast to the CoMFA of pure antagonists, a good CoMFA model with $r^2 = 0.983$ and $q^2 = 0.555$ (Eq. 3) was developed using the results from 13 chemicals with both agonist and antagonist activities (Fig. 5). As described above, although vinclozolin has a unique structure among 13 compounds, it was included in Eq. 3. A similar equation (2 components, $r^2 = 0.949$ and $q^2 = 0.502$) was obtained excluding vinclozolin. Of importance, sterically unfavorable yellow regions in CoMFA for pure antagonists appeared around Asn705, Arg752, and Thr877 in the AR-LBD (Fig. 4), while there were no yellow regions in the same areas for agonists/antagonists (Fig. 5). This implies that ligands without a bulky group at these regions are able to interact with the AR-LBD and do not preclude three residues, Asn705, Arg752, and Thr877, from taking the appropriate positions so that the AR–ligand complex can become transcriptionally active. However, when ligands with pure AR antagonist activity bind to AR-LDB, they likely contact with one (or more) of these three amino acids, resulting in a transcriptionally inactive form of the AR–ligand complex.

Since other yellow areas existed around the 4–7 positions on steroids A and B-ring in Figures 4 and 5, ligands with a bulky group in these areas may have lower AR binding activity. Electrostatically positive blue regions above the steroid B ring suggest that the presence of a group with a negative charge such as an oxygen atom at these regions is unfavorable for interaction with AR.

In Figure 5, red negative electrostatic regions appeared near the 3-keto and 17β-OH groups of DHT capable of forming H-bond with Gln711, Arg752, Asn705, and Thr877. This finding was basically consistent with other reported interactions.[12,18,19] Moreover, the green regions around Asn705 and Thr877 in AR-LBD in

Table 9. Summary of the CoMFA statistical results CoMFA equations for the AR antagonist activity of test compounds $pIC_{50} = A$ + [CoMFA field terms]

A	CN[a]	*n*[b]	*s*[c]	r^2[d]	Cross-validated[e]		RC[h]		Exclusion	Eq. no.[k]
					s_{cv}[f]	q^2[g]	Steric.[i]	Electro.[j]		
—	3	36	—	—	0.829	0.091	—	—		1
3.82	3	35	0.244	0.825	0.976	0.332	43.3	56.7	Procymidone	2
4.45	3	13	0.171	0.983	0.868	0.555	42.3	57.7		3
4.05	5	49	0.205	0.944	0.644	0.446	38.5	61.5		4

[a] Number of latent variables (components).
[b] Number of compounds.
[c] Standard error.
[d] Correlation coefficient.
[e] Obtained from the leave-one-out cross-validation.
[f] Standard error.
[g] Correlation coefficient.
[h] Relative contribution (%).
[i] Steric effects.
[j] Electrostatic effects.
[k] Equation number.

