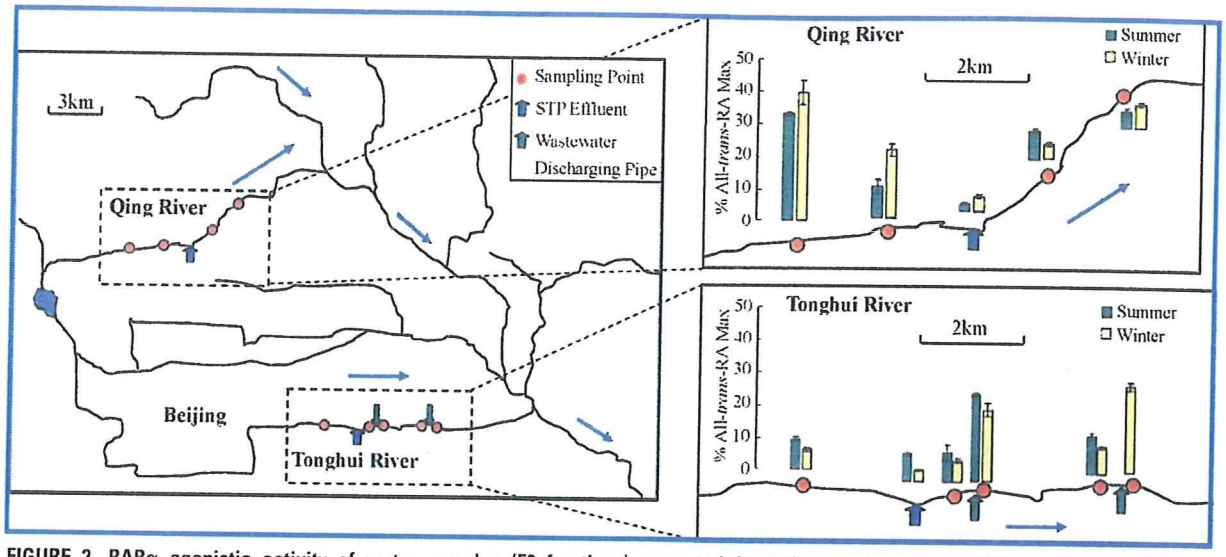

FIGURE 2. RARα agonistic activity of water samples (F2 fractions) extracted from the Tonghui River and Qing River at different distances from the corresponding STPs. The concentration factor for river water is 5000. Response magnitude stands for the percentage of the average maximum response observed for a 100 nmol/L all-*trans*-RA standard (% All-*trans*-RA Max).

two F3 samples taken from upstream sites (4 and 2 km upstream) of the Qing River in the summer caused significant responses in RARα agonistic activity tests, and this activity was comparable to that of the F2 fraction, while no detection was found in the sample taken in the winter, showing an obvious seasonal variation. However, no activity was detected in the F3 fractions of STP samples which were also taken in the summer. The reason for this difference of F3 fractions between STP and river samples is not clear, and needs to be investigated further.

Identification of RAR Agonists in Sewage Treatment Plants. As noted above, the major RARα agonists in both STP waters and river waters were present in the F2 fraction,

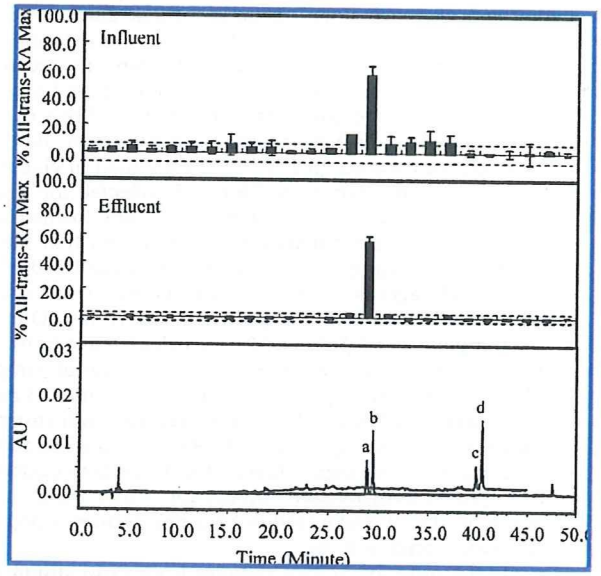

FIGURE 3. RARα agonistic activity profiles produced from F2 fractions of Gaobeidian STP influent (upper panel) and effluent (middle panel) using a bioassay-directed HPLC fractionation method. The concentration factors for influent and effluent are 10,000 and 25,000, respectively. The lower panel presents the chromatograph of several RAR agonists in HPLC with UV detector, including (a) 13-*cis*-4-oxo-RA, (b) all-*trans*-4-oxo-RA, (c) 13-*cis*-RA, and (d) all-*trans*-RA and 9-*cis*-RA. The detection wavelength is 350 nm for endogenous RAs.

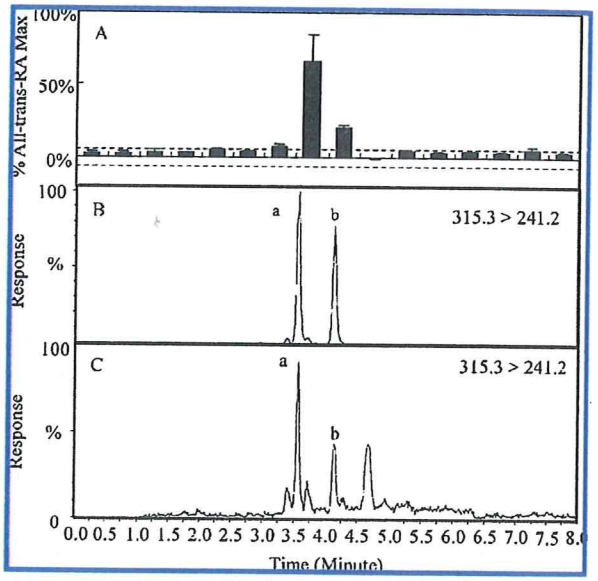

FIGURE 4. (A) RARα agonistic activity profiles of HPLC F15 fraction from Gaobeidian STP influent using bioassay-directed UPLC fractionation methods; (B) UPLC/MS/MS chromatograms of (a) all-*trans*-4-oxo-RA and (b) 13-*cis*-4-oxo-RA in mixed standards; and (C) UPLC/MS/MS chromatograms of (a) all-*trans*-4-oxo-RA and (b) 13-*cis*-4-oxo-RA in an influent sample of Gaobeidian STP.

a midpolar fraction. Thus, we further investigated the specific RAR agonists in aquatic environments using a bioassay-directed HPLC fractionation method. We fractionated F2 fractions of SPE samples from the effluents of all seven STPs and one influent, and found that the major RARα agonistic activities in all the samples occurred at the 15th fraction (HPLC F15) corresponding with a retention time between 28 and 30 min (Figure 3), indicating that this fraction is responsible for the main RARα agonistic activities in the STP samples.

To characterize the chemicals causing the response in the bioassay, we tested the RAR α, β, and γ agonistic activities of the HPLC F15 to get the phenotype of this fraction, and tried to compare the phenotype with those of already-known RAR agonists. As shown in Figure S4, both the RAR agonistic

TABLE 1. Concentrations (ng/L) of all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA (n = 3), and the Chemical-Derived all-*trans*-RA Equivalents (ATRA-EQ_{cal}) in the Seven STP Influents and Effluents

	all-*trans*-4-oxo-RA		13-*cis*-4-oxo-RA		ATRA-EQ_{cal}	
	influent	effluent	influent	effluent	influent	effluent
Gaobeidian	5.9 ± 0.2	0.5 ± 0.1	2.7 ± 0.1	0.4 ± 0.1	23	2.0
Beixiaohe	4.7 ± 1.1	0.5 ± 0.0	2.5 ± 0.6	1.1 ± 0.4	18	2.3
Fangzhuang	10.4 ± 1.1	0.9 ± 0.1	7.1 ± 1.8	0.8 ± 0.2	41	3.7
Xiaohongmen	5.3 ± 0.2	nd[a]	3.1 ± 0.4	1.0 ± 0.4	21	0.8
Wujiacun	4.8 ± 0.6	nd	2.3 ± 0.1	nd	19	0.5
Jiuxianqiao	4.8 ± 0.7	nd	2.6 ± 0.1	0.7 ± 0.1	19	0.7
Qinghe	4.9 ± 0.7	nd	2.7 ± 0.0	nd	19	0.5

[a] nd = no detection.

activity profile of HPLC F15 in three subtypes (sensitivity decreased in the order of $\alpha > \gamma > \beta$) and its maximal activities are highly similar to those of all-*trans*-RA, suggesting that the RAR agonists in HPLC F15 might have a chemical structure similar to all-*trans*-RA. Therefore, the swine serum and human urine samples were also fractionated by HPLC, and significant RARα agonistic activities were also detected in the HPLC F15 of both samples. These results indicated that the RAR agonists in wastewater are endogenous RAs. To identify the potential chemical, three isomers of RA including all-*trans*-RA, 13-*cis*-RA, and 9-*cis*-RA were injected into the HPLC system. However, the peaks of the three isomers of RA did not overlap with HPLC F15 (Figure 3), indicating that these RAs were not the causative chemicals for the RAR agonistic activity in wastewater.

Thus, we tested two commercially obtainable metabolites of RAs, all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA, which were reported to exhibit relatively high RAR-ligand affinity (*26, 27*). In our yeast assay for RARα-mediated activity, the EC_{50} for all-*trans*-4-oxo-RA was determined to be 0.32 nmol/L, much lower than all-*trans*-RA (1.35 nmol/L), while the EC_{50} for 13-*cis*-4-oxo-RA (3.2 nmol/L) was higher than that of all-*trans*-RA, as shown in Figure S5. The EC_{50} for all-*trans*-4-oxo-RA or 13-*cis*-4-oxo-RA relative to that of all-*trans*-RA was defined as its all-*trans*-RA equivalency factor (RAEF), which was calculated to be 3.87 and 0.46, respectively. As shown in Figure 3, the peaks of two isomers of 4-oxo-RA were observed at the retention times of 28.9 min (13-*cis*-4-oxo-RA) and 29.5 min (all-*trans*-4-oxo-RA), which overlapped with HPLC F15, suggesting that the 4-oxo-RAs might be the agonists of RAR in the samples. To further identify the causal chemicals inducing the RARα agonistic activity, UPLC fractionation for HPLC F15 from Gaobeidian STP influent was carried out, and the RARα activities of the fractions were also determined. As shown in Figure 4, the two bioactive fractions were found at the retention times of 3.5−4.0 min and 4.0−4.5 min (Figure 4A), overlapping with all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA standards at 3.55 and 4.13 min, respectively (Figure 4B). In the influent sample (Figure 4C), two peaks were also found at 3.55 and 4.13 min by detecting a 315 m/z to 241 m/z MRM transition, which were highly suspected to be all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. Figure S6 shows the MS/MS spectra of the base peak ion of m/z 315 (ESI positive ion mode) in two bioactive fractions, and we found that the mass spectra at 3.55 and 4.13 min are highly similar to those of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA standards (Figure S2), suggesting that all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA contributed to the RAR agonistic activity in the STP.

