In vitro profiling of endocrine disrupting effects of phenols

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ARTICLE INFO

Article history:

Received 3 April 2009

Accepted 11 September 2009

Available online 16 September 2009

Keyword:

Phenols, Estrogen receptor

Androgen receptor

Progesterone receptor

Estrogen-related receptor

Yeast two-hybrid assay

ABSTRACT

Some phenols have been suspected to modulate the endocrine systems of wildlife and humans, but less is known about their interactions with different types of nuclear receptors. In this study, the ability of 2-*tert*-butylphenol, 2-isopropylphenol, 4-*tert*-octylphenol (4-*t*-OP), 2,4-dichlorophenol (2,4-DCP), 3,4-dichlorophenol (3,4-DCP), pentachlorophenol (PCP), bisphenols A (BPA), tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA) and 4-phenylphenol to activate estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR) and estrogen-related receptor (ERR) were determined using a set of recombined yeast strains. It was found that 4-*t*-OP, 3,4-DCP, PCP, BPA, TBBPA, TCBPA and 4-phenylphenol were ER α agonists, while 4-*t*-OP, PCP and 4-phenylphenol showed ER α antagonistic activities. 2-*tert*-Butylphenol, 4-*t*-OP, 2-isopropylphenol, 2,4-DCP, 3,4-DCP, BPA, TCBPA and 4-phenylphenol were antagonists for AR, whereas none of the compounds studied were found to be an AR agonist. TCBPA, TBBPA and PCP were PR antagonists, and 2-*tert*-butylphenol, 3,4-DCP, 4-*t*-OP, 4-phenylphenol and 2-isopropylphenol were weak inhibitors on expression under control of the PR. None of the phenols were PR agonists. 2-*tert*-Butylphenol, 4-*t*-OP and PCP were ERR γ inverse agonists, while 2,4-DCP, 3,4-DCP, PCP, BPA, TBBPA and TCBPA exhibited the ability to reverse the ERR inhibition induced by 4-hydroxytamoxifen. Based on the functional agonistic or antagonistic receptor-mediated effects, we further discussed the possible action mechanisms of these phenols as endocrine disrupting chemicals.

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1. Introduction

Over the past 20 years there has been increasing concern by both the public and researchers that phenols and many other related environmental chemicals have the ability to modulate the neuro-endocrine system and thus adversely affect human and wildlife reproduction (Terasaka et al., 2006). Several possible mechanisms of action, including direct hormone agonists or antagonists that could mimic or inhibiting endogenous hormone action, by modulating the production of endogenous hormones, or by altering hormone receptor have been suggested (Sonnenschein and Soto, 1998). Thus, hormone receptors have become one of the subjects of research on endocrine disruptors in general, but most of the research has focused primarily on estrogen receptor (ER) agonists. However, the endocrine system is more complex and presents a large number of potential endocrine disrupting chemical (EDC) target sites (Kristensen et al., 2006). Thus, to accurately predict the potential effects of mixtures of endocrine disruptors, it is important to study the interaction of EDCs with different types of hormone receptors and through indirect mechanisms of effect.

The steroid receptors, including the ER, androgen receptor (AR) and progesterone receptor (PR) are classified as members of the subfamily within the nuclear receptor super-family 3 (Nuclear Receptors Nomenclature Committee, 1999). It is well known that androgens are important physiologically in male mammals, which stimulates or controls the development and maintenance of masculine characteristics by binding to AR (Meisel and Sachs, 1994). Recent studies have shown that several chemicals may exert anti-androgenic effect by interfering with AR (Sohoni and Sumpter, 1998). For example, *p,p'*-dichlorodiphenylethane (*p,p'*-DDE), bisphenol A (BPA), octylphenol (OP) and nonylphenol (NP) have demonstrated in vitro AR-mediated anti-androgenic activities (Xu et al., 2005). Besides estrogens and androgens, progesterone is also a key regulator of proliferation and differentiation in reproductive tissues (Graham and Clarke, 1997). The effects of progesterone are mediated via the PR. Several pieces of evidence indicate that some synthetic chemicals in the environment are able to inhibit the binding of the progesterone to PR (Wang et al., 2005). But only a limited number of environmental chemicals with anti-progesterone-like properties have been identified (Klotz et al., 1997), while little information is available on progesterone-like or anti-progesterone activity and on the relationship between estrogenic, androgenic and progesterone activity in the environmental chemicals (Wang et al., 2005).