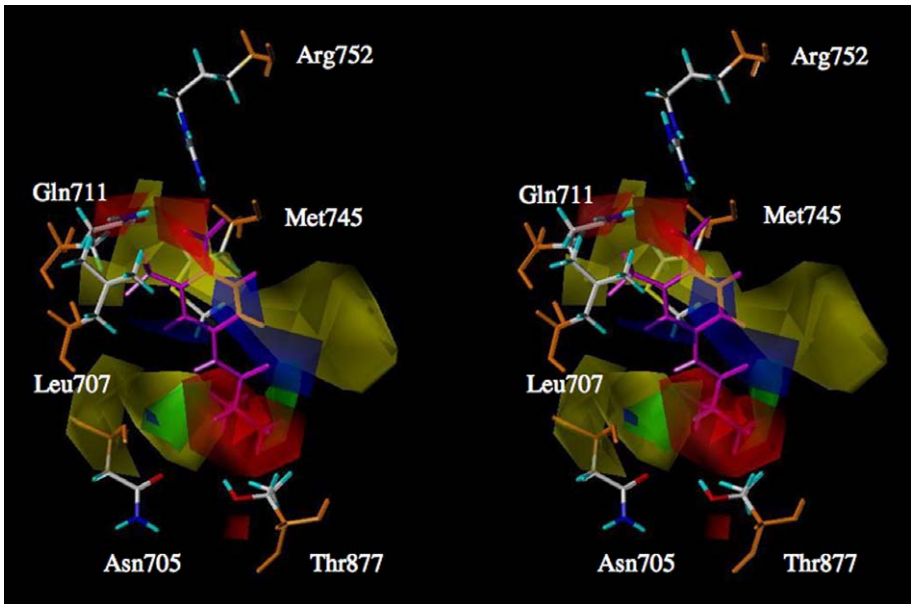


Figure 4. Stereoviews of contour diagrams of steric and electrostatic fields with flutamide (magenta) according to Eq. 2 in Table 9 for AR pure antagonists. See the text for an explanation of the colors. Amino acid residues, Asn705, Arg752, Thr877, Leu707, Gln711, and Met745, in the AR ligand-binding site are also displayed.

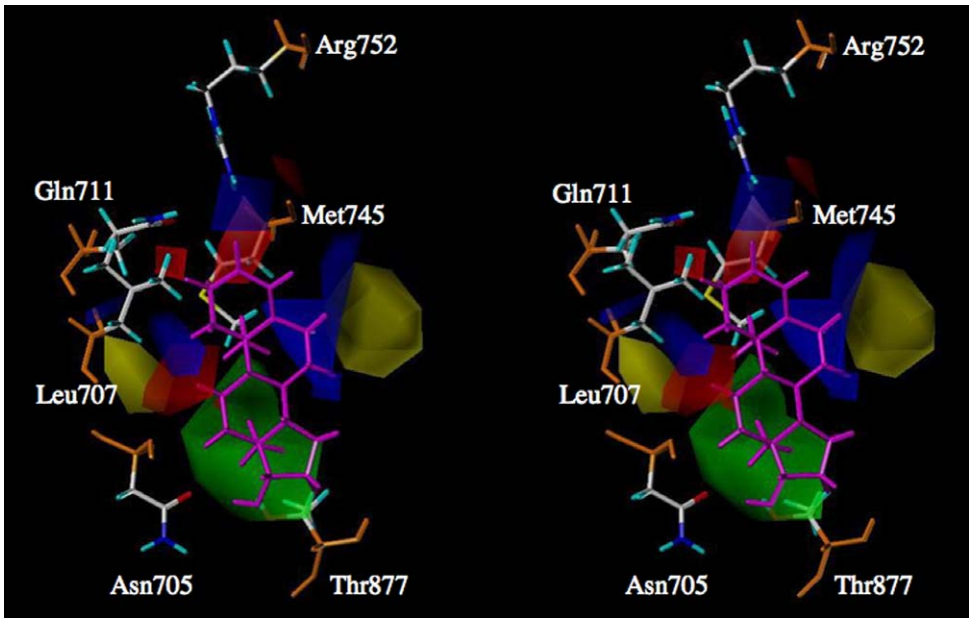


Figure 5. Stereoviews of contour diagrams of steric and electrostatic fields with testosterone (magenta) according to Eq. 3 in Table 9 for AR agonists/antagonists. See the text for an explanation of the colors. Amino acid residues, Asn705, Arg752, Thr877, Leu707, Gln711, and Met745, in the AR ligand-binding site are also displayed.

Figures 4 and 5 indicate that ligands should reach the green areas to have binding affinity; however, the green regions for agonists/antagonists (Fig. 5) are closer to Asn705 and Thr877 than those for pure antagonists (Fig. 4). These results suggest the importance of the distance between two groups having H-bond ability such as the 3-keto and 17β-OH groups of DHT to interact with both Arg752 and Asn705/Thr877 and switch on the transcription process. This finding is consistent with our previously proposed 'Near 10 Å Polar Interaction Rule.'[34]

2.3. Importance of hydrophobic interaction

Bohl et al.[19] indicated that the hydrophobic pocket of the AR-LBD is created by two hydrophobic amino acid residues, Val746 and Met742; however, in this study, three residues, Gln711, Met745, and Leu707, appear to be responsible for hydrophobic interactions with the ligands at the pocket (Fig. 4). This inconsistency comes from the difference between the analysis methods, that is, analysis in our study was performed

based on the natural hAR-LBD crystal structure, while Bohl et al.[19] used their own docking model of hAR-LBD based on homology to the human progesterone receptor. Ligands such as flutamide and fenitrothion with trifluoromethyl and methyl groups, respectively, adjacent to the nitro group increased their AR antagonist activity. Even though the CF_3 group of flutamide seems to be surrounded by yellow sterically prohibited regions (Fig. 4), these relatively small substituents are predicted to be closely surrounded by the hydrophobic pocket created by the amino acid residues.

