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4-Alkylphenols and related chemicals show similar effect on the function of human and rat estrogen receptor α in reporter gene assay

Hong Sun, Xiao-Lin Xu, Jian-Hua Qu, Xia Hong, Yu-Bang Wang, Li-Chun Xu, Xin-Ru Wang *

Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing 210029, China

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Abstract

Alkylphenols (APs) are widely used as important industrial materials and have attracted lots of attention because of their potential estrogenic activities. In this study, we developed human estrogen receptor α (hERα) and rat estrogen receptor α (rERα) mediated reporter gene assays and compared the estrogenic activity of APs and related chemicals based on the two ERα. Human breast cancer cell line MCF-7 was co-transfected with Gal4-fused hERα and corresponding reporter plasmid; African green monkey kidney cell line CV-1 was co-transfected with rERα and reporter gene. Both assays showed acceptable response to natural estrogen 17β-estradiol (E2) with EC_{50} of 0.16 nM and 4.7 nM. Then the estrogenic activity of 4-APs, 4-phenylphenol and bisphenol-A were evaluated and compared with the effects of E2. The data suggested that test APs and related chemicals possessed weakly estrogenic activity and the activity of test APs increased with the increase of substituent size. This structure–activity relationship helped to infer the activity of chemicals with similar feature. Furthermore, test APs showed similar effect on the function of hERα and rERα. This consistency helped to extrapolate *in vivo* rodent data to human being when performing risk assessment of endocrine disruptors.

Keywords: Phenylphenol; Bisphenol-A; Estrogenic activity; Structure–activity relationships

1. Introduction

Alkylphenols (APs) are natural components of crude oil and important materials for alkylphenol ethoxylates (APEs) which are commonly used in many herbicides, paints, industrial cleaning and degreasing agents, and large quantities of these chemicals enter the seas (Ioppolo-Armanios et al., 1992; Meier et al., 2007). APEs enter the aquatic environment primarily via industrial and domestic wastewater. They can be biodegraded to lower ethoxymer and subsequently generate more persistent and lipophilic APs under anaerobic conditions (Thiele et al., 1997; Ying et al., 2002). There has been increasing concern over the presence of APs in the environment because of their toxicity and estrogenic activity (Comber et al., 1993). Historically, large quantities of APEs have also been used in offshore petroleum production, both as detergents for washing platforms and as additives in the production process. APEs are now banned in many European countries. However, they are still widely used in the USA and Asia as high performance and cost effective products (Renner, 1997; Meier et al., 2007).

Recently, many studies have been done to evaluate the distribution and concentration of APs in water in China and other countries (Ferrara et al., 2005; Chen et al., 2006; Song et al., 2006; Voutsa et al., 2006). Most studies focused on their estrogenic properties which were evaluated by their effect on the functions of ER. Both hERα and rERα were used in these studies. The hERα was used in traditional E-screen assay or hERα mediated transcriptional activation assay; and the rERα was used in ER binding

* Corresponding author. Tel.: +86 25 86862939; fax: +86 25 86527613.
E-mail address: xrwang@njmu.edu.cn (X.-R. Wang).

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assay and many in vivo assays such as uterotrophic assay. All these experiments were acceptable, but few researches were performed to compare the effects of 4-APs and related chemicals on the functions of these two ERα.

In the present study, we developed hERα and rERα mediated reporter gene assays, and compared the estrogenic activity of 4-propylphenol, 4-*tert*-butylphenol, 4-pentylphenol, 4-octylphenol, 4-phenylphenol and bisphenol-A (BPA), which were all composed of a phenol-ring and a substituent group. 4-Propylphenol was the first APs which were found possessing estrogenic activity. Dodds and Lawson (1938) reported that 4-propylphenol caused vaginal cornification, as occurs during a normal oestrus cycle, thus mimicking the activity of estradiol. 4-*tert*-butylphenol was detected in cosmetic products and proved to stimulate the expression of vitellogenin protein (Gagliardi et al., 1989; Meier et al., 2007). 4-Pentylphenol could stimulate the proliferation of MCF-7 cells in E-screening assay (Kwack et al., 2002). 4-Octylphenol was widely used to produce APE, and had been found in food, river, and sea water (Fernandes et al., 2003; Chen et al., 2006; Ogawa et al., 2006). And our previous studies have proved that octylphenol damaged the function of rat's testis (Bian et al., 2006) showed anti-androgenic activity (Xu et al., 2005). Phenylphenol possessed the similar structure with APs. It was one of the top 10 active ingredients in household pesticides and the second most widely used ingredient for indoor applications (Grossman, 1995). 4-Phenylphenol was reported to possess strongest estrogenic activity among different isomers of phenylphenol, with the activity close to that of BPA (Routledge and Sumpter, 1997; Paris et al., 2002).