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Table 1
Estrogen receptor α (ER α), androgen receptor (AR), progesterone receptor (PR), estrogen-related receptor γ (ERR γ) agonistic and antagonistic potency of the tested chemicals using a serious of yeast assay. Each value represents the mean $\pm$ standard error of the mean ($n = 3$). REC20 = the concentration inducing 20% of the maximum effect, RIC20 = the concentration reducing 20% of the maximum effect, RC20 = the concentration reversing 20% of the inhibition by 4-hydroxytamoxifen.^a

	ER α		AR		PR		ERR γ	
	REC20 (mol/L)	RIC20 (mol/L)	REC20 (mol/L)	RIC20 (mol/L)	REC20 (mol/L)	RIC20 (mol/L)	REC20 (mol/L)	RIC20 (mol/L)
<i>Alkylphenols</i>								
2- <i>tert</i> -butylphenol	ND	ND	ND	1.1E–05	ND	1.5E–06	ND	>1.0E–04
4- <i>t</i> -OP	2.1E–7	6.6E–6	ND	1.6E–06	ND	1.2E–06	ND	3.5E–06
2-Isopropylphenol	ND	ND	ND	1.6E–06	ND	1.8E–05	ND	ND
<i>Chlorinated phenols</i>								
2,4-DCP	ND	ND	ND	1.0E–05	ND	ND	1.0E–04	ND
3,4-DCP	1.0E–4	ND	ND	1.3E–05	ND	2.4E–06	>1.0E–04	ND
PCP	>1.0E–5	6.2E–7	ND	ND	ND	7.6E–07	1.0E–05	3.0E–06
<i>Bisphenols</i>								
BPA	1.0E–5	ND	ND	1.2E–06	ND	ND	1.0E–04	ND
TBBPA	>1.0E–5	ND	ND	ND	ND	7.8E–08	>1.0E–06	ND
TCBPA	>1.0E–5	ND	ND	1.0E–05	ND	2.7E–08	>1.0E–06	ND
<i>Phenylphenol</i>								
4-Phenylphenol	2.4E–6	3.1E–7	ND	1.4E–05	ND	1.4E–06	ND	ND

^a $n \geq 3$; ND: no detection.

Because no endogenous ligands have been identified for estrogen-related receptors (ERRs), they are classified as a subfamily of orphan nuclear receptors closely related to ER α and ER β (Horard and Vanacker, 2003). ERRs and ERs have a high degree of amino acid sequence similarity and identity in both their DNA-binding (DBD) and ligand-binding (LBD) domains. ERRs can bind to functional estrogen response elements (EREs) in ER target genes, suggesting possible similarities between ERR and ER action (Takayanagi et al., 2006). Some synthetic chemicals including BPA, 4-hydroxytamoxifen (4-OHT), diethylstilbestrol, 4-NP, and the endogenous hormone, estrone have been reported to be able to bind to human ERR γ (Takayanagi et al., 2006).

Regarding various EDCs, many phenols suspected as xenoestrogens, such as NP, OP and BPA, are a constant concern from the environmental point of view due to their large distribution. For example, aquatic biology can be exposed to these phenols not only because of the discharge from industrial and municipal wastewater treatment plant effluents, but also due to direct discharge (Suzuki et al., 2004). The endocrine disruption effects of these chemicals have been reported, for example, 4-*t*-OP, BPA, 2,4-dichlorophenol (2,4-DCP) have been long acknowledged to be estrogenic chemicals having the ability to interact with human ER (Nishihara et al., 2000). However, less is known about the interaction of these phenols with other nuclear receptors such as AR, PR or ERR. Based on different structures and different types (e.g. alkylphenols, chlorinated phenols, bisphenols and phenylphenols), 10 phenolic compounds were selected (listed in Table 1). This study was conducted to determine the effects of these specific phenols on nuclear receptors. For this purpose, the recombinant yeast assays were carried out to investigate the effects of phenols (listed in Table 1) on ER, AR, PR and ERR-mediated transcription of β -galactosidase in vitro in reporter yeasts. These test systems could be used for the rapid, high-throughput screening of chemicals for their agonist and antagonist properties for ER, AR, PR and ERR.