2.4. Difference between AR agonists and antagonists

It was reported that AR AF2 binds to the ^{23}FQNLF^{27} in the AR NH_2-terminal region.[11] This interaction is androgen-dependent and was suggested to be important for transcriptional function.[9] In the crystal structure of AR-LBD-R1881 bound to AR 20–30 peptides containing FQNLF, FQNLF was hydrogen-bonded by Glu897 (H12) and Lys720 (H3).[11] Figure 6 shows the structure of AR-LBD in which important residues were highlighted. It was suggested by CoMFA results that compounds with both agonist and antagonist activities are capable of interacting with AR-LBD without preventing Asn705, Arg752, and Thr877 from taking the appropriate positions. The three critical residues for agonist activity, Asn705, Arg752, and Thr877, are located in H3, H5, and H11 in AR-LBD, respectively. If bulky groups of a ligand hinder Asn705 and Arg752, these residues may interfere with the interaction of the ligand with H3. The position of Thr877 may affect the conformational change of H12. As a result, Glu897 (H12) and Lys720 (H3) are unlikely to interact with ^{23}FQNLF^{27} in the AR NH_2-terminal region, leading to the inactive form of AR.

2.5. How do ER agonists act as AR antagonists?

Since well-known ER agonists like DES, octylphenol, and bisphenol A acted as pure AR antagonists, we aimed to elucidate the nature of the receptor binding domain by comparing the cavity surface areas between the ligand binding sites of AR and ER. Each cavity surface area was calculated based on the ligand-AR[8] and ligand-ER crystal structures[23] with their own ligand (R1881 and estradiol, respectively) by the MOLCAD option of SYBYL.[35] Figure 7 shows the cavity surface at the ligand-binding sites of AR (cyan) and ER (yellow) with DES. The area of the ligand in AR was slightly thinner than that in ER when the surface areas of the AR and ER were superimposed. Although DES has two OH groups as H-bond interaction sites with AR, two diethyl moieties of DES sticking out from AR-LBD made interaction of the OH groups with Asn705 and Thr877 of AR difficult, likely causing its AR antagonist activity. On the other hand, DES was well accommodated in ER-LBD (Fig. 7). This finding was also suggested by the CoMFA of pure antagonists. Since the ethyl moieties of DES reached the yellow sterically forbidden regions around Asn705 and Thr877 in AR-LBD, it would be an obstacle for taking an active conformer of the ligand-AR complex. Other pure AR antagonists with ER agonist activity like octylphenol and bisphenol A also protruded from the binding site surface area in AR like DES (data not shown). These observed results suggested that the bulky substituents of ligands may not fit the AR binding pocket surrounded by Asn705, Arg752, and Thr877 and as consequence, AR-ligand complexes fail to make a complete transcriptionally active conformer, for which the precise positioning of H12 and other helices is required. This means that such a subtle difference of ligand-binding cavities between AR and ER determines whether a ligand acts as an agonist and/or antagonist in each receptor. In the case of estriol, the hydroxy group at the 16-position may also interfere with H-bond formation of the hydroxy group at the 17-position. This is one possible reason why estriol acts as an AR pure antagonist. Moreover, X-ray crystal structure data[8,23] suggested that hydrophobic amino acids such as Leu346, 349, 384, 387, 391, 428, 525, Ile424, and Phe404 in the ERα-LDB play an important role in the hydrophobic interaction of ligands. As a result, ligands that interact with these amino acids behave as ER agonists, whereas hydrophobic