The HPLC F15 samples in the seven STPs, of which the RARα agonistic activities were reported above, were also analyzed by UPLC-ESI-MS/MS, and the concentrations in the influent and effluent samples without recovery correction are listed in Table 1. The concentrations of all-*trans*-4-oxo-RA were in the range of 4.7 to 10.4 ng/L in influents and below detection limit (<0.2 ng/L) to 0.9 ng/L in effluents, while the concentrations of 13-*cis*-4-oxo-RA were in the range of 2.3 to 7.1 ng/L in influents and below detection limit (<0.4 ng/L) to 1.1 ng/L in effluents. Based on the concentrations of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA and their RAEF values, the chemical-derived all-*trans*-RA equivalents (ATRA-EQ_{cal}) were calculated to be in the ranges of 18 ng/L (Beixiaohe STP) to 41 ng/L (Fangzhuang STP) and 0.5 ng/L (Wujiacun STP) to 3.7 ng/L (Fangzhuang STP) in influent and effluent, respectively.

For the river water samples, the concentrations of all-*trans*-4-oxo-RA were in the range of below detection limit (<0.2 ng/L) to 1.0 ng/L in summer and <0.2 to 1.8 ng/L in winter, while the concentrations of 13-*cis*-4-oxo-RA were in the ranges of below detection limit (<0.4 ng/L) to 1.6 ng/L in summer and <0.4 to 1.5 ng/L in winter (Tables S5 and S6). It should be noted that the ATRA-EQ_{cal} of samples taken at 4 and 2 km upstream Qing River in summer were much lower than the corresponding ATRA-EQ values derived from bioassay (Table S4), suggesting that there would be other unidentified RAR agonists in the river water and need to be studied further.

Previous studies have reported that all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA widely exist in human and animal serum (*36, 37*) and can be eliminated from the body through urinary excretion (*38*). In another study, Li et al. also detected the glucuronide conjugate of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in urine of rats that had been fed with all-*trans*-RA, 9-*cis*-RA, and 13-*cis*-RA previously (*39*). Thus, it is possible that all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA can be produced through deconjugation of their glucuronides in wastewater treatment plants or rivers. In this study, however, we did not aim at the fates of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in STP and their receiving river waters, and there is a need for further study.

All-*trans*-4-oxo-RA has been found to be much more active than all-*trans*-RA in causing microcephaly in *Xenopus laevis* embryos (*27*). In this study, the causal chemicals inducing RAR activity in the environment were identified for the first time, and there is a need to further investigate their occurrence, fates, and ecotoxicity to assess their ecological risk.

Acknowledgments

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Supporting Information Available

Additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Effects of coumestrol on lipid and glucose metabolism as a farnesoid X receptor ligand ☆

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ABSTRACT

In the course of an effort to identify novel agonists of the farnesoid X receptor (FXR), coumestrol was determined to be one such ligand. Reporter and *in vitro* coactivator interaction assays revealed that coumestrol bound and activated FXR. Treatment of Hep G2 cells with coumestrol stimulated the expression of FXR target genes, thereby regulating the expression of target genes of the liver X receptor and hepatocyte nuclear factor-4α. Through these actions, coumestrol is expected to exert beneficial effects on lipid and glucose metabolism.

The farnesoid X receptor (FXR; NR1H4) is a member of a nuclear receptor superfamily expressed predominantly in the intestine, kidney, and liver. Upon activation by bile acids, which are endogenous FXR ligands, FXR regulates the expression of genes involved in bile acid homeostasis, such as cholesterol-7α hydroxylase [1] and ileal bile acid binding protein [2]. The identification of phospholipid transfer protein (PLTP) [3] and apolipoprotein A-I [4] as FXR target genes suggests that FXR also controls triglyceride (TG) metabolism. Recent studies using FXR-deficient mice, in which serum glucose levels are elevated, demonstrated that FXR also has a regulatory role in glucose homeostasis [5]. In addition, FXR activation induces the expression of the small heterodimer partner (SHP; NR0B2) [6,7], which acts as a corepressor for several nuclear receptors, including liver X receptor α (LXRα; NR1H3) and hepatocyte nuclear factor-4α (HNF-4α; NR2A1) [8,9]. LXRα is a sterol-responsive transcription factor that regulates the expression of sterol response element-binding protein 1c (SREBP1c) [10,11], which in turn regulates lipogenic enzymes. The best-characterized HNF-4α target genes are those involved in lipid transport and glucose metabolism. Thus, FXR regulates lipid and glucose metabolism through the FXR-SHP cascade.

In general, nuclear receptors regulate gene expression in response to ligand binding through the direct recruitment of cofactors [12–14]. In the presence of agonists, the receptor binds coactivators (e.g., TIF2), and in turn associates with additional proteins to form a complex that activates transcription [15]. In the ab-

Abbreviations: ABCA1, ATP-binding cassette transporter A1; AP, alkaline phosphatase; apoB, apolipoprotein B; apoC-III, apolipoprotein C-III; BAP, bacterial alkaline phosphatase; CDCA, chenodeoxy cholic acid; CoA-BAP, coactivator-bacterial alkaline phosphatase; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FAS, fatty acid synthase; FXR, farnesoid X receptor; GST, glutathione-*S* transferase; HNF-4α, hepatocyte nuclear factor-4 α; LBD, ligand binding domain; LXRα, liver X receptor α; MTP, microsomal triglyceride transfer protein; NID, nuclear receptor interaction domain; NR, nuclear receptor; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; PLTP, phospholipid transfer protein; RT-PCR, reverse-transcription polymerase chain reaction; SHP, small heterodimer partner; SREBP1c, sterol response element-binding protein 1c; TG, triglyceride.

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sence of agonists, nuclear receptors bind corepressors (e.g., NCoR) and form a complex that represses transcription [16]. The coactivator-bacterial alkaline phosphatase (CoA-BAP) system developed by Kanayama et al. is a new cell-free assay system for evaluating ligand activity that is based on the ligand-dependent interactions between nuclear receptors and cofactors [17]. Using this system to screen food compounds and their derivatives, we determined that coumestrol is a potent FXR agonist. We show here that coumestrol activates FXR, and thereby regulates the expression of genes involved in lipid and glucose metabolism.

Materials and methods

Materials. Coumestrol, chenodeoxy cholic acid (CDCA), and 17-β-estradiol were purchased from Sigma–Aldrich Co. (St. Louis, MO). An FXR ligand, GW4064, was custom synthesized.

CoA-BAP system. LBD protein (ligand binding domain of nuclear receptors) fused to glutathione-*S* transferase (GST) and NID protein (nuclear receptor interaction domain of cofactors) fused to BAP were prepared as described previously [17]. The purified GST-hFXR LBD protein sample, diluted in 0.1 M $NaHCO_3$ (pH 8.4), was incubated on 96-well plates (MaxiSorp; Nalge Nunc Internationals, Roskilde, Denmark). After overnight incubation at 4 °C, excess protein was removed. An appropriate concentration of a test chemical was then added. After 1 h of incubation at 4 °C, excess test chemical was aspirated and purified hTIF2 NID-BAP fusion protein was applied. After 1 h incubation at 4 °C and subsequent washing, *p*-nitrophenyl-phosphoric acid was added to each well as a BAP substrate, and the enzyme reaction was performed at 30 °C. After an appropriate incubation period, the alkaline phosphatase (AP) activity was measured spectrophotometrically at 405 nm on a microtiter plate reader.

Cell culture. The human hepatoma cell line Hep G2 and the embryonic kidney cell line HEK293T were cultured as previously described [18]. The breast cancer cell line MCF-7 was maintained in minimal essential medium (MP Biomedicals, Irvine, CA) supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Reporter assays. The expression plasmids pGAL4-hFXR and pFLAG-hFXR [19] and reporter plasmid pSHPp-Luc [18] were previously described. HEK293T cells (12-well plate) were transfected with 300 ng of a reporter plasmid (pUAS-Luc or pSHPp-Luc) and 100 ng of a hFXR expression plasmid (pGAL4-hFXR or pFLAG-hFXR), together with 100 ng of a SV40-gal internal control plasmid using the calcium phosphate transfection method. Twenty-four hours after transfection, the cells were treated with test chemicals in minimal essential medium (phenol red free) supplemented with 5% charcoal-stripped fetal bovine serum. After 24 h exposure to the chemicals, luciferase activities were quantified using a Luciferase Assay Systems (Promega, Madison, WI) according to the manufacturer's instructions. β-Galactosidase activity was used as an internal control.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from Hep G2 cells or MCF-7 cells using an RNA extraction reagent (ISOGEN; Nippon Gene, Toyama, Japan) and reverse-transcribed using TaqMan reverse transcriptase reagents (Applied Biosystems, Weiterstadt, Germany). Fluorescent real-time PCR was performed using SYBR green PCR master mix on an ABI 7700 system. The sequences of the primers used for quantitative RT-PCR are shown in Supplementary Information Table 1. The mRNA expression data were normalized to 36B4 mRNA levels. TaqMan Gene Expression Assays were used for pS2/TFF1 (Applied Biosystems).

ApoB ELISA assay. Hep G2 cells were cultured in medium containing 5% lipoprotein-deficient serum and treated with test chemicals for 3d. At 18 h before the end of the 3d treatment, the media were replaced with serum-free media. At the end of the treatment the media were collected and apoB secretion was measured using an ApoB Microwell ELISA Assay Kit (ALerCHEK Inc., Portland, ME) according to the manufacturer's instructions. ApoB secretion was normalized by cell protein determined using a bicinchoninic acid assay (Pierce Chemical Co., Millford, IL).

Results

Ligand-dependent interaction between nuclear receptor and cofactor

To assess the FXR agonist activity of the test compounds, we used an in vitro coactivator interaction assay, the CoA-BAP system. We confirmed that a natural FXR ligand, CDCA, and a synthetic ligand, GW4064, enhanced the AP activity in a dose-dependent manner. Coumestrol increased the AP activity in the FXR-TIF2 system (Fig. 1). In the absence of FXR LBD (GST alone), there was no apparent increase in the AP activity.

Effects of coumestrol on transcriptional activity of FXR

To investigate the effect of coumestrol on FXR transcriptional activity, we used a GAL4 system using a fusion protein that included a GAL4 DNA-binding domain and a full-length hFXR. Micromolar concentrations of coumestrol increased the reporter activity dose-dependently, while CDCA (100 μM) and GW4064 (0.1 μM) caused approximately 30- and 80-fold inductions, respectively (Fig. 2A).

To further examine the effect of coumestrol on FXR transcriptional activity, a reporter assay was performed using the human SHP promoter-containing reporter gene. The reporter activity was induced by 50 μM coumestrol to 2.0-fold over that in non-treated cells, while CDCA (100 μM) and GW4064 (1 μM) enhanced the reporter activity 4- and 3-fold, respectively (Fig. 2B).

Effects of coumestrol on the expression of genes involved in lipid and glucose metabolism

Although the GAL4 system in Fig. 2A revealed that coumestrol is a less potent ligand than CDCA, another reporter assay in Fig. 2B showed comparable effects between them. To evaluate the effect of coumestrol on lipid and glucose metabolism, we performed

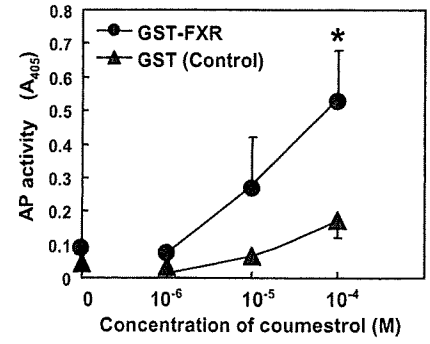

Fig. 1. Assessment of FXR ligand activity using a CoA-BAP system. The CoA-BAP assay was performed with increasing amounts of coumestrol, as described in the Materials and methods. Ligand-dependent interactions between GST-hFXR LBD and hTIF2 NID-BAP were determined as AP activity. AP activity without GST fusion protein was measured as background. Means ± SD ($n = 3$) are shown. $^*P < 0.05$ compared with control (GST alone).