2. Materials and methods

2.1. Chemicals

17 β -Estradiol (E₂, 97%, CAS No. 50-28-2), dimethyl sulfoxide (DMSO, 99.5%, CAS No. 67-68-5), BPA (99%, CAS No. 80-05-7), 4-*t*-OP (97%, CAS No. 140-66-9), pentachlorophenol (PCP, 99%, CAS No. 87-86-5) and 4-OHT (98%, CAS No. 68047-06-3) were pur-

chased from Sigma Chemical (St. Louis, MO, USA). 2-*tert*-Butylphenol (99%, CAS No. 88-18-6), 2-isopropylphenol (97%, CAS No. 88-69-7), tetrabromobisphenol A (TBBPA, 97%, CAS No. 79-94-7), 2,4-DCP (99%, CAS No. 120-83-2), 3,4-dichlorophenol (3,4-DCP, 99%, CAS No. 95-77-2) and 4-phenylphenol (97%, CAS No. 92-69-3) were purchased from Acros Organics (Geel, Belgium). Dihydrotestosterone (DHT, CAS No. 521-18-6) and tetrachlorobisphenol A (TCBPA, CAS No. 79-95-8) were obtained from Tokyo Chemical Industry (Osaka, Japan). Progesterone (CAS No. 57-83-0) was purchased from the MP Biomedical, Eschwege, Germany. For all compounds, stock solutions were prepared in DMSO. Final chemical concentration in the DMSO stock was 0.2 mol/L.

2.2. Yeast strain

Yeast strains respectively transformed with the ER α gene, ERR γ gene or AR gene were constructed in our laboratory using yeast two-hybrid assay system and selected by growth on synthetic dextrose (SD) medium (lacking tryptophan and leucine, SD/-Leu/-Trp) according to the method described in our previous paper (Li et al., 2008a,b). Briefly, we introduced two expression plasmids, bait plasmid and hunter plasmid, into yeast cells.

Another yeast strain transformed with the PR gene was a kind gift of Dr. Kevin W. Gaido (Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, USA). Yeast (*Saccharomyces cerevisiae*) stably transfected with the human PR gene and expression plasmids carrying a progesterone response element, while the reporter LacZ gene encoding the enzyme β -galactosidase was used. The yeast strain was grown in synthetic complete (SC) medium with tryptophane, but lacking histidine and leucine (SC/-His/-Leu) following the prescription of Gaido et al. (1997).

The ER α , AR, PR and ERR γ yeast used in this study had been detected, in our previous study, to have high ability of interacting with known agonists and antagonists (Table 2), thus high sensitivity for screen chemicals with ant/agonistic activity (Li et al., 2008a,b).

2.3. β -Galactosidase assay

The yeast assay was conducted as described by Ma et al. (2005). Yeast strains were grown overnight at 30 °C, 130 rpm with vigorous shaking. Each test chemical was serially diluted in DMSO. Five microliters of serial dilutions of test samples were combined with

Table 2

Overview of the four in vitro bioassays used to determine the possible endocrine disrupting potency on seven different endpoints. ER α = estrogen receptor α , AR = androgen receptor, PR = progesterone receptor, ERR γ = estrogen-related receptor γ , E₂ = 17 β -Estradiol, 4-OHT = 4-hydroxytamoxifen, DHT = dihydrotestosterone, REC50 = the concentration inducing 50% of the maximum effect, RIC50 = the concentration causing a 50% inhibition of the maximum effect.

Bioassay	Reference material	Endpoint	REC50 or RIC50 (mol/L)
Two-hybrid ER α -Laz	E ₂	Estrogenic activity through ER α	2.5×10^{-10a}
	4-OHT	Antiestrogenic activity through ER α in the presence of E ₂	1.2×10^{-6a}
Two-hybrid AR-Laz	DHT	Androgenic activity through AR	1.3×10^{-8b}
	Flutamide	Anti-androgenic activity through AR in the presence of DHT	9.8×10^{-6b}
PR-Laz	Progesterone	Progestagenic activity through PR	5×10^{-10b}
	RU486	Antiprogestagenic activity through PR in the presence of progesterone	6.6×10^{-6b}
Two-hybrid ERR γ -Laz	4-OHT	Inhibition of the constitutive activity through ERR γ	7.9×10^{-6b}

^a According to Li et al. (2008a).