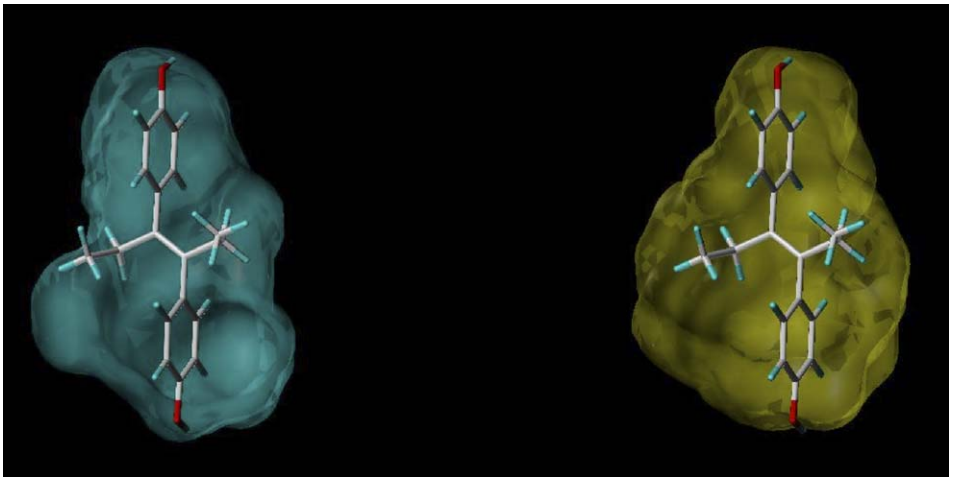


Figure 6. The cavity surface at the ligand-binding sites of AR (cyan) and ER (yellow) calculated based on the ligand-AR and ligand-ER crystal structures with DES.