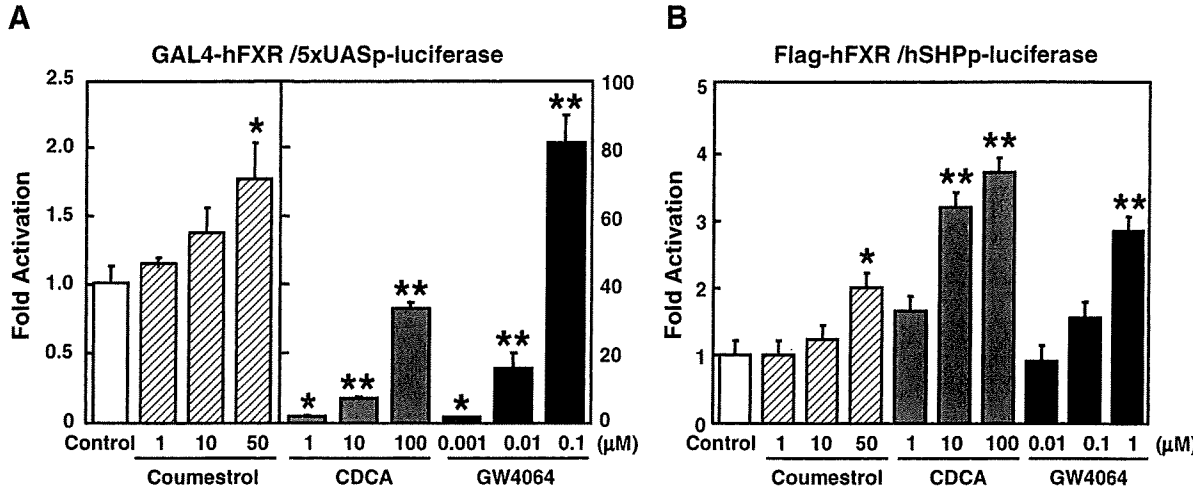

Fig. 2. Coumestrol stimulates FXR transcriptional activity. HEK293T cells were cotransfected with a pGAL4-hFXR expression plasmid and a pUAS-Luc reporter plasmid together with a SV40-gal internal control plasmid and treated for 24 h with coumestrol (1, 10, or 50 μM), CDCA (1, 10, or 100 μM), GW4064 (0.001, 0.01, or 0.1 μM), or DMSO (control) (A). HEK293T cells were cotransfected with a pFLAG-hFXR expression plasmid and a reporter plasmid of SHPp-Luc, together with a SV40-gal internal control plasmid, and treated with coumestrol (1, 10, or 50 μM), CDCA (1, 10, or 100 μM), GW4064 (0.01, 0.1, or 1 μM), or DMSO (control) (B) for 24 h before assaying luciferase activity. Means ± SD ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared vehicle-treated cells.

quantitative RT-PCR to measure the mRNA levels of metabolism-related genes. The expression levels of FXR target genes, such as SHP and PLTP, were upregulated in Hep G2 cells treated with coumestrol as well as CDCA for 24 h (Fig. 3A), suggesting that the GAL4 system in Fig. 2A underestimated coumestrol functions. Coumestrol treatment decreased the expression of LXR target genes, such as SREBP1c and ATP-binding cassette transporter A1 (ABCA1), SREBP1c target fatty acid synthase (FAS) (Fig. 3B), and HNF-4α target genes, such as microsomal triglyceride transfer protein (MTP), apolipoprotein B (apoB), apolipoprotein C-III (apoC-III), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) (Fig. 3C). To exclude the possibility that the upregulation of SHP by coumestrol was accomplished via the estrogen receptor (ER) [20], we evaluated the estrogen responsiveness of Hep G2 cells. In ER-positive MCF-7 cells treated with 10 nM 17-β-estradiol, expression of the ER target gene pS2/TFF1 increased over 14-fold compared to untreated controls, whereas neither pS2/TFF1 nor SHP were induced by 17-β-estradiol in Hep G2 cells (data not shown).

Reduced apolipoprotein B secretion from Hep G2 cells

To examine whether the suppression of MTP and apoB gene expression leads to reduced apoB secretion, we determined the

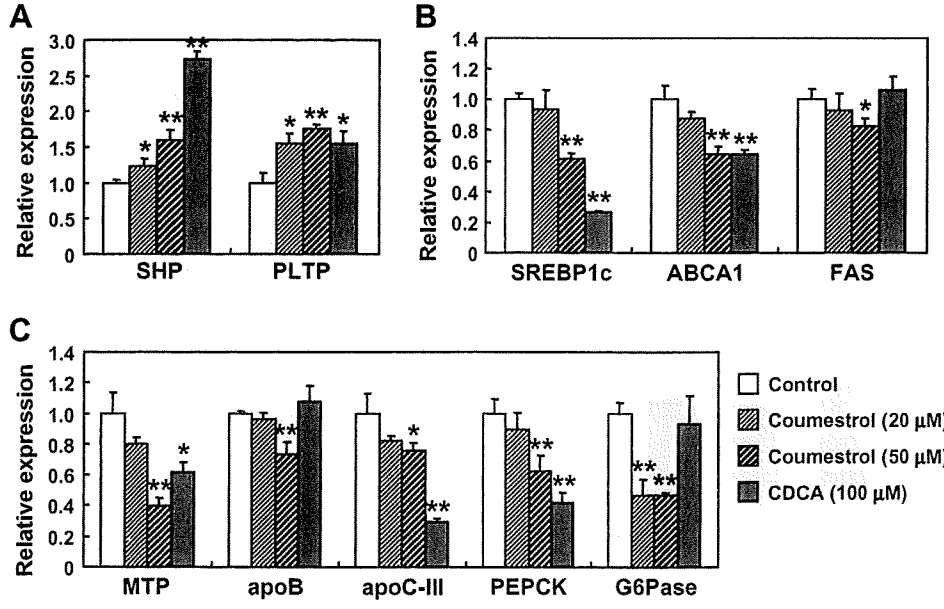

Fig. 3. Coumestrol regulates the expression of genes that are involved in lipid and glucose metabolism. Hep G2 cells were incubated with DMSO (control), coumestrol (20 or 50 μM), or CDCA (100 μM) for 24 h. Hepatic expression levels of FXR target genes (SHP and PLTP) (A), LXR target genes (SREBP1c, ABCA1, and FAS) (B), and HNF-4 target genes (MTP, apoB, apoC-III, PEPCK and G6Pase) (C) were measured using quantitative RT-PCR. Data are presented as the fold-change in gene expression relative to that in DMSO-treated control cells. Means ± SD ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated cells.

levels of apoB secreted in the culture medium of Hep G2 cells incubated with coumestrol. Coumestrol (50 μM) reduced apoB secretion by 50% compared to the control, comparable to the decrease observed with CDCA (Fig. 4). Although CDCA treatment had no significant effect on apoB gene expression (Fig. 3C), it seems likely that MTP activity should be a determinant for apoB-containing lipoprotein secretion.

Discussion

Coumestrol is a coumestan phytoestrogen present in soy sprouts and alfalfa [21]. Previous studies demonstrated that coumestrol binds ERα and ERβ [22], effectively lowers total serum cholesterol in ovariectomized rats [23], and prevents ovariectomy-induced bone loss [24]. These effects of coumestrol are generally considered to be due to its estrogenic activity. ERα directly binds the SHP promoter and enhances transcription [20]. This raises the possibility that coumestrol enhanced SHP gene expression in Hep G2 cells through activation of ERα in Fig. 3. Consistent with the previous finding that Hep G2 cells lack any ER activities [20], 17-β-estradiol stimulated the expression of pS2/TFF1, an ER target gene, only in the ER-positive cell line MCF-7, but not in Hep G2 cells. These findings indicate that coumestrol increases SHP gene expression through the direct activation of FXR.

The present study shows a novel regulatory mechanism by which coumestrol exerts its beneficial effects through FXR activation. Reporter gene and *in vitro* coactivator interaction assays revealed that this compound is a potent FXR agonist, thereby bringing about the increased expression of FXR target genes. The subsequent increase in SHP suppresses the expression of LXR and HNF-4α target genes. At the cellular level, coumestrol decreased apoB secretion, as reflected by the downregulation of MTP and apoB gene expression. These results indicate that coumestrol exerts various effects as a bifunctional ligand via an FXR-mediated pathway as well as through an ER-mediated pathway.

In the current experiments, high levels of coumestrol concentration (~50 μM) were required to exert significant effects resembling endogenous FXR ligand, CDCA (100 μM). However, given that both of them were effective at the comparable concentration, coumestrol is likely to be one of potent FXR ligands. Cholic acid, another endogenous FXR ligand, prevents hepatic TG accumulation, very-low density lipoprotein secretion, and elevated serum TG in mouse models of hypertriglyceridemia [25]. Recent *in vivo* studies also demonstrate that FXR ligands are potentially useful targets for the treatment of cholestatic liver disease [6] and dyslipidemia [26]. Taken together, our findings suggest that coumestrol might improve metabolic disorders such as hypertriglyceridemia, fatty liver disease and diabetes, at least partly through the activation of FXR.

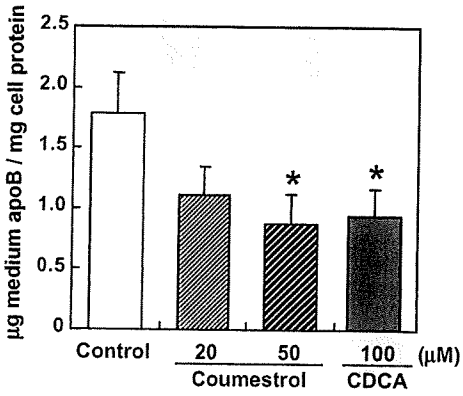

Fig. 4. Coumestrol reduced apoB secretion from Hep G2 cells. Hep G2 cells were cultured in medium containing DMSO (control), coumestrol (20 or 50 μM), or CDCA (100 μM) for 3 d, and apoB secretion into media for the last 18 h was measured using ELISA. Means ± SD ($n = 3$) are shown. $^*P < 0.05$ compared with vehicle-treated cells.

A recent report demonstrated that coumestrol functions as a naturally occurring agonist of the pregnane X receptor [27]. In the report, this compound was evaluated as a week agonist of several nuclear receptors including FXR using a GAL4 system tha must be a similar system we used. It is possible that coumestro greatly regulated gene expression of FAS, apoB and G6Pase rathe than CDCA (Fig. 3) by taking advantage of the ligand activities o multiple nuclear receptors.

In conclusion, the results of the present study indicate tha coumestrol activates FXR and regulates the expression of genes involved in lipid and glucose metabolism, suggesting that coumestrol exerts beneficial effects on metabolic disorders, including hypertriglyceridemia and diabetes. Moreover, the results provide evidence that the CoA-BAP system, in combination with various other analyses, is a practical and useful new strategy for identifying novel bioactive substances.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.136.