^b According to Li et al. (2008b).

995 μ L of medium containing 5×10^3 yeast cells/mL resulting in a test culture. Two hundred microliters of the test cultures were transferred into each well of the 96-well plate and incubated at 30 °C with vigorous orbital shaking (800 rpm) for 2 h, then the cell density of the culture was measured at 600-nm wavelength (TECAN GENios A-5002, Salzburg, Austria).

A 50 μ L test culture was transferred to a new 96-well plate and after addition of 120 μ L of Z-buffer (16.1 g/L Na₂HPO₄·7H₂O; 5.5 g/L NaH₂PO₄·H₂O; 0.75 g/L KCl; 0.246 g/L MgSO₄·7H₂O) and 20 μ L chloroform, the assays were carefully mixed (vortex 25 s). The enzyme reaction was initiated by addition of 40 μ L *o*-nitrophenyl- β -D-galactopyranoside (13.3 mmol/L, dissolved in Z-buffer) and incubated at 30 °C for 60 min. The reactions were terminated by the addition of 100 μ L Na₂CO₃ (1 mol/L). After centrifugation at 12,000g for 15 min (Sigma Laborzentrifugen 2K15, Osterode, Germany), 200 μ L of the supernatant was transferred into a new 96-well plate and the absorbance at 420 nm was determined. The β -galactosidase activity was calculated according to reports by Ma et al. (2005), β -galactosidase activities are expressed as the means and standard deviations of the results from three independent transformed yeast clones and for each clone, all samples were evaluated in triplicate. The antagonistic activity of samples was tested by co-incubation of yeast strain with nature ligand which produced a sub-maximal stimulatory response (2×10^{-10} mol/L E₂, 5×10^{-8} mol/L DHT and 1×10^{-9} mol/L progesterone, respectively; Li et al., 2008).

To ensure that increased/reduced activities in the bioassay were caused by true agonistic/antagonistic responses but not by cytotoxicity, viability was measured. After exposure, cell viability was determined spectrophotometrically as a change of cell density (OD_{600 nm}) in the assay medium.

2.4. Data analysis

For some compounds yielding responses lower than 50% of the positive control value, the concentration inducing 50% of the maximum effect (REC50) or the concentration causing a 50% of the maximum effect (RIC50) was not calculated. Then, the concentration resulting in 20% of the maximum effect (REC20) or the concentration reducing 20% of the maximum effect (RIC20, inducing 80% of the maximum effect) was calculated for test compounds. The REC20 value, which means the concentration reversing 20% of the inhibition by 4-OHT, was specifically used for evaluation the compounds with reversing ERR γ activities. For compounds yielding responses less than 20% of the positive control value, but showing a significant difference from the negative control, the REC20 or RIC20 values were greater than the minimum value (>minimum value) having significant difference (Table 1). The dose-response curve was generated using a four-parameter logistic model based on the Marquardt–Levenberg algorithm (Sigmaplot 4.0, SPSS, Chicago,

IL, USA) (Rehmann et al., 1999). The four-parameter logistic equation gives a dose response curve fitted to the measured values REC20 as well as the opportunity to calculate RIC20 values for antagonistic assays.

3. Result and discussion

3.1. Gene expression modulation via the ER α

To study ER agonistic activity, two-hybrid ER α yeast was tested in the presence of increasing concentrations (1×10^{-9} – 1×10^{-4} mol/L) of phenolic endocrine disrupting compounds. 4-*t*-OP, 3,4-DCP, BPA and 4-phenylphenol was found to be ER α agonists that significantly induced expression of a reporter, β -galactosidase, in a dose-dependent manner (Fig. 1A). The REC20 value of 4-*t*-OP, 3,4-DCP, BPA and 4-phenylphenol were 2.1×10^{-7} , 1.0×10^{-4} , 1.0×10^{-5} and 2.4×10^{-6} mol/L. PCP, TBBPA and TCBPA only showed ER α -binding activity at the highest concentration without cytotoxicity. The REC20 value of these compounds could not be calculated and these compounds could not be included in Fig. 1A.