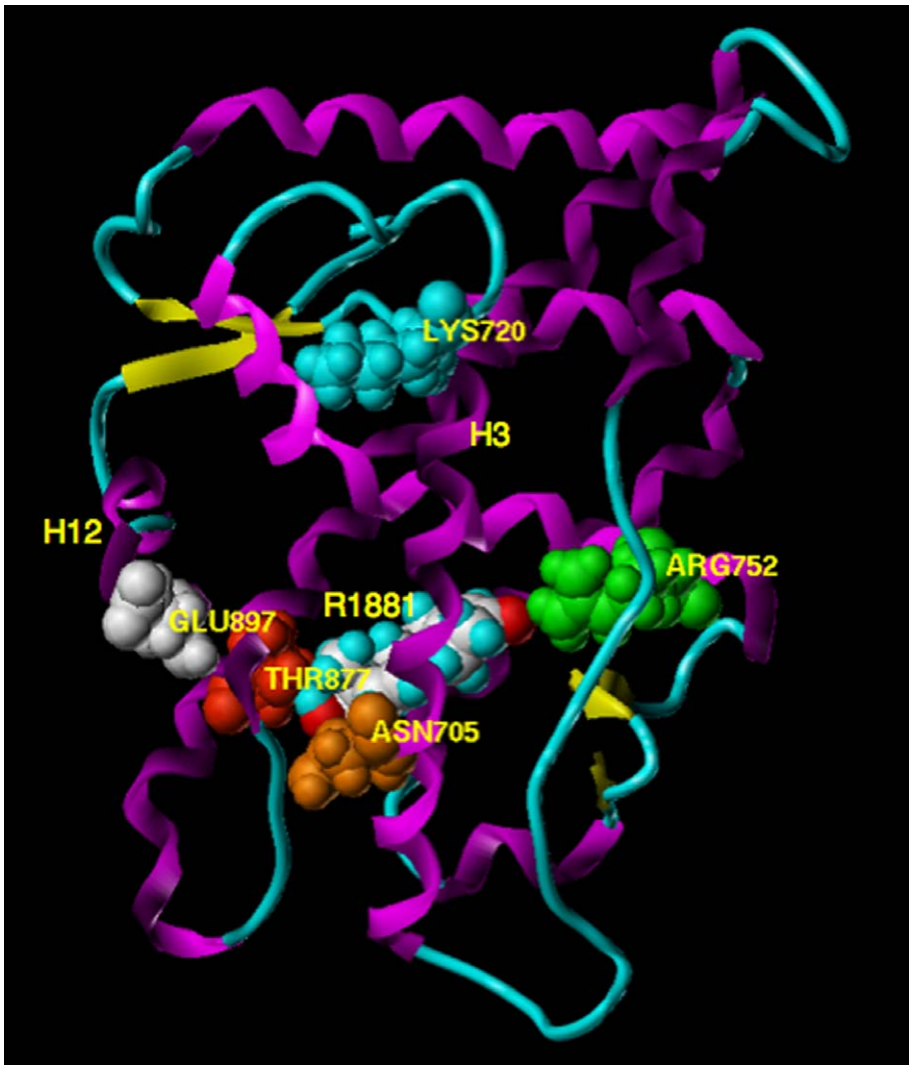


Figure 7. Structure of the AR-LBD-R1881 (space-filled atoms colored by atom types; white carbon, cyan hydrogen, and red oxygen) complex. Helices of AR are depicted as the ribbon structure. The critical three residues for agonist activity, Arg752 (green), Asn705 (orange), and Thr877 (red/orange), as well as the residues, Lys720 (cyan) and Glu897 (white), which interact with the FQNLF peptide, were labeled and depicted as space-filled atoms.

amino acids such as Leu701, 704, 707, 873, 880 and Phe764, 876, 891 in the AR-LBD play a significant role in steric hindrance and ligands that interact with these amino acids behave as AR antagonists.

3. Conclusions

We clarified whether chemicals act as an AR agonist and/or antagonist and how they increase their activity. In addition, the difference between ER agonists and AR antagonists was explained based on the cavity surface at the ligand-binding sites of AR and ER.

The precise structural requirements for AR agonists and/or antagonists are depicted as follows.

(1) In general, ligands should have a strong hydrogen bonding and/or electrostatic interaction ability like nitro groups at the position corresponding to the 3-keto group of DHT to interact with AR-LBD. Moreover, trifluoromethyl and methyl groups adjacent to the nitro group in flutamide and fenitrothion, which can induce the interaction with the hydrophobic pocket surrounded by Gln711, Met745, and Leu707 in AR-LBD, play 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 hydrogen bonding and/or electrostatic interaction ability corresponding to 3-keto and 17β-OH groups should be near 10 Å to maintain a favorable interaction in AR ligand-binding domain.

(3) The length axis of antagonists should be less than or more than 10 Å so as not to make a hydrogen bonding and/or electrostatic interaction with

Asn705 and Thr877 in the steroid D ring anchoring pocket, preventing the correct positioning of H3 and H12.

The results of this study could provide useful information for not only a rational drug design with improved activity for partial androgen insensitivity syndrome but also prioritization of a large number of man-made chemicals and prediction of their AR-mediated action in a screening program. Future works, especially animal studies, are necessary to determine whether androgenic and/or antiandrogenic compounds found in this study influence androgen functions by modulating transcription within target organs through the interaction with AR in vivo.

4. Experimental

4.1. Chemicals

All test compounds obtained from Wako (Kyoto, Japan) and Sigma–Aldrich Chemical Co. (St. Louis, MO) were of analytical grade and used without any further purification.

4.2. Androgen reporter gene assay

The stable MDA-kb2 human breast cancer cell line was kindly gifted by Dr. Gray, L. E. Jr. (U.S. EPA, Research Triangle Park, NC). These cells have an endogenous human androgen receptor (AR) and MMTV-neo-luciferase gene construct and elicit an increase in luciferase activity in the presence of an androgen. The assay procedures were described in detail previously.[5–7] Briefly, the MDA-kb2 cell line was maintained in Leibovitz's L-15 medium (GIBCO/BRL, USA) supplemented with 10% (v/v) resin-stripped fetal bovine serum (Hyclone, USA) and 1% (v/v) (final concentration) antibiotic-antimycotic agents (GIBCO/BRL, USA) at 37 °C. For each replicate, cells were plated in quadruplicate in 96-well plates (Coster, USA) at a density of 10^5 cell/well and then were incubated for 4 h at 37 °C. Following incubation, the cells were treated with various concentrations of test chemicals from 10^{-8} to 10^{-4} M to determine the AR agonist and antagonist activity in the absence and presence of 0.2 nM DHT, respectively. For the dose–response of natural ligands, cells were treated with concentrations of chemicals from 3×10^{-12} to 3×10^{-5} M. A vehicle control, ethanol or dimethylsulfoxide, was included in each experiment. The final concentration of ethanol and dimethylsulfoxide in the medium was 0.1%. After 24 h incubation, treated cells were rinsed with phosphate-buffered saline and were lysed with 25 μl of lysis buffer (Promega, USA). After 30 min, 25 μl of D-luciferin potassium salt (1 mM) and 25 μl of reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, and 0.5 mg/ml bovine serum albumin) were added to each well. Relative light units (RLU) of luciferase activity were immediately measured using a microtiter plate luminometer (Luminoskan, Labsystem, USA).