Our study was undertaken to compare the effect of the above APs on the function of hERα and rERα. For this purpose, we co-transfected ERα and their corresponding reporter gene plasmid into mammalian cell lines, getting estrogen-response cell. Then the activity of APs, phenylphenol and BPA were evaluated and compared with the effects of E2. After comparing the relative activity of test chemicals, we found that there was a general trend of increase in estrogenicity of test chemicals with increased substituent size, and moreover, test 4-APs and related chemicals showed similar effect on the function of hERα and rERα.

2. Materials and methods

2.1. Chemicals

Test chemicals were the highest grade available for the environmental analysis. The source, purity and CAS of chemical are listed in Table 1. E2 (purity > 99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chemical structures of the materials tested in this study are shown in Fig. 1. Stock solutions of the chemicals were prepared in absolute ethanol at a concentration of 0.01 M, stored at −20 °C, and diluted to desired concentrations in phenol red-free DMEM medium (Sigma) immediately before use. The final ethanol concentrations in the culture medium did not exceed 0.1% (v/v) that did not affect cell yields.

2.2. Plasmids

We constructed the recombinant ER plasmid pGal4-ERdef based on the pGal4-L-TRβ vector which was from Professor Ronald M. Evans (Gene Expression Laboratory, Howard Hughes Medical Institute, San Diego, CA, USA) and the HEG0/pSG5 vector which was from Professor Pierre Chambon (The Institute for Genetics and Cellular

Table 1
Data on test chemicals

Chemicals and abbreviation	Supplier	CAS	Purity (%)
4-Propylphenol	Sigma	645-56-7	>99
4-*tert*-Butylphenol	Sigma	98-54-4	>99
4-Pentylphenol	Sigma	14938-35-3	>98
4-Phenylphenol	Sigma	92-69-3	>99
4-Octylphenol	TCI[a]	1806-26-4	>99
Bisphenol A	Sigma	80-05-7	>99
Diethylstilbestrol (DES)	Sigma	56-53-1	>99
ICI 182,780	Tocris[b]	129453-61-8	>99

[a] Tokyo Chemical Industry Co. (Japan).
[b] Tocris, Cookson Ltd., Bristol, UK.

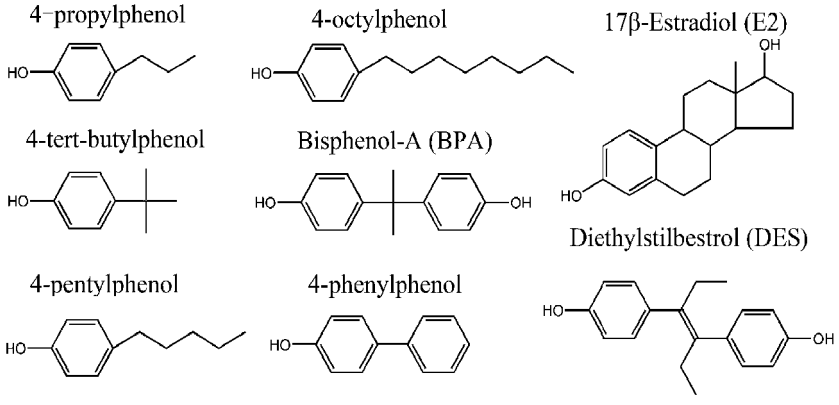

Fig. 1. Structures of related chemicals.