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Screening and detection of the *in vitro* agonistic activity of xenobiotics on the retinoic acid receptor

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Abstract

The retinoic acid receptors (RARs) play key roles in various biological processes in response to endogenous retinoic acids. However, excessive embryonic exposure to specific ligands for each subtype of the RAR was reported to induce specific developmental abnormalities. We measured the RAR agonistic activity of 543 chemicals using an assay system adopting yeast cells transfected with the human RARγ and a coactivator. Eighty-five of the 543 chemicals, including 16 organochlorine pesticides, 14 styrene dimers, 9 monoalkylphenols and 6 parabens, exhibited RARγ agonistic effects in this assay. In particular, monoalkylphenols having a 6–9 carbon alkyl group *para* to the phenolic hydroxyl group possessed high affinity for the RARγ, and their activities were 1.363–0.446% of that of *all-trans* RA. *para*-Alkylphenols chlorinated at the *ortho* position also were about as active or more active than their unchlorinated analogs. In addition, all tested styrene dimers showed positive effects, and the activity of 1-phenyltetralin, the strongest in this category, was 1.169% that of *all-trans* RA. A number of chemicals having binding affinity for the RARγ were revealed in this study (both newly identified and confirmed), further comprehensive studies of *in vitro* and *in vivo* effects via the RARs are required for the reliable risk assessment of chemicals. *In vitro* receptor binding studies represent an important step in hazard identification and suggest a potential mechanism of action, which can be an important step in risk assessment and in particular for screening studies to identify potential toxicity and inform mechanistic studies.

Keywords: Retinoic acid receptor; Two-hybrid yeast assay; Monoalkylphenol; Styrene dimer; Paraben; Organochlorine pesticide

1. Introduction

The retinoic acid (RA) receptors (RARs) are, like the steroid hormone and thyroid hormone receptors (TR), nuclear receptors that respond to specific natural ligands, *all-trans* RA and 9-*cis* RA. Both RAs, which are oxidative metabolites of vitamin A, are essential for cellular proliferation, development and differentiation in vertebrates and therefore play crucial roles in normal growth and homeostasis. However, both deficiency and excess of vitamin A are harmful. Vitamin A deficiency during gestation results in diverse embryonic malformations in various organs (Zile, 1998, 2001), while excess RA has been reported to trigger teratogenic actions in the developing embryo via the RARs. Ligands specific for each subtype of the RARs (α, β and γ) have been reported to induce specific deformities in rodent embryos (Elmazar et al., 1996, 2001). A ligand for the α-subtype causes defects of the ear, mandible and limb, a β-subtype ligand causes defects of the urinary system and liver, and a γ-subtype ligand causes ossification deficiencies and defects of the sternebrae and vertebral body.

We have developed several yeast two-hybrid systems transduced with the ligand binding domains of nuclear receptors and a coactivator for the receptors for detecting and measuring the activity of chemicals (Nishikawa et al., 1999; Shiraishi et al., 2000). In prior works, it has been found that there are a number of industrial/environmental

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compounds with the capability to activate or inactivate nuclear receptors such as the estrogen receptors (ERs) and the TRs, and some compounds show unexpected activity; i.e., activity that would not be readily predicted from the structure of the compounds (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). These studies indicated that chemicals encountered in daily life or through accidental exposure bind with nuclear receptors and suggested potential toxicity, mechanisms of action and therefore potential risks to human health. In our laboratory, yeast assays with nuclear receptors derived from the Japanese medaka fish (*Oryzias latipes*) as well as the human have also demonstrated the receptor activation of environmental water samples taken from contaminated rivers, lakes and seas (Mispagel et al., 2005; unpublished data, Shiraishi et al., 2006) and thereby show that organisms in the environment may be exposed to potentially harmful chemicals and/or bioactive substances originating from human activity.

The objective of the present study was to screen a wide range of xenobiotic and other compounds for agonistic effects on the RAR and to quantify their activities. We prepared a yeast two-hybrid system to detect transcriptional activation via the human RARγ and assessed 543 compounds including industrial chemicals, agrochemicals, natural compounds, medicines and cosmetic chemicals. An assay for each human RAR subtype was derived from previously reported yeast assays and optimized for high-throughput screening. Because the yeast cells transfected with the γ-subtype showed the lowest luminescence intensity when inactivated and the highest reactivity to an endogenous ligand, *all-trans* RA, we selected the RARγ type of assay for the present investigation. As the RAR agonistic effect was detected in different categories of chemicals, we provide the measured activities of the positively-reacting compounds grouped according to chemical structure.

2. Materials and methods

2.1. Compounds

The 543 compounds examined in this study are listed in Table 1, grouped according to their intended uses and chemical structures. The compounds were purchased from Accu Standard, Inc. (New Haven, CT, USA), Acros Organics N.V. (Geel, Belgium), Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany), Cosmo Bio Co., Ltd. (Tokyo, Japan), Dr. Ehrenstorfer GmbH (Augsburg, Germany), GL Science, Inc. (Tokyo, Japan), Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Katayama Chemical Industries Co., Ltd. (Osaka Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Maruzen Petrochemical Co., Ltd. (Tokyo, Japan), MP Biochemicals (Solon, OH, USA), PerkinElmer, Inc. (Wellesley, MA, USA), Scientific Polymer Products, Inc. (Ontario, NY, USA), Sigma–Aldrich Corp. (St. Louis, MO, USA), Steraloids, Inc. (Newport, RI, USA), Tocris Bioscience (Ellisville, MO, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Toronto Research Chemicals Inc. (North York, ON, Canada) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or were gifts from researchers who had synthesized them for other purposes.

2.2. Yeast two-hybrid assay

The transcriptional agonistic activities of compounds to the RAR were measured with a reporter assay using yeast cells (*Saccharomyces cerevisiae* Y190). An expression plasmid for the ligand binding domain of the human RARγ and the coactivator pGAAD424-TIF-2 was introduced into yeast cells that carried the β-galactosidase reporter gene (Nishikawa et al., 1999). The assay was performed using a chemiluminescent reporter gene method (for β-galactosidase) employing a 96-well culture plate, based on a yeast two-hybrid estrogenicity assay (Shiraishi et al., 2000, 2003).

Yeast cells were preincubated for 24 h at 30 °C with shaking in modified SD medium lacking tryptophan and leucine (5.8% yeast nitrogen base, 0.75% dextrose, 0.013% L-valine, 0.00435% L-phenylalanine, 0.00261% L-isoleucine, L-lysine HCl and L-tyrosine, 0.00174% L-adenine hemisulfate salt, L-arginine HCl, L-histidine HCl monohydrate, L-methionine and L-uracil) and the cell density was adjusted to an absorbance of 1.65–1.80 at 595 nm. A dimethylsulfoxide (DMSO) solution of each test compound was stored at −80 °C until just before examination and was serially twofold diluted with the medium. An aliquot of the diluted solution (120 μl) was poured to two wells of a black 96-well culture plate for chemiluminescence measurement, and then the yeast cell suspension (60 μl) was added. At least seven serial two-fold concentrations of each chemical from 10 μM to 156 nM were tested; lower concentrations were tested for chemicals showing high RAR agonistic activity. The solution in every well contained 1% DMSO. After vortex mixing, the plate was incubated at 30 °C under conditions of high humidity for 4 h. A solution (80 μl) for inducing chemiluminescence from released β-galactosidase, consisting of reaction buffer (30 μl) containing GalactLux substrate (AURORA GAL-XE, MP Biochemicals) and a 1:1 mixture (50 μl) of zymolyase 20 T and 100 T solutions for enzymatic digestion (Kirin Brewery Co, Ltd., Tokyo, Japan), was added to each well. The plate was incubated at 37 °C for 1 h and then placed in a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan), and a light emission accelerator solution (AURORA GAL-XE, 50 μl) was added to each well using the luminometer pump. The chemiluminescence produced by released β-galactosidase in each well was measured.

All test compounds were evaluated in a minimum of three separate assays which were performed in duplicate. For comparative estimates of the ability of test compounds to activate the RAR, *all-trans* RA, an endogenous agonist