When the test chemicals were co-administered with 2×10^{-10} mol/L of E₂, some of them suppressed the β -galactosidase activity (Fig. 1B). The inhibition by 4-*t*-OP, PCP and 4-phenylphenol demonstrated a dose-effect relationship. The RIC20 value of 4-*t*-OP, PCP and 4-phenylphenol were 6.6×10^{-6} , 6.2×10^{-7} and 3.1×10^{-7} mol/L.

The test chemicals were also demonstrated that they did not affect the viability and proliferation of yeast cell, alone or with 2×10^{-10} mol/L E₂ assay (data not shown). This indicates that no cytotoxic effect existed in the tested concentration range.

Recently, Nishihara et al. (2000) used a yeast two-hybrid assay system to test 517 chemicals including 4-*t*-OP, 3,4-DCP, BPA, PCP, TBBPA and TCBPA as potential agonists to the rat ER, which were similar to our result. Moreover, in the present study, 4-phenylphenol induced the activity of human ER α when its concentration increased to 10^{-7} mol/L, which was consistent with the report by Dhooze et al. (2006) also using a yeast assay.

The data shown here suggests that 4-*t*-OP, PCP and 4-phenylphenol were able to interact with significant affinity with ER α showing antagonistic activity. Whereas few results concerning antiestrogenic activity of these chemicals have been reported previously, Jung et al. (2004) proposed that PCP may be antiestrogenic rather than estrogenic when tested by in vitro assays including the yeast two-hybrid assay, MCF-7 cells and ER competitive binding assay, which supports our results. This study revealed that 4-*t*-OP, PCP and 4-phenylphenol showed inhibition of estrogenic activity and these compounds also showed agonistic activity (Table 1), suggesting the antiestrogenic activity upon binding to ER. In addition, Soto et al. (1997) tested the estrogenicity of some

Fig. 1. (A) Estrogen receptor α (ER α) agonistic activity of test chemicals determined by the yeast strain ER α -GRIP1. (B) ER α antagonistic activity of test chemicals determined by the yeast strain ER α -GRIP1. The chemical's ant/agonistic activity is represented as the percent inhibition or induction activity relative to the maximum induced by 17 β -estradiol (E_2 , 2×10^{-10} mol/L). Values are presented as the average $\pm$ standard error ($n = 3$).

xenoestrogens using the E-SCREEN assay, and reported that some full agonists were devoid of antagonistic effects and partial agonists were also partial antagonists.

3.2. Gene expression modulation via the AR

Our result showed that none of the test compounds caused any AR transactivation (data not list). 2-*t*-Butylphenol, 4-*t*-OP, 2-isopropylphenol, 2,4-DCP, 3,4-DCP, BPA, TCBPA and 4-phenylphenol were found to be AR antagonists and inhibited the β -galactosidase activity induced by DHT in a concentration dependent manner (Fig. 2). However, the inhibition effects of PCP and TBBPA were not found. The RIC20 values of test chemicals with antagonistic activity were listed in Table 1. Among these compounds, 4-*t*-OP, 2-isopropylphenol and BPA were more effective inhibitor, with RIC20 values lower than 1.0×10^{-5} mol/L (Table 1).

In this study, we found that 2-*t*-butylphenol, 4-*t*-OP, 2-isopropylphenol, 2,4-DCP, 3,4-DCP and 4-phenylphenol were anti-androgens. 4-*t*-OP was also reported to have an inhibitory effect on the androgenic activity of DHT by means of androgen-responsive luciferase reporter gene assay using MDA-kb2 human breast cancer cells (Tamura et al., 2006). For 4-phenylphenol, 4-hydroxyl group