and Molecular Biology, University of Louis Pasteur, France). The following primer pairs were designed to amplify the portion of human ERdef domain: forward, 5′-ATTCGGTACCATGAAAGGTGGATACGA-3′; reverse, 5′-GCTGCAATAAACAAGTTCTG-3′. The ERdef fragment was verified by DNA sequencing and then digested by Kpn*I* and BamH*I*. At the same time, the pGal4-L-TRβ vector was digested by Kpn*I* and BamH*I*, removing the portion of L-TRβ. Then the ERdef fragment and pGal4 fragment of pGal4-L-TRβ were ligated to create the new plasmid pGal4-ERdef. The plasmid phRL-tk (used as internal control for transfection efficiency and the cytotoxicity of test chemicals), containing *Renilla* luciferase gene, was purchased from Promega (Madison, WI, USA). The Gal4 responsive luciferase reporter plasmid pUAS-tk-Luc containing four copies of the Gal4 binding site was also from Professor Ronald M. Evans.

The luciferase reporter plasmid pERE-TATA-Luc+ and rat ERα expression vector rERα/pCI was provided by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The plasmids were constructed as previously described (Takeyoshi et al., 2002).

2.3. MCF-7 cell assay

The MCF-7 cell line was a gift from Professor Xiao Han (Nanjing Medical University, China) and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen Corp., Carlsbad, CA, USA), 0.01 mg/ml bovine insulin (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) at 37 °C in an atmosphere containing 5% CO_2. The host cells were plated in 24-well microplate (Corning) at a density of 1.0×10^5 cells per well in the phenol red free DMEM medium containing 10% charcoal-dextran-stripped FBS (CDS-FBS). Following 12 h incubation, MCF-7 cells were transfected with 0.5 μg pUAS-tk-Luc, 0.2 μg of pGal4-ERdef and 0.1 μg of phRL-tk, using 5 μg Sofast™ (Sunma Company, Xiamen, China) transfection reagent per well. After an incubation period of 12 h, the transfection medium was removed before various concentrations of E2 or test chemicals dissolved in medium were added for measurement of their agonistic activity. Medium with 0.1% ethanol was used as a vehicle control.

2.4. CV-1 cell assay

The CV-1 cell line was obtained from Chinese Cell Center in Beijing, and maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Twelve hours after plated in the 24-well micro-plate at the density of 1.0×10^5 cells per well in the phenol red free DMEM medium containing 10% CDS-FBS, the CV-1 cells were transfected with 0.5 μg of pERE-TATA-Luc+, 0.2 μg of rERα/pCI and 0.1 μg of phRL-tk per well. Then the cells were treated with test chemicals.

2.5. Reporter gene assays

The cells were harvested after treated with test chemicals for 24 h. After rinsed three times with phosphate-buffered saline (PBS, pH 7.4), the cells were lysed with 1× passive lysis buffer (Promega). Then the cell lysates were analyzed immediately using a 96-well plate luminometer (Berthold Detection System, Pforzheim, Germany). The amount of luciferase and *Renilla* luciferase was measured with the Dual-luciferase reporter assay system kit (Promega) following the manufacturer's instructions. Then the value of luciferase for each lysate was normalized to the *Renilla* luciferase activity. At last, the relative transcriptional activity was converted to fold induction above the vehicle control value (*n*-fold).

2.6. Relative potencies

The estrogenic activity of test chemicals was recorded as REC_{20}, which was defined as the concentration of test compound showing 20% of the maximal activity of E2. EC_{50} (median effective concentration) and maximum induction fold of candidate chemicals were also calculated.

3. Results

3.1. Cytotoxicity of the tested chemicals

The concentrations of test chemicals were determined by MTT assay before performing reporter assay. No cytotoxic effect could be observed by microscopic examination throughout the transfection assay. Furthermore, the cytotoxicity of the chemicals was assessed by co-transfecting the cells with the plasmid phRL-tk. There was no significant difference between the tested groups and the vehicles control group in the expression of *Renilla* luciferase (data no shown). All these results suggested that no cytotoxic effect existed in the tested concentration ranges.