Table 1
List of 543 compounds tested in a yeast two-hybrid assay for the RARγ

Compounds		
Industrial chemicals (252)	Bisphenols and related chemicals (Continued)	Parabens (13)
Aromatic hydrocarbons (10)	4-Hydroxyphenyl isopropanol	3-Hydroxybenzoic acid
Biphenyl	4-Hydroxyphenyl isobutyl methyl ketone	4-Hydroxybenzoic acid
2-Terphenyl	6-Hydroxy-1-(4-hydroxyphenyl)-1,3,3-trimethylindane	Methyl 4-hydroxybenzoate
3-Terphenyl	4-Cumylphenol	Ethyl 4-hydroxybenzoate
4-Terphenyl	2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman	Propyl 4-hydroxybenzoate
n-Butylbenzene	6-Hydroxy-3-(4-hydroxyphenyl)-1,1,3-trimethylindane	Isopropyl 4-hydroxybenzoate
n-Octylbenzene	2,4-Bis(4-hydroxycumyl)phenol	Butyl 4-hydroxybenzoate
n-Nonylbenzene	4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman	Isobutyl 4-hydroxybenzoate
1,3-Diphenylpropane	4-Propenylphenol	n-Amyl 4-hydroxybenzoate
Benzylbiphenyl	4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene	n-Hexyl 4-hydroxybenzoate
Triphenylmethane	2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman	Benzyl 4-hydroxybenzoate
		2-Ethylhexyl 4-hydroxybenzoate
Polycyclic aromatic hydrocarbons (14)		n-Dodecyl 4-hydroxybenzoate
Naphthalene		
Acenaphthene	Dyes (21)	Phenols and related chemicals (37)
Fluorene	Azobenzene	4-Cyanophenol
Phenanthrene	N-Acetyl-5-hydroxy-tryptamine	4-Hydroxybenzaldehyde
Anthracene	Acid Alizarin Violet N	4-Methoxyphenol
Pyrene	1-Acetyl-4-(4-hydroxyphenyl)-piperazine	2-Cyclopentylphenol
Fluoranthene	Anthrarobin	4-Cyclopentylphenol
Chrysene	Anthraflavic acid	2,4-Dichlorophenol
Benz[a]anthracene	cis-1,3-O-Benzylideneglycerol	3,4-Dichlorophenol
Benzo[a]pyrene	Chlorophenol Red	4-Hydroxycinnamic acid
Benzo[e]pyrene	2,5-Dihydroxyphenylacetic γ-lactone	2-Hydroxybiphenyl
Benzo(k)fluoranthene	4-(2,4-Dinitroanilino)-phenol	4-Hydroxybiphenyl
Benzo(b)fluoranthene	6-Fluoro-4-hydroxy-coumarin	4-Cyclohexylphenol
Dibenz(a,h)anthracene	Hoechst 33258	4-Benzylphenol
	6-Hydroxy-1,3-benzoxathiol-2-one	4-Hydroxybezophenone
Arsenic compounds (13)	2-Hydroxy-9-fluorenone	Phenyl salicylate
Sodium meta-arsenite	4-Hydroxyindole	2-Iodophenol
Dimethyl arsinic acid	4-Hydroxy-6-methyl-2-pyrone	4-Iodophenol
Methyl arsonic acid	3-Hydroxy-1H-phenalen-1-one	1,1-Bis(4-hydroxyphenyl)-propane
Arsenic(III)oxide	2-(4-Hydroxyphenyl)-5-pyrimidinol	4-(1-Adamantyl)phenol
Phenyldimethylarsine oxide	1-Hydroxypyrene	2-(1-Adamantyl)-4-methylphenol
Phenylmethylarisine oxide	Indigo, carmine	2,4-Dibromophenol
Phenyl arsonic acid	Indigo, synthetic	2,6-Dibromophenol
Diphenylmethylarsine oxide		3-(4-Hydroxyphenyl)propionic acid N-hydroxysuccinimide ester
Diphenyl arsinic acid	Metals (5)	Hexestrol
Triphenyl arsine	Tributyltin(IV)chloride	4,4-Bis-(4-hydroxyphenyl)valeric acid
Sodium arsenate (dibasic) 7H2O	Triphenyltin(IV)chloride	Phenolphthalein
Triphenyl arsine oxide	Dibutyltin(IV)dichloride	4,4-(1,3-Adamantane-diyl)diphenol
10,10'-Oxybis(phenoxyarsine)	Lead (II) chloride	2,4,6-Tribromophenol
	Lead (II) acetate trihydrate	3,5-Diiodosalicylic acid
Bisphenols and related chemicals (37)		Butylphenoxy acetic acid
Octafluoro-4,4'-biphenol	Monoalkyl phenols and related chemicals (27)	2,3-Dichlorophenoxy acetic acid
2,2',6,6'-Tetramethylbisphenol A	2-n-Propylphenol	2-Chloro-4-butylphenoxy acetic acid
4,4'-Thiodiphenol	3-n-Propylphenol	4-Nonylphenyl 2-hydroxyethyl ether
Bisphenol A	4-n-Propylphenol	2,6-Dichloro-4-butylphenoxy acetic acid
2,2',6,6'-Tetrabromobisphenol A	2-Isopropylphenol	2-Chloro-4-nonylphenyl 2-hydroxyethyl ether
2,2',6,6'-Tetrachlorobisphenol A	3-Isopropylphenol	2-Chloro-4-octylphenoxy acetic acid
Bisphenol B	4-Isopropylphenol	4-Nonylphenyl 2-(2hydroxy-ethyloxy)ethyl ether
2-Chlorobisphenol A	4-n-Butylphenol	2,6-Dichloro-4-nonylphenyl 2-hydroxyethyl ether
2,2'-Dichlorobisphenol A	2-s-Butylphenol	
2,6-Dichlorobisphenol A	4-s-Butylphenol	
2,2',6-Trichlorobisphenol A	2-t-Butylphenol	Phthalates (9)
4,4'-Methylenebisphenol (Bisphenol F)	3-t-Butylphenol	Diethyl phthalate
4,4'-Sulfonyldiphenol (Bisphenol S)	4-t-Butylphenol	Di-n-propyl phthalate
3,4-(1-Methyl-1-phenylethyl)diphenol	4-n-Pentylphenol	Di-n-butyl phthalate
2,4'-Isopropylidenediphenol	4-t-Pentylphenol	Di-n-pentyl phthalate
4,4'-(1,3-Dimethylbutylidene)bisphenol	4-n-Hexylphenol	Benzyl n-butyl phthalate
Bisphenol E	4-n-Heptylphenol	Dicyclohexyl phthalate
2,4'-Dihydroxydiphenyl sulfone (Bisphenol S iso.)	4-n-Octylphenol	Di-n-hexyl phthalate
2,2'-Isopropylidenediphenol	4-t-Octylphenol	Dibenzyl terephthalate
4,4'-Cyclohexyliden bisphenol	4-n-Nonylphenol	Di-2-ethylhexyl phthalate
4,4'-(1-Ethyl-2-methyltrimethylene)bisphenol	4-Nonylphenol (mixed isomers)	
4,4'-Diisobutylethylidenediphenol	4-Dodecylphenol (mixed isomers)	
4-Hydroxyacetophenone	2-Chloro-4-butylphenol	
4-Hydroxy-4'-isopropoxy diphenylsulfone	2,6-Dichloro-4-butylphenol	
4,4'-Isopropoxy diphenylsulfone	2-Chloro-4-octylphenol	
4-[1-(4-Hydroxyphenyl)-1-methylethyl]-1,2-benzoquinone	2,6-Dichloro-4-octylphenol	
	2-Chloro-4-nonylphenol	
	2,6-Dichloro-4-nonylphenol	

Table 1 (*continued*)

Compounds		
Industrial chemicals (Continued)	Agrochemicals (124)	Organophosphates (Continued)
Styrene dimers (14+1)	Amides (2)	EPN
1,4-Diphenyl-2-butene	Mepronil	Ethion
1-Methyl-1-phenylindan	Flutolanil	Glyphosate
1-Methyl-3-phenylindan		Iprobenfos
1-Phenyltetralin	Benzimidazoles (2)	Isofenphos
2,3-Diphenyl-1-butene	Carbendazim	Isofenphos oxon
2,4-Diphenyl-1-butene	Thiabendazole	Isoxathion
cis-1,4-Diphenyl-1-butene		Isoxathion oxon
trans-1,4-Diphenyl-1-butene	Carbamates (12)	Leptophos
cis-2,4-Diphenyl-2-butene	Aldicarb	Malathion
trans-2,4-Diphenyl-2-butene	Benfuracarb	MEP oxon
cis-1,2-Diphenylcyclobutane	Benomyl	Parathion
trans-1,2-Diphenylcyclobutane	Carbaryl (NAC)	Phenthoate
trans-1,3-Diphenyl-1-butene	Mancozeb	Piperophos
trans-1,3-Diphenylcyclobutane	Maneb	Prothiophos
Polystyrene standard (mixed styrene dimer isomers)	Methomyl	Thenylchlor
	Metiram	Tolclofos-methyl
Styrene trimer (16)	Molinate	
1,2,4-Triphenylcyclohexane	Thiobencarb	Pyrethroids (16)
1,3,5-Triphenyl-1-hexene	Zineb	Allethrin
1,3,5-Triphenylcyclohexane	Ziram	cis-Permethrin
1,4,5-Triphenyl-1-hexene		Cycloprothrin
1a-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Diphenyl ethers (7)	Cyfluthrin
1a-Phenyl-4a-(2-phenylethyl)tetralin	Acifluorfen	Cyhalothrin
1a-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Aclonifen	Cypermethrin
1e,2e,4a-Triphenylcyclohexane	Bifenox	Esfenvalerate
1e-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Chlomethoxynil	Ethofenprox
1e-Phenyl-4a-(2-phenylethyl)tetralin	Chlomitrofen (CNP)	Fenpropathrin
1e-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	CNP-amino	Fenvalerate
1-Methyl-1,2,4-triphenylcyclopentane	Nitrofen	Flucythrinate
1-Methyl-1,3,4-triphenylcyclopentane		Fluvalinate
1-Methyl-3-phenyl-2-(1-phenylethyl)indan	Organochlorines (30)	Permethrin
2,4,6-Triphenyl-1-hexene	Aldrin	Resmethrin
2,4,6-Triphenyl-2-hexene	β-Benzene hexachloride (β-BHC)	Tralomethrin
	Chlordane	trans-Permethrin
Others (35)	cis-Chlordane	
4-Nitrotoluene	trans-Chlordane	Triazines (4)
1-Naphthol	Chlordecone	Atrazine
2-Naphthol	o,p'-DDD	Dimethametryn
4-Hydroxy-1-indanone	o,p'-DDE	Metribuzin
5,6,7,8-Tetrahydro-1-naphthol	o,p'-DDT	Simazine (CAT)
5,6,7,8-Tetrahydro-2-naphthol	p,p'-DDD	
Sodium pyrithione	p,p'-DDE	Ureas (2)
1,2-Benzothiazol-3-one	p,p'-DDT	Diuron
Biphenyl ether	Dicofol	Pencycuron
2-n-Octyl-4-isothiazolin-3-one	Dieldrin	
N,N',N''-Trishydroxyethylhexahydro-s-triazine	α-Endosulfan	Others (22)
Benzyl-2-naphthylether	β-Endosulfan	Alachlor
4-Nonylcatechol	Endrin	Amitrole
1-(4-Hydroxyphenyl)-1-nonanol	Fthalide	Bromofenoxim
Methyl o-benzoylbenzoate	Heptachlor	Buprofezin
1,2-Bis(3-methylphenoxy)ethane	cis-Heptachlor epoxide	Captan
Triphenylborane	trans-Heptachlor epoxide	Chlorothanonil
1-Nitropyrene	Hexachlorobenzene	Counachlor
2-Iodobenzoic Acid	1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	Dazomet
Dipyrithione	Methoxychlor	1,2-Dibromo-3-chloropropane (DBCP)
6-Bromoharman	Mirex	Dichlofluanid
Phenyl-1-hydroxy-2-naphthoate	trans-Nonachlor	(2,4-Dichlorophenoxy)acetic acid
β-Naphthoflavone	Oxychlordane	2-(3-Chlorophenoxy)-propionic Acid
Zinc pyrithione	Pentachlorophenol (PCP)	1,3-Dichloropropene
Triphenylborane-pyridine complex	Toxaphene	Fluazifop-butyl
6,8-Dibromoharman	2,4,5-Trichlorophenoxyacetic Acid	Oxadiazon
meso-Stilbene dibromide		Pendimethalin
Copper quinolate	Organophosphates (27)	Probenazole
Di(2-ethylhexyl)adipate	Bromophos-ethyl	Pyroquilon
Octachlorostyrene	Bromopropylate	Tebuconazole
3,6,8-Tribromoharman	Butamifos	Tebufenozide
Triphenylborane-octadecylamin complex	Chlorpyrifos	Trifluralin
Perfluorobutansulfonate (potassium salt)	Chlorpyrifosmethyl	Vinclozolin
Chlorhexidine Hydrochloride	Cyanofenphos	
1,2,5,6,9,10-Hexabromocyclododecane	Diazinon	
	Diazinon oxon	
	Dichlofenthion	
	Edifenphos	

(*continued on next page*)

Table 1 (*continued*)