has been demonstrated to be effective in phenylphenols to repress AR transcription (Paris et al., 2002). Kitamura et al. (2005) reported isopropylphenol was a potent anti-androgen with RIC₅₀ value of 6.2×10^{-5} mol/L, which was consistency with our research. In the present study, 2,4-DCP was found to have anti-androgenic activity, which also was supported by the study of Tamura et al. (2006). However, Kim et al.'s (2005) work suggested that 2,4-DCP (1.0×10^{-10} mol/L) exhibited synergistic androgenic activities by co-treatment with DHT. This result may be due to the different concentration selected for the measurement (1.0×10^{-10} mol/L vs. 1.0×10^{-5} mol/L) and suggests that the disturbing effect of 2,4-DCP on AR may be related to the exposure concentration. It has been reported that 3,4-DCP showed estrogenic activity (Table 1), in addition, Sohoni and Sumpter (1998) suggested that many oestrogens possess combining activity to AR. So, we detected the ant/agonistic activity of this compound and found that 3,4-DCP did inhibit AR-mediated reporter that co-treatment with DHT. However, PCP was inactive for AR, suggesting PCP was not an agonist or antagonist of androgen, which was supported by Sun et al. (2006).

In the present study, we found that BPA was not an AR agonist, but was an AR antagonist. In previous studies, BPA has been demonstrated to have AR-mediated anti-androgenic activities using in vitro yeast-based assay (Lee et al., 2003). Sun et al. (2006) also reported that it was failed to find the AR agonistic effect of BPA, but found that BPA was an effective inhibitor of androgen-induced AR transcriptional activity (IC₅₀ value of 2.14×10^{-6} mol/L). The AR antagonistic activities of TCBPA have been investigated while the data contradicted each other. Sun et al. (2006) proved that TCBPA was an anti-androgen (IC₅₀ value of 1.0×10^{-5} mol/L). However, Kitamura et al. (2005) reported that TCBPA was not an androgen antagonist. In our study, TCBPA exhibited weaker anti-androgenic activities (RIC₂₀ of 1.0×10^{-5} mol/L) than did BPA. In addition, the antagonistic effect was not measured for TBBPA, which was consistent with the values reported by Kitamura et al. (2005).

These results suggest that many test compounds including some alkylphenol, chlorophenol, phenylphenol, BPA and BPA derivatives, act as AR antagonists. Once bound to AR, the androgen antagonists are transported into the nucleus and prevent endogenous androgens from regulating androgen-dependent transcription. The AR-compound interaction may be crucial contributor to adverse effects on male reproductive system. For example, exposure of fish to some anti-androgens has been associated with gonadal changes (induction of intersex), reduced spermatogenesis, demasculinization, and reduced sperm counts (Kiparissis et al., 2003).

3.3. Gene expression modulation via the PR

The potential activity of phenols via PR was investigated using recombinant PR yeast, in which none of the above-mentioned compounds showed agonistic activity (data not list). Antagonist activity was determined by co-incubation with 1×10^{-9} mol/L progesterone. In the results, TCBPA, TBBPA and PCP showed strong antagonistic effects with the RIC₂₀ value of 2.7×10^{-8} , 7.8×10^{-8} and 7.6×10^{-7} mol/L, respectively (Fig. 3, Table 1). At 1.0×10^{-6} mol/L and greater, 2-*t*-butylphenol, 3,4-DCP, 4-*t*-OP, 4-phenylphenol and 2-isopropylphenol decreased the β -galactosidase expression obviously, then the RIC₂₀ values for these compounds were determined and listed in Table 1. In contrast, 2,4-DCP and BPA were not antagonists of the PR.

Our results have demonstrated both 4-*t*-OP and PCP to be antagonists. The effect of these compounds on the PR has previously been investigated and it was confirmed that 4-*t*-OP and PCP inhibited progesterone-induced reporter gene activity in a

Fig. 2. Typical androgen receptor (AR) antagonistic activity of test chemicals determined by the yeast strain AR-GRIP1. The chemical's antagonistic activity is represented as the percent inhibition activity relative to the maximum induced by dihydrotestosterone (DHT, 5×10^{-8} mol/L). Values are presented as the average $\pm$ standard error ($n = 3$).