4.3. Data analysis

Data were expressed as the mean fold induction compared to that of the vehicle control, that is, fold induction = RLU (test chemical)/RLU (vehicle control). When a test chemical has no activity, the obtained fold induction is 1. When a test chemical has AR agonist activity, its fold induction increases as its concentration increases in the absence of DHT. Likewise, when a test chemical has AR antagonist activity, its fold induction decreases as its concentration increases in the presence of 0.2 nM DHT.

The data were analyzed by Student's *t*-distribution with Excel (Microsoft, USA) and *p* values less than 0.05 were considered significant. The value of IC_{50} for antagonist activity was the concentration of the test chemical producing 50% inhibition of 0.2 nM DHT-induced luciferase activity.[6] Since observation with a microscope of the damage to treated cells was correlated with decreased fold induction values in the absence of DHT, fold induction values less than 0.85 were suggested as potential other mechanisms that can disrupt luciferase assay. Therefore, fold induction values less than 0.85 were assigned for cell toxicity and were not used to calculate antagonist activity in the presence of DHT in this assay. Unless otherwise noted, the measured activities presented in this study represented means ± SD resulting from at least three separate experiments with quadruplicate wells for each treatment dose.

4.4. Molecular modeling

All computations were performed using the molecular modeling software package SYBYL, version 6.9.[35] To select the initial conformation of compounds, we started from the coordinates of X-ray crystallographic data for each compound obtained from the Cambridge Structure Database,[36] if available. Compounds not contained in the Database were constructed from the structures of similar compounds. The coordinates of the modified parts of these structures were calculated using the SYBYL standard values for bond lengths and angles. A systematic search in SYBYL was applied to all rotatable bonds. The low-energy conformer of each compound obtained by a systematic search was then optimized by the semiempirical PM3 method.[37] For the optimized coordinates of all compounds, atomic charges were calculated using MNDO.[38] Molecular electrostatic potentials of the molecules were computed from the MNDO atomic charges and used in CoMFA studies.

4.5. Alignment

We assumed the fully optimized conformation of the molecules to be the active conformation for binding. The optimized conformation of testosterone was selected as the reference standard because the optimized conformation of testosterone in AR-LBD was available in the reference,[26] even though DHT was used as the reference androgen in this assay. Steroidal compounds, DES, chlorinated compounds like dieldrin, phthalate esters,