Compounds		
Natural compounds and related chemicals (109)	Natural compounds (continued)	Medicines and cosmetic-related chemicals (58)
Natural compounds (72)	Melatonin	Medicines (30)
cis-Stilbene	Naringenin	Allyl-thiourea
trans-Stilbene	Naringin	Amiodarone
Azulene	Progesterone	5α-Androstane
Dibenzyl	Resveratrol	Benzophenone
Acridine	Retene	Benzoyl peroxide (BPO)
Flavone	9-cis-Retinoic Acid	Camphorquinone
Biochanin A	13-cis-Retinoic acid	Cinnarizine
Quercetin Dihydrate	all-trans-Retinal	Clofibrate
Genistein	all-trans-Retinoic acid	Clomiphene
17β-Estradiol	all-trans-Retinol	Cyproterone acetate
Zearalenone	3,5-Diiodo-L(+)-tyrosine dihydrate	Dexamethasone
Daidzein	T3 (3,5,3'-Triiodothyronine)	Dienestrol
β-Estradiol 3-Sulfate	T4 (3,3',5,5'-Tetraiodothyronine)	Diethylstilbestrol (DES)
β-Estradiol 3,17-Disulfate	3,3',5'-Triiodo-L-Thyronine	Dimethylaminoethyl methacrylic acid
β-Estradiol 17-(β-D-Glucuronide)	Vitamin A acetate	Dimetyl-p-toluidine
β-Estradiol 3-(β-D-Glucuronide)		Ethynylestradiol
Testosterone	Natural product-related chemicals (37)	Flutamide
Kaempferol	6,8-Dichlorochrysin	Hydroxypropionic Acid
Abietic Acid	6,8-Dichloroapigenin	4-Hydroxytamoxifen
Phloretin	3',8-Dichlorodaidzein	ICI 182780
Apigenin	3',5',8-Trichlorodaidzein	3-Iodo-L-tyrosine
4',5,7-Trihydroxyflavanone	6,8-Dichlorogenistein	Methyltrienolone
Coumestrol	6,8-Dichloronaringenin	Mibolerone
Genistin	6,8-Dichlorocatechin	5-Propyl-2-thiouracil
Daidzin	2-Chloroestrone (E1)	6-n-Propyl-2-thiouracil
17α-Estradiol	4-Chloroestrone (E1)	Spironolactone
Chrysin	2,4-Dichloroestrone (E1)	Tamoxifen
Luteolin	2,4,16,16-Tetrachloroestrone (E1)	3,3',5,5'-Tetraiodothyroacetic acid
Indole-3-Carbinol	10-Chloro-1,4-estradiene-3,17-dione	Thiamazole
Hesperetin	2-Chloro-17β-estradiol (E2)	3,3',5-Triiodothyroacetic acid
β-Sitosterol	4-Chloro-17β-estradiol (E2)	
Equol	2,4-Dichloro-17β-estradiol (E2)	Cosmetic-related chemicals (28)
Enterolactone	2-Chloroestriol (E3)	4-Aminobenzoic Acid
Formononetin	4-Chloroestriol (E3)	2-phenoxyethanol
β-Ecdysterone	2,4-Dichloroestriol (E3)	Ethyl 4-aminobenzoate
α-Ecdysterone	2-Chloro-17α-ethinylestradiol (EE2)	2-Ethylhexyl-4-p-dimethylamino-benzoate
Juvabione	4-Chloro-17α-ethinylestradiol (EE2)	2-Hydroxyethyl salicylate
Cyasterone	2,4-Dichloro-17α-ethinylestradiol (EE2)	4-t-Butylphenyl salicylate
Murisuterone A	4-Androstene-3,17-dione	4-Octylphenyl salicylate
Allylisothiocyanate	16,16-Dichloro-4-androstene-3,17-dione	Salicylic Acid 2-Ethylhexyl Ester
Catechin	1,4-Androstadiene-3,17-dione	2,4-Dihydroxy-benzophenone
Dehydroabietic acid	4,6-Cholestadien-3-one	2-Hydroxy-4-methoxy-benzophenone
5α-Dihydrotestosterone	2-Bromoestrone	2,2'-Dihydroxy-4-methoxy-benzophenone
Estriol	4-Bromoestrone	2,2',4,4'-Tetrahydroxybenzophenone
Estrone	2,4-Dibromoestrone	2,2'-Dihydroxy-4,4'-dimethoxy-benzophenone
Fisetin hydrate	2-Bromo-17β-estradiol	2-Hydroxy-4-n-octyloxy-benzophenone
Flavanone	4-Bromo-17β-estradiol	2-Hydroxy-4-methoxy-benzophenone-5-sulfonic acid
Galangin	2,4-Dibromo-17β-estradiol	4-t-Butyl-4'-methoxy-dibenzoylmethane
Harmol hydrochloride dihydrate	2-Bromoestriol	2-(2'-Hydroxy-5'-methylphenyl)-benzotriazole
Hesperidin	4-Bromoestriol	2-(2'-Hydroxy-5'-tert-butylphenyl)-benzotriazole
Hinokitiol	2,4-Dibromoestriol	2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-benzotriazole
Hydrocortisone	2-Bromo-17α-ethinylestradiol	2-(2'-Hydroxy-3',5'-di-tert-amylphenyl)-benzotriazole
6-Hydroxyflavanone	4-Bromo-17α-ethinylestradiol	2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole
7-Hydroxyflavanone	2,4-Dibromo-17α-ethinylestradiol	2-(5-Chloro-2-benzotriazolyl)-6-tert-butyl-p-cresol
16α-Hydroxyestrone		3-(4-Methylbenzylidane)-camphor
11-Ketotestosterone		2-Ethylhexyl-4-Methoxycinnamate
		(±)-α-Tocopheryl Acetate
		Octamethylcyclotetrasiloxane
		Decamethylcyclopentasiloxane
		Dodecamethylcyclohexasiloxane

of RAR, was used as a standard. A DMSO solution of *all-trans* RA was stored in a shielding container at −80 °C until just before examination to prevent degradation, and seven serial twofold concentrations of *all-trans* RA were examined for every culture of yeast cells. A dose-response curve of the luminescence intensity of each compound was described, and two activity values were calculated from the power approximate expression. The EC × 10 was defined as the concentration of a test solution producing luminescence intensity 10 times that of the blank control, and the REC20 (20% relative effective concentration) was the concentration showing 20% of the activity of 10^{-8} M *all-trans* RA. Activity relative to RA was then calculated by dividing the REC20 of *all-trans* RA by that of a test compound. As there were no high volatile compounds in this study, loss of compounds by volatilization over an incubation period was not taken into consideration.

3. Results

3.1. Assay characteristics

The sensitivity and reproducibility of the RAR yeast assay were assessed using the endogenous ligand, *all-trans*

RA. As a chemiluminescence method is used in which an artificial substrate for β-galactosidase is added to sensitively detect transcriptional activation via the human RAR, the large amount of substrate did not allow luminescence intensity to reach a plateau, even at high concentrations of *all-trans* RA (Fig. 1). Therefore, the half-maximal effective concentration (EC_{50}) commonly used in this kind of *in vitro* assay was not reasonable to evaluate the agonistic ability of test chemicals in the present method. Instead of the EC_{50}, the EC × 10 of *all-trans* RA as defined above was 5.41 ± 1.73 nM (mean ± SD, 22 experiments, Table 2). Chemiluminescence intensity at 10^{-8} M *all-trans* RA was 21.2 ± 7.2 times that of the blank control, and then the REC20 was 2.19 ± 0.20 nM (Table 2).

3.2. Positive substances

Eighty-five of the 543 tested compounds, at their highest concentrations (10 μM), exhibited transcriptional agonistic activity via the human RARγ of at least 20% that of 10^{-8} M *all-trans* RA. Table 2 lists the positive substances in order of RARγ activation potency grouped into categories as for Table 1. The range of molecular weights was 164 (4-*n*-pentylphenol) to 444 (*trans*-nonachlor), and the range of total carbon number was 7 (chlorpyrifosmethyl) to 20 (*all-trans* retinol). Many of the tested organochlorine pesticides, styrene dimers, monoalkylphenols and parabens were found to be RARγ active.

3.3. Organochlorine pesticides

Sixteen of the tested 30 organochlorines had a positive effect on RARγ transfected yeast cells. γ-BHC was the most potent in this category and the activities of this compound and seven other organochlorines were over 0.1% (1/1000) of that of *all-trans* RA. The numbers of chlorine atoms in the active compounds were 6–9, while organochlorines out of this range had no effect on the RARγ.

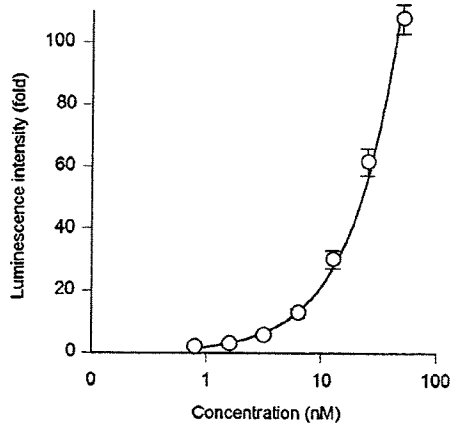

Fig. 1. Response of a yeast two-hybrid assay transfected with the human RARγ and the coactivator to the endogenous ligand, *all-trans* retinoic acid. Values are presented as n-fold induction over the vehicle control and as the mean ± SE of eight independent duplicate experiments.

3.4. Styrene dimers and trimers

All tested styrene dimers and a styrene dimer mixture of unspecified composition exhibited agonistic activity on the RARγ (Fig. 2), but styrene trimers had no effect. 1-Phenyltetralin and 1-methyl-3-phenylindan were particularly active styrene dimers with activities of over 0.6% of that of *all-trans* RA, while five other compounds had agonistic activities over 0.1%.

3.5. Monoalkylphenols

There were several highly active monoalkylphenols with 4-*n*-heptylphenol being the most potent compound tested in this study. All active monoalkylphenols had their alkyl chains *para* to the phenolic hydroxyl group, and the five most active compounds in this category had an alkyl group containing 6–9 carbons and their activities were over 0.4% of that of *all-trans* RA. The ranking of phenols having a linear alkyl group was heptyl (7 carbons) > hexyl (6) > octyl (8) > pentyl (5) > nonyl (9) > dodecyl (12) (Fig. 3). Branching of the alkyl group altered the potency of the phenols (Fig. 4). Comparison of REC20 values showed that an unspecified mixture of isomers of 4-nonylphenol was 4.7 times as active as 4-*n*-nonylphenol and that 4-tert-octylphenol was 2.2 times as active as 4-*n*-octylphenol. However, 4-tert-pentylphenol was 2.8-fold weaker than 4-*n*-pentylphenol. Moreover, six ring-chlorinated monoalkylphenols exhibited positive effects and had about the same or more potency than their unchlorinated analogs.

3.6. Parabens and other chemicals

Some alkyl *p*-hydroxybenzoates (parabens) with an alkyl group of 4–8 carbons were also positive to the RARγ. Of six positives, *n*-hexyl 4-hydroxybenzoate, *n*-pentyl 4-hydroxybenzoate and benzyl 4-hydroxybenzoate showed the highest agonistic activities with values over 0.1% that of *all-trans* RA. Phenols having a cyclic hydrocarbon side-chain *para* to the phenolic hydroxyl group (except 2-(1-adamantyl)-4-methylphenol), a hydroxyethyl ether of 4-nonylphenol and its chlorinated derivative were also positive to the RARγ, but with the exception of 4-(1-adamantyl)phenol, their activities were under 0.1% of that of *all-trans* RA. There were six active substances in the group that we have categorized as 'bisphenol-related compounds', namely, chlorinated bisphenol A and compounds found as impurities in industrial grade bisphenol A, but their activities, with the exception of 4-cumylphenol, were comparatively low. As shown in Table 2, there were also several active substances in the other categories, but most of these had a low potency for the RARγ. However, three diphenyl ethers, aclonifen, nitrofen and chlornitrofen, and the