Fig. 3. Typical progesterone receptor (PR) antagonistic activity of test chemicals determined by the yeast strain PR. The chemical's antagonistic activity is represented as the percent inhibition activity relative to the maximum induced by progesterone (1×10^{-9} mol/L). Values are presented as the average $\pm$ standard error ($n = 3$).

dose-dependent manner with RIC50 value of 2.5×10^{-6} and 1.0×10^{-6} mol/L, respectively (Tran et al., 1996). Tran et al. (1996) also confirmed that 2,4-DCP had not any ant/agonistic activity. But, the fact that 2-*t*-butylphenol, 3,4-DCP, 4-phenylphenol and 2-isopropylphenol inhibited of PR-controlled β -galactosidase expression, suggesting that they are PR antagonists, has not been reported previously.

The agonistic or antagonistic effect of BPA had been investigated. The result showed that BPA did not act with PR (Tran et al., 1996), which was confirmed by our research. TBBPA and TCBCPA, halogenated derivatives of BPA, have been studied on their action with ER, but there was little work to study their action with PR. Our results have demonstrated both TBBPA and TCBCPA to be strong PR antagonists (RIC20 value lower than 1.0×10^{-7} mol/L). The ability of TBBPA and TCBCPA to inhibit progesterone-induced gene transcription in the yeast may reflect the ability of these compounds to compete with progesterone for binding to PR.

Although to date, only a limited number of environmental chemicals with anti-progesteronic properties have been identified, the disruption effects, especially the antagonistic effects, of environmental samples (e.g. soot from fossil fuel combustion) have been demonstrated (Wang et al., 2005). Recently other researchers have stressed the importance of some progesterones to fish, in which they act as pheromones crucial for eliciting physiological and behavioral responses in the sex (Sumpter, 2005). In addition, some studies have demonstrated that functional interactions between estrogen and progesterone are fundamental to maintaining some physiological process and suggested that progestins can oppose the effects of estrogens in some systems (Kraus et al., 1995). Thus, research on the agonistic and antagonistic activities of the chemicals provide endocrine disrupting mechanistic insight in the level at which these compounds are likely to disturb the PR system and induce/inhibit PR mediated cellular responses.

3.4. Modulation gene expression via the $ERR\gamma$

Our result showed that 4-*t*-OP and PCP were strong $ERR\gamma$ inverse agonists with RIC20 values of 3.5×10^{-6} and 3.0×10^{-6} mol/L, respectively (Fig. 4A). The inhibition effect of 2-*t*-butylphenol

Fig. 4. (A) Typical estrogen-related receptor γ ($ERR\gamma$) inverse agonistic activity of test chemicals determined by the yeast strain $ERR\gamma$ -GRIP1. (B) Effect of test chemicals on the inverse agonist activity of 4-hydroxytamoxifen (4-OHT) in yeast strain $ERR\gamma$ -GRIP1. The chemical's inverse agonistic activity is represented as the percent inhibition activity relative to the high constitutive activity in yeast strain $ERR\gamma$ -GRIP1. Values are presented as the average $\pm$ standard error ($n = 3$).

was so weak that the RIC20 value could not be determined (Table 1). However, other compounds did not decrease β -galactosidase expression compared with negative control (DMSO).

Then 1.0×10^{-5} mol/L 4-OHT, which inhibited about 20% ERR γ constitutive activity at this concentration, was chosen as standard inverse agonist to test the inhibitory effect of test compounds on the inverse agonist activity of 4-OHT (Li et al., 2008b). The result showed that 4-OHT's activity was suppressed by 2,4-DCP, 3,4-DCP, PCP, BPA and TBBPA (Fig. 4B) in a concentration dependent manner. Especially for 2,4-DCP, PCP and BPA almost all activity induced by 4-OHT was reversed by co-administrated with these chemicals. The activity induced by TCBPA was so weak that the significant difference with control (DMSO) only be found at high concentration (1.0×10^{-6} mol/L). In comparison, 2-*t*-butylphenol, 4-*t*-OP, 4-phenylphenol and 2-isopropylphenol did not reverse the inverse agonist activity of 4-OHT.