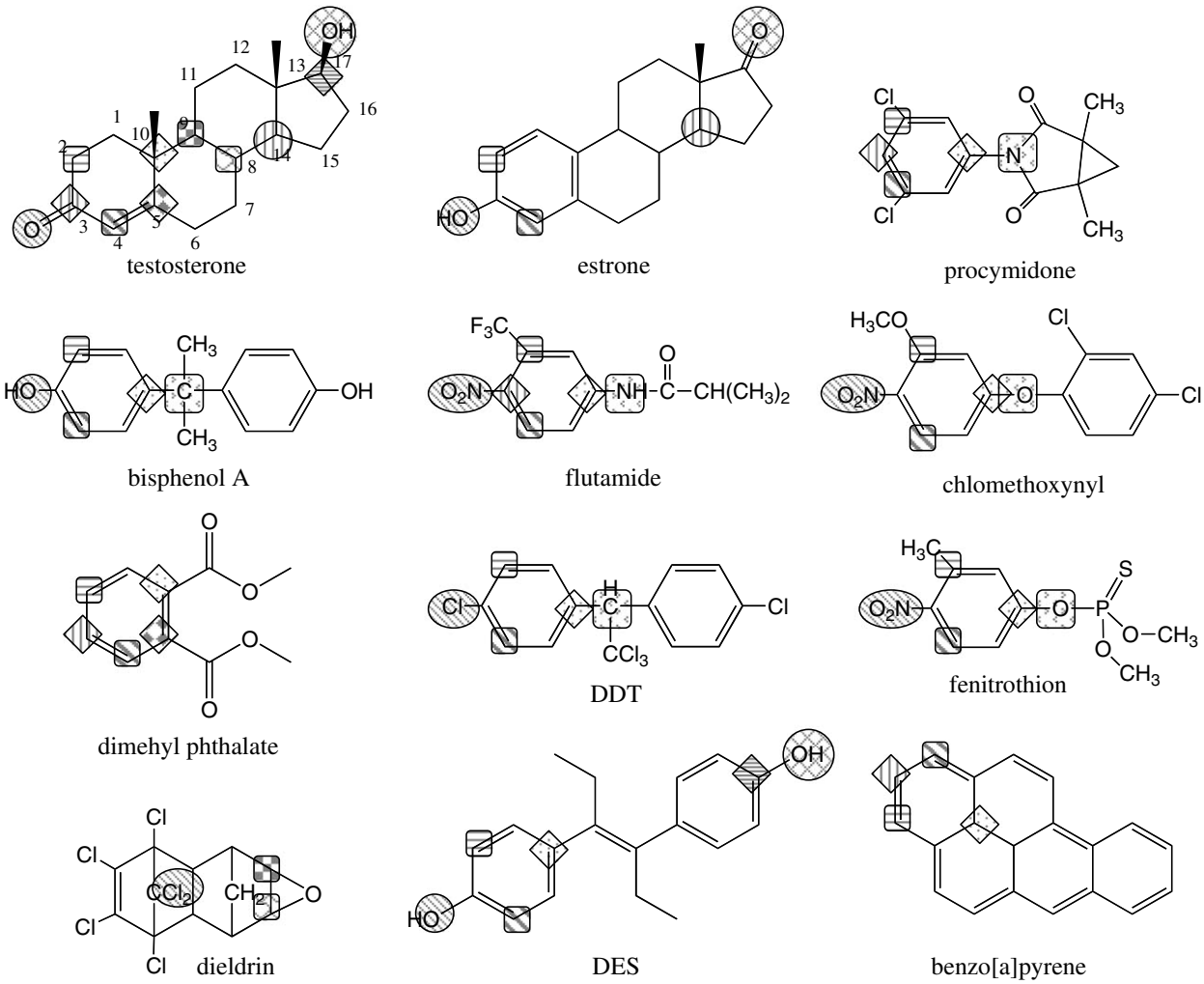


Figure 8. Atoms used for superimposition of representative compounds. Pairs of atoms are indicated by the same symbols.

and polyaromatic hydrocarbons were superimposed on testosterone. A benzene ring of bisphenol A or flutamide, included in the most potent AR antagonists, was superimposed on the steroid A ring of testosterone. Phenols, DDT derivatives, and diphenylethers were superimposed on bisphenol A. Compounds with N–C(=O) bond(s) were superimposed on flutamide. Atoms used for the superimposition of representative compounds are indicated in Figure 8. Regarding compounds containing two benzene rings, two alignments were attempted, that is, the benzene rings were superimposed on the corresponding position to a benzene ring of flutamide, respectively. The alignment which gave the better CoMFA equation was adopted.

4.6. Correlation by CoMFA

Analyses were conducted with the 'Advanced CoMFA' module of SYBYL. The procedure was similar to that described in our previous report.[39] The superimposed sets of stable conformers were placed in a lattice of 23 Å × 26 Å × 19 Å ($X = -6$ to 17, $Y = -15$ to 11, $Z = -14$ to 5) with 2 Å spaces automatically generated by the CoMFA routine in SYBYL. The potential energy fields of each stable conformer were calculated at the lattice intersections. To calculate the coulombic electrostatic potential at each lattice point, the charge of +1.0 as a probe and the atomic charges for each of the molecules were used. The steric interaction (Lennard-Jones) potential at the lattice points was calculated using the sp^3-carbon atom as a probe. The data matrix was analyzed by the partial least squares method.[40] The results of the analysis were expressed as correlation equations with the number of latent variable terms, each of which was a linear combination of original independent lattice variables. In order to show favorable and unfavorable potential regions, the variables were displayed as contour diagrams of coefficients of the corresponding field descriptor terms at each lattice intersection. We initially selected the number of compounds in the set as the number of the cross-validation (the leave-one-out method) and then analyzed using the optimum number of latent variables deduced from the cross-validation tests without actual cross-validation.

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