Table 2
Responsiveness of a RARγ yeast assay to active compounds

Compounds	CAS No.	EC×10 (± SD, ×10^{-6} M)	REC20 (± SD, ×10^{-6} M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (55)				
Aromatic hydrocarbons (4)				
2-Terphenyl	84-15-1	5.03 ± 2.43	1.77 ± 1.14	0.165
n-Octylbenzene	2189-60-8	20.09 ± 6.88	4.51 ± 2.52	0.065
1,3-Diphenylpropane	1081-75-0	8.68 ± 0.81	4.74 ± 1.10	0.062
Triphenylmethane	519-73-3	19.73 ± 4.76	5.34 ± 1.10	0.055
Bisphenols and related chemicals (6)				
2,2'-Dichlorobisphenol A	-	18.95 ± 7.05	6.15 ± 1.61	0.048
2,2',6-Trichlorobisphenol A	-	16.66 ± 5.30	7.85 ± 1.56	0.037
4-Cumylphenol	599-64-4	2.73 ± 0.58	1.68 ± 0.67	0.174
2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman	-	6.53 ± 0.44	3.97 ± 1.33	0.074
2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman	-	7.26 ± 0.96	4.57 ± 2.15	0.064
4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman	472-41-3	6.98 ± 1.33	5.11 ± 2.00	0.057
Monoalkyl phenols and related chemicals (15)				
4-n-Heptylphenol	1987-50-4	0.49 ± 0.26	0.21 ± 0.11	1.363
4-t-Octylphenol	140-66-9	0.78 ± 0.41	0.29 ± 0.13	0.997
4-n-Hexylphenol	2446-69-7	0.69 ± 0.23	0.42 ± 0.13	0.695
4-Nonylphenol (mixed isomers)	84852-15-3	1.36 ± 0.70	0.62 ± 0.25	0.476
4-n-Octylphenol	1806-26-4	1.70 ± 0.56	0.66 ± 0.41	0.446
4-n-Pentylphenol	14938-35-3	3.43 ± 0.70	1.85 ± 0.44	0.159
4-n-Nonylphenol	104-40-5	4.61 ± 1.01	2.92 ± 1.10	0.100
4-Dodecylphenol (mixed isomers)	27193-86-8	5.45 ± 0.84	3.62 ± 0.72	0.081
4-t-Pentylphenol	80-46-6	9.92 ± 2.29	5.24 ± 1.33	0.056
2-Chloro-4-octylphenol	-	0.61 ± 0.37	0.23 ± 0.05	1.286
2,6-Dichloro-4-octylphenol	-	0.70 ± 0.16	0.28 ± 0.14	1.041
2-Chloro-4-nonylphenol	-	1.70 ± 0.96	0.69 ± 0.06	0.422
2,6-Dichloro-4-nonylphenol	-	2.77 ± 1.25	1.35 ± 0.41	0.217
2,6-Dichloro-4-butylphenol	-	4.64 ± 0.32	3.05 ± 0.96	0.096
2-Chloro-4-butylphenol	-	17.33 ± 4.12	7.94 ± 1.04	0.037
Parabens (6)				
n-Hexyl 4-hydroxybenzoate	1083-27-8	1.24 ± 0.26	0.75 ± 0.26	0.391
n-Amyl 4-hydroxybenzoate	6521-29-5	2.95 ± 0.26	1.92 ± 0.58	0.153
Benzyl 4-hydroxybenzoate	94-18-8	3.18 ± 0.55	2.26 ± 0.64	0.130
Isobutyl-4-hydroxybenzoate	4247-02-3	5.54 ± 0.93	4.08 ± 1.36	0.072
2-Ethylhexyl 4-Hydroxybenzoate	5153-25-3	5.58 ± 0.76	4.27 ± 0.90	0.069
Butyl-4-hydroxybenzoate	94-26-8	10.35 ± 2.66	6.37 ± 1.80	0.046
Phenols and related chemicals (7)				
4-(1-Adamantyl)phenol	29799-07-3	1.30 ± 0.80	0.54 ± 0.33	0.547
2-Chloro-4-nonylphenyl 2-hydroxyethyl ether	-	4.93 ± 1.33	3.21 ± 0.31	0.091
4-Cyclohexylphenol	1131-60-8	5.48 ± 1.00	3.23 ± 1.06	0.091
2-(1-Adamantyl)-4-methylphenol	41031-50-9	4.90 ± 0.68	3.82 ± 0.55	0.077
4-Nonylphenyl 2-hydroxyethyl ether	-	7.61 ± 2.34	4.35 ± 0.76	0.067
4-Benzylphenol	101-53-1	9.39 ± 3.55	4.74 ± 1.07	0.062
Hexestrol	5635-50-7	22.01 ± 3.51	7.00 ± 2.18	0.042

aromatic hydrocarbon 2-terphenyl showed agonistic activities over 0.1% of that of *all-trans* RA.

4. Discussion

The reactivity, reproducibility and dose-dependency of the RARγ yeast assay, using *all-trans* RA, as a standard, were satisfactory for assessing a wide range of chemicals as described here. The EC_{50} of *all-trans* RA in a reporter gene assay using RARγ-cotransfected HeLa cells and the IC_{50} in a competitive binding assay using RARγ-transfected COS-1 cells were reported to be 2.5 nM and 8 ± 1 nM, respectively (Bernard et al., 1992; Allenby et al., 1994). Although there is no simple comparison between these reports and our results, the responsivity of our assay system, which can be represented as EC × 10 or REC20 values, is within a similar range. Taking into account its simplicity and rapidity, application of this assay using easily managed yeast cells to the toxicological evaluation of chemicals is appropriate and reasonable as a step

Table 2 (*continued*)

Compounds	CAS No.	ECx10 (± SD, $\times 10^{-6}$ M)	REC20 (± SD, $\times 10^{-6}$ M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (continued)				
Styrene dimers (14+1)				
1-Phenyltetralin	-	0.65 ± 0.02	0.25 ± 0.16	1.169
1-Methyl-3-phenylindan	-	1.22 ± 0.13	0.46 ± 0.28	0.632
trans-1,2-Diphenylcyclobutane	20071-09-4	3.37 ± 0.80	1.33 ± 0.75	0.220
1-Methyl-1-phenylindan	79034-12-1	4.77 ± 1.98	2.16 ± 0.81	0.135
cis-1,2-Diphenylcyclobutane	7694-30-6	5.43 ± 2.03	2.20 ± 1.12	0.133
2,4-Diphenyl-1-butene	16606-47-6	4.62 ± 0.69	2.32 ± 0.52	0.126
2,3-Diphenyl-1-butene	-	5.51 ± 2.81	2.40 ± 1.35	0.122
cis-1,4-Diphenyl-1-butene	-	6.22 ± 2.78	2.95 ± 1.38	0.099
trans-1,3-Diphenyl-1-butene	-	6.04 ± 1.91	2.99 ± 1.39	0.098
trans-1,3-Diphenylcyclobutane	-	7.09 ± 2.15	3.23 ± 1.39	0.091
1,4-Diphenyl-2-butene	-	8.29 ± 4.00	3.54 ± 1.62	0.083
cis-2,4-Diphenyl-2-butene	-	8.95 ± 2.77	3.89 ± 1.76	0.075
trans-1,4-Diphenyl-1-butene	-	8.62 ± 3.77	4.09 ± 1.84	0.072
trans-2,4-Diphenyl-2-butene	-	14.33 ± 4.49	6.83 ± 2.40	0.043
Polystyrene standard (mixed styrene dimer isomers)	-	7.54 ± 2.96	3.97 ± 0.77	0.074
Others (2)				
Octachlorostyrene	29082-74-4	21.93 ± 9.01	4.77 ± 3.47	0.061
Benzyl-2-naphthylether	613-62-7	21.98 ± 6.32	6.79 ± 0.37	0.043
Agrochemicals (22)				
Carbamate (1)				
Thiobencarb	28249-77-6	16.74 ± 1.64	5.48 ± 0.39	0.054
Diphenyl ethers (3)				
Aclonifen	74070-46-5	2.74 ± 0.62	1.33 ± 0.82	0.220
Nitrofen	1836-75-5	4.45 ± 1.31	2.18 ± 0.61	0.134
Chlornitrofen (CNP)	1836-77-7	7.77 ± 3.31	2.82 ± 1.22	0.104
Organochlorines (16)				
1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	58-89-9	0.89 ± 0.27	0.44 ± 0.14	0.668
Endrin	72-20-8	1.22 ± 0.79	0.85 ± 0.21	0.346
Heptachlor	76-44-8	2.09 ± 0.77	0.87 ± 0.37	0.336
Oxychlordane	27304-13-8	3.87 ± 0.99	1.90 ± 0.03	0.154
Chlordane	57-74-9	3.23 ± 1.89	1.93 ± 1.15	0.152
Toxaphene	8001-35-2	3.35 ± 1.64	2.59 ± 0.96	0.113
cis-Heptachlor epoxide	1024-57-3	3.46 ± 1.82	2.85 ± 0.60	0.103
cis-Chlordane	5103-71-9	5.63 ± 4.03	2.94 ± 1.33	0.100
trans-Chlordane	5103-74-2	7.15 ± 3.70	3.56 ± 1.80	0.082
β-Endosulfan	33213-65-9	9.06 ± 0.87	4.36 ± 2.71	0.067
Dieldrin	60-57-1	7.47 ± 4.60	4.70 ± 1.80	0.062
trans-Nonachlor	39765-80-5	7.74 ± 5.15	4.92 ± 0.30	0.060
Aldrin	309-00-2	8.88 ± 6.14	5.43 ± 0.91	0.054
β-Benzene hexachloride (β-BHC)	319-85-7	12.85 ± 3.15	5.69 ± 0.77	0.052
α-Endosulfan	959-98-8	8.02 ± 2.67	6.07 ± 2.40	0.048
trans-Heptachlor epoxide	1024-57-3 (trans)	8.49 ± 5.06	6.30 ± 1.58	0.047
Organophosphates (2)				
Cyanofenphos	13067-93-1	8.83 ± 1.51	3.42 ± 1.89	0.086
Chlorpyrifosmethyl	5598-13-0	13.81 ± 3.47	7.08 ± 2.11	0.041
Natural compounds and related chemicals (7)				
all-trans-Retinol	68-26-8	2.81 ± 0.17	1.67 ± 0.77	0.175
Flavanone	487-26-3	10.65 ± 1.14	4.23 ± 0.26	0.069
cis-Stilbene	645-49-8	10.16 ± 5.57	4.99 ± 2.58	0.059
Dibenzyl	103-29-7	17.95 ± 3.31	6.66 ± 2.10	0.044
4-Chloro-17β-estradiol	-	16.23 ± 3.77	4.35 ± 1.75	0.067
2,4-Dichloro-17β-estradiol	-	13.26 ± 1.52	4.79 ± 1.38	0.061
4-Bromo-17β-estradiol	-	12.49 ± 4.72	5.52 ± 3.91	0.053
Medicines and cosmetic-related chemicals (1)				
Cosmetic-related chemical (1)				
3-(4-Methylbenzylidane)-camphor	36861-47-9	15.98 ± 4.04	5.92 ± 1.85	0.050

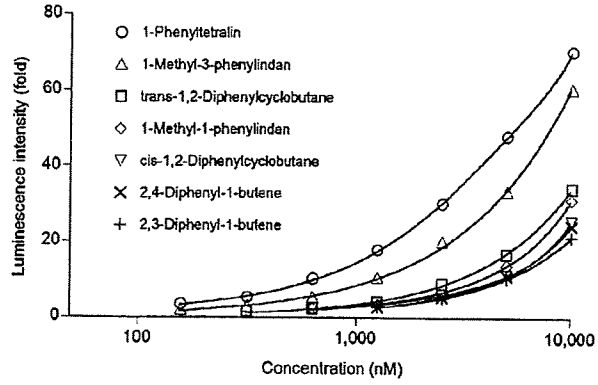

Fig. 2. Dose-response curves of styrene dimers in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

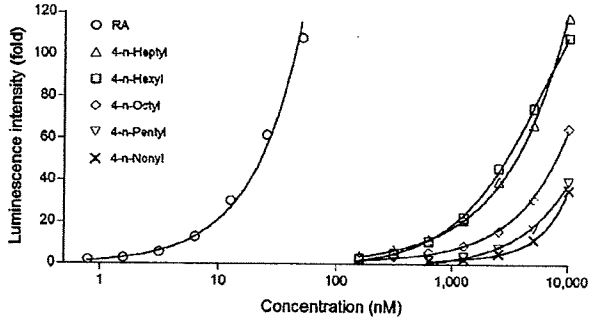

Fig. 3. Dose-response curves of monoalkylphenols having a linear alkyl group in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

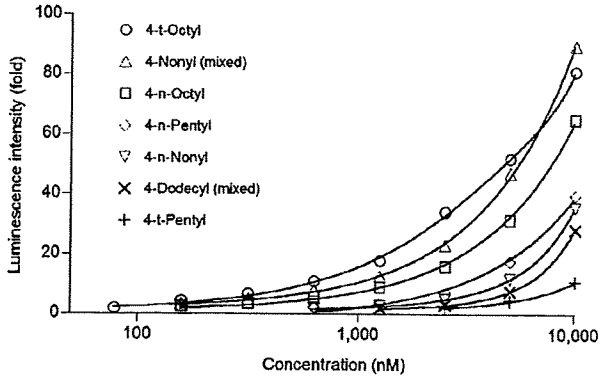

Fig. 4. Activity comparison of monoalkylphenols having a linear alkyl group with a branched group in a RARγ yeast two-hybrid assay. Results are presented as the averages of a minimum of three duplicated experiments.

in hazard identification with implications for mechanism of action. As RAs are known to undergo isomerization and oxidation when exposed to light and air (Bempong et al., 1995), some of the variability in the activity of *all-trans* RA can probably be attributed to the lability of the reagent to, in particular, photodegradation. However, the ready availability of an endogenous ligand for use in the assay more than compensates for this instability.