ERR shares a high amino acid sequence homology with ER. Considering the fact that ERR can bind to several functional EREs, attempts were made to search for ant/agonists of ERR. In the present study, 4-*t*-OP and PCP were found to be strong inverse agonists for ERR γ . In the previous work, the compounds, toxaphene and chlordane, have been reported to behave as antagonists of ERR α -1 and modulate estrogen biosynthesis (Yang and Chen, 1999). Meanwhile, the PCP and other compounds, 2,4-DCP, 3,4-DCP, BPA and TBBPA, could interact with ERR γ and showed a strong effect on the inverse agonist activity of 4-OHT. An interesting phenomena was found that PCP was proved as both ERR γ inverse agonist and inhibitor of inverse agonist induced by 4-OHT in this study. In combination, PCP and 4-OHT might be substantially less toxic than either drug alone. Their combined toxicity may be due to independent effects of each compound, which is need for further investigate. Previous work had been shown that BPA could antagonize the inhibition activity of 4-OHT and such potential functional cross-talk between the ERR γ and ER systems might used to explain some of the estrogenic activities of BPA (Takayanagi et al., 2006). Thus, similar to BPA, the interaction with the ERR γ was a possible mechanism for the estrogenic activity for these compounds, including 2,4-DCP, 3,4-DCP, PCP, BPA, TBBPA and TCBPA.

3.5. Action sites

The results in this study suggested that the tested alkylphenols, including 2-*tert*-butylphenol, 4-*t*-OP and 2-isopropylphenol, mainly acted with AR, PR and/or ERR γ . 4-*t*-OP was the only chemical which exhibited both of ER α agonistic and antagonistic activity. Some xenoestrogens showing both ER agonistic and antagonistic effects had been reported, which also were confirmed that the antagonistic activity of these compounds, although statistically significant, was biologically marginal (Soto et al., 1997). In an *in vivo* test, the vitellogenin was detected in serum of the male medaka fish exposed to 4-*t*-OP in all treatment group in steadily increasing concentrations (Gronen et al., 1999), proving the estrogenic activity of 4-*t*-OP in fish. Endocrine disrupters are widely known for their estrogenic and androgenic effects by binding to ER or AR. But, the mechanisms of action of endocrine disrupters are actually complicated. For 4-*t*-OP, it was found that the obvious interrupting effect was inhibiting the gene expression via the PR at 1.0×10^{-6} mol/L. Interestingly, similar results obtained with 2-*tert*-butylphenol and 2-isopropylphenol which may interact with PR showing strong anti-progesteronic activity. Therefore, although disruption of the progesterone activity was rarely addressed in the past, in contrast to estrogenic and anti-androgenic activities, it could well contribute to trigger a wide range of hormonal and/or anti-hormonal effect *in vivo* (Molina-Molina et al., 2006).

For BPA, TBBPA and TCBPA, they acted as ER α agonists as well as AR and/or PR antagonists, but the abilities to interact with ER α , AR and PR were significantly different. TBBPA and TCBPA exhibited obvious estrogenic activity at 1.0×10^{-5} mol/L, the highest concentration without cytotoxicity in our studies. However, the lowest concentration to observe the anti-progesteronic activities of TBBPA and TCBPA were detected at 1.0×10^{-8} mol/L. Although anti-progesteronic activity of BPA was not found, BPA showed significant AR antagonistic activity with RIC20 values of 1.2×10^{-6} mol/L. Moreover, we confirmed that all tested bisphenols were also inhibitors that reversed the antagonistic effect induced by 4-OHT at the level of ERR γ , which can be used to explain the estrogenic activity of these compounds (Takayanagi et al., 2006).

In the present study, some chlorinated phenols and phenylphenol were also detected. The results showed that these phenols acted with more than one receptor. Taken together, all of the results suggest that the tested chemicals might exhibit multiple hormonal activities, which renders difficult interpretation of their mechanism of action. Although the potency of these phenols to behave as a hormone agonists or antagonists is low compared to the natural ligands, their ability to act via more than one mechanism might contribute to their biological effects in the intact organism, because the final response will likely be determined by the interactions of all the implicated pathways (Molina-Molina et al., 2006). Thus, for a detailed risk assessment of phenols concerning endocrine disruption in humans and animals, more data, especially *in vivo* data, are needed.

Acknowledgements

This study was supported by National Natural Science Foundation of China (20677075), the National Basic Research Program of China (2003CB415005), and Natural Science Foundation of Beijing Municipality (8061004).

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