Many of the compounds active to the RARγ (organochlorines and styrene dimers were notable exceptions) were *para*-alkyl-substituted phenols. Phenols of this type are manufactured on a very large scale for many industrial purposes and have been reported to possess estrogenic activity, with the degree of activity depending on the length and branching of the alkyl substituent. Of the 4-*n*-alkylphenols, 4-*n*-nonylphenol had the highest binding affinity for the human ER (Tabira et al., 1999). 4-Alkylphenols having an alkyl group composed of 3–12 carbons, including branched groups (both secondary and tertiary), exhibited ER transactivation activity in a recombinant yeast assay (Routledge and Sumpter, 1997). There were some differences in the responsivities of the present RAR assay to alkyl and other phenols from those of the ER. The most potent activator of the RARγ was 4-*n*-heptylphenol and phenols with four or less carbons in their alkyl groups had no effect regardless of its position or branching. Alkylphenols having a branched alkyl group with many carbons, such as the mixed isomers of nonylphenol and tert-octylphenol, were more potent than analogs with a linear alkyl group, whereas of the pentylphenols with less carbon atoms in their alkyl groups, tert-pentylphenol is weaker than its analog with a linear alkyl group. This suggests that the overall length of a side-chain but not the actual number of carbons it contains may influence the potency of alkylphenols to the RARγ and that there may be an optimal length.

Ring-chlorinated alkylphenols had somewhat higher activity than their unchlorinated analogs, and bisphenol A, which had no positive effect on the RARγ, acquired agonistic activity by chlorination. This parallels observations in previous studies of estrogenicity where the estrogenic activities of chlorinated bisphenol A were stronger both *in vitro* and *in vivo* than those of the unchlorinated compound (Takemura et al., 2005; unpublished data, Shiraishi et al., 2000). Halogenation of chemicals might thus activate them or boost their actions on nuclear receptors. This is also indicated by observations in the present study that chlorination and bromination activated 17β-estradiol (E2) to the RARγ to a small extent. These findings suggest that not only in industrial manufacturing but in effluent processing, halogenation treatment does not reduce the unexpected activity of chemicals on nuclear receptors, but on the contrary might enhance the harmful effects.

We present here the first evidence that styrene dimers but not trimers have the ability to transactivate the RAR. The dimer 1-phenyltetralin was one of the most active chemicals revealed in this study. Furthermore, it was apparent that the fewer carbons linking the benzene rings of the dimers, the higher the agonistic activity (Figs. 2 and 5). Therefore, limited overall molecular length seems

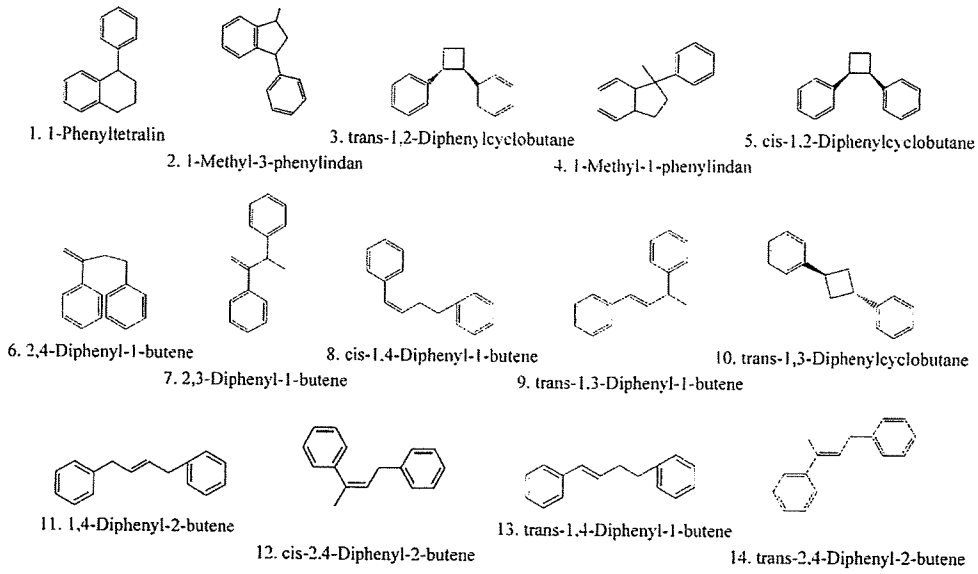

Fig. 5. Structural formulas of styrene dimmers.

to correlate with potency in styrene dimers. The lack of activity of styrene trimers may also arise from their length or size as the upper limit for the molecular weights of positive compounds (except halogenated compounds and organophosphates) was 286.5 (*all-trans* retinol), whereas the styrene trimers have molecular weights of 312. It is interesting that 2-terphenyl and 1,3-diphenylpropane have about the same activity as styrene dimers with similar structures (trans-1,2-diphenylcyclobutane and trans-2,4-diphenyl-2-butene, respectively). The binding affinity of these chemicals for the RAR may depend on molecular length, like alkylphenols as discussed above. Styrene oligomers also have binding affinity for the human ER (Ohyama et al., 2001; Kitamura et al., 2003). Importantly, it has been reported that styrene trimers exhibit *in vivo* disruption of endocrine systems and that embryonic exposure obstructs genital organ development and disrupts the endocrine function of male rat offspring (Ohyama et al., 2007). Polystyrene resins contain substantial amounts of styrene dimers and trimers, and extraction tests with polystyrene food containers have shown that these compounds leak into the food, water and oil with which they are in direct contact (Kawamura et al., 1998a,b,c). Because all styrene dimers tested in this study had a positive effect on the RARγ and selective ligands for the RAR subtypes are teratogenic in the developing embryo, the developmental toxicity of styrene oligomers via the RAR as well as the ER should be investigated.

Organochlorine pesticides were previously reported to transactivate RARβ and γ, but not RARα in human RAR reporter cell lines (Lemaire et al., 2005). The tested five organochlorines had dose-dependent effects on the RARγ and the order of potency was endrin > dieldrin > aldrin > chlordane > endosulfan, although even the activity of endrin was only approximately 1/12000 of that of the standard RAR agonist, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl] benzoic acid. Our results indicated that many organochlorines, including those five compounds examined, also have the potential to activate the RARγ. The present yeast assay detected the activities of these compounds at lower concentrations than the reported cell line. In addition, our assay results indicated that binding affinity for the RAR partially depends on the number of chlorine atoms in the molecule and that there seems to be an optimal range of their chlorine number. Many studies have shown that a number of pesticides, including organochlorines, possess estrogenic activity (Hodges et al., 2000; Kojima et al., 2004), but their potencies for the ER do not correspond with those for the RAR. For example, the ranking of the activities of organochlorines for the ERs in Chinese hamster ovary cells are *o,p'*-DDT (4.5×10^{-8} M, test compound concentration showing 20% of the agonistic activity of 10^{-10} M 17β-estradiol) > β-BHC (3.5×10^{-7} M) > methoxychlor (5.6×10^{-7} M) for the ERα, and β-BHC (1.1×10^{-7} M) > *o,p'*-DDT (1.2×10^{-7} M) > δ-BHC (1.1×10^{-6} M) for the ERβ (Kojima et al., 2004), whereas none of these compounds showed a significant effect on the RARγ in our assay system. Because persistent and/or harmful pesticides such as organochlorines are now prohibited in developed countries, but still used in developing regions, detailed investigations of their toxicity via nuclear receptors, as well as a global restriction on their use, are required.

The present study illustrated that a number of compounds possessed unexpected transcriptional activation effects on the human RARγ in a yeast two-hybrid system, as we have already shown for the ERs and TRs (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). It is common for xenobiotics to have low affinity for nuclear receptors relative to the natural ligands, as we

reported here. However, there are a number of examples (principally from the ER, which is better studied than the RAR) where man-made compounds produce adverse effects in living organisms via nuclear receptors. Agonistic ligands for nuclear receptors such as the RARs and ERs have been confirmed the ability to experimentally induce developmental abnormalities (Elmazar et al., 1996, 2001; Kamata et al., 2006), and there are medically or environmentally documented cases of their teratogenicities (Lammer et al., 1985; Fry, 1995; Sumpter, 1998). For example, in previous studies, the REC20 of 2,2′,4′6′-tetrachlorobiphenyl-4-ol, one of monohydroxylated polychlorinated biphenyls, was 24 nM in our human ERα yeast assay, and its activity relative to E2 was 1.7% (Arulmozhiraja et al., 2005). *In ovo* exposure to this compound at a dose of 100 ng/g egg or more caused shortening of the oviduct in female Japanese quails and a dose of 500 ng/g or more caused a reduction testis weight in males after sexual maturation (Kamata et al., 2006). This dose range in ovo was approximately 100 times higher than a positive control, diethylstilbestrol, producing *in vitro* effect equivalent to E2. Recently, the existence of environmental pollutants having binding affinity for less researched but important nuclear receptors, such as the retinoid X receptors (RXR) and peroxisome proliferator-activated receptors (PPAR), and consequential disorders in organisms has been reported (Nishikawa et al., 2004; Abbott et al., 2007; Takacs and Abbott, 2007). Both tributyltin and triphenyltin used in antifouling paints exhibited agonistic activity in yeast assays transfected with the human RXR (α, β or γ) at a concentration of 10 nM or more, and this concentration was somewhat lower than that of 9-*cis* RA, the natural ligand of RXR (Nishikawa et al., 2004). One injection of 1 μg triphenyltin/g wet weight induced the differentiation and growth of male genital tracts in female gastropods, *Thais clavigera*, which was also stronger than 9-*cis* RA (Nishikawa et al., 2004). Thus, these reports are typical cases that *in vitro* affinity of chemicals for nuclear receptors is well correlated with their *in vivo* potential, and therefore, measurement of the relative activity of chemicals using *in vitro* assays seems to be valuable to estimate the *in vivo* effects of them.

There is great concern that substances released into the environment and/or used in everyday life may influence human and wildlife health. Detection of chemicals with an affinity for the various receptors is an important step in hazard identification. This data can be used to direct and prioritize additional research and *in vivo* studies on chemicals that bind nuclear receptors for possible receptor-mediated toxicity and endocrine disrupting activity. As described here, yeast assay systems including a RAR assay are useful for high-throughput screening of substances active to nuclear receptors.

5. Conflict of interest

There are no conflicts of interests involved in this study.

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