3.2. Response of MCF-7 assay to known ER agonist DES and antagonist ICI 182,780

MCF-7 cell reporter system showed appropriate response to the natural estrogen E2 and a known ER agonist DES. Both chemicals induced luciferase activity in a concentration-dependent manner in the test concentrations (Fig. 2). For E2, the maximal induction of 17.9-fold of vehicle control was achieved at concentration of 10^{-9} M. From the dose–response curve, the median effective concentration (EC_{50}) value of E2 was 0.16 nM. And for DES, the maximal induction of 17.8-fold of vehicle control was achieved at 10^{-8} M and greater, with EC_{50} of 0.28 nM.

To characterize the response of MCF-7 reporter system to anti-estrogen, ICI 182,780 was added the medium with

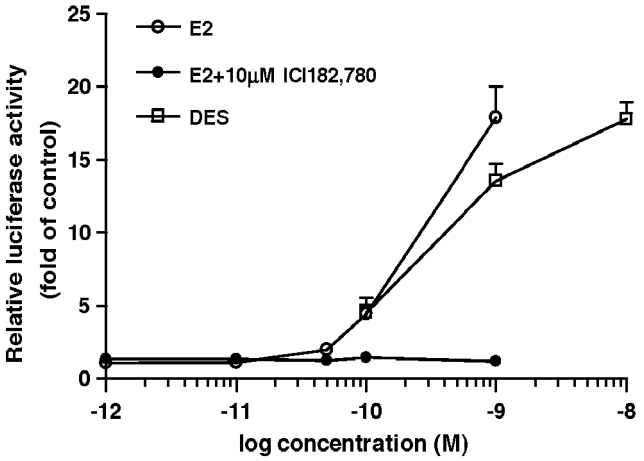

Fig. 2. The estrogenic activity of E2 and DES examined in MCF-7 assay. MCF-7 cells were transiently transfected with pUAS-tk-Luc, pGal4-ERdef and phRL-tk. Then we treated the cells with a various concentration of E2 and DES. The absolute ethanol solvent was used as control. Values were means ± SE of three independent experiments and the estrogenic activity were presented as fold of control. 10 μM ICI 182,780 completely inhibited luciferase expression induced by E2.

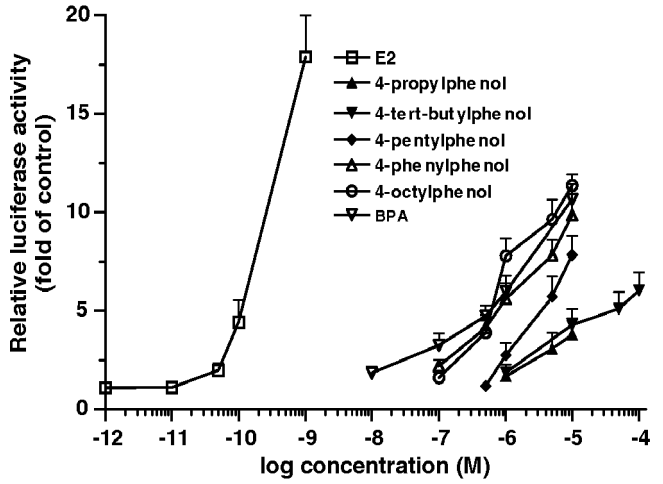

Fig. 3. The estrogenic activity of test chemicals in MCF-7 cell assay. Values were means ± SE of three independent experiments and the estrogenic activity were presented as fold of control.

various concentrations of E2. As a result, 10 μM ICI 182,780 completely inhibited the E2-inducing luciferase expression (Fig. 2).

3.3. Estrogenic activities of 4-APs in MCF-7 reporter assay

When the test APs were administered to the transfected cells, each of them induced the expression of luciferase (Fig. 3). The REC_{20}, EC_{50} value and the maximum induction of each chemical are compared in Table 2. We found that the size of alkyl group was related with estrogenic activity. The rule was that the expression of luciferase increased with each additional carbon in the substituent, from 3 to 8 carbon atoms, but all test alkylphenol chemicals were much less potent than E2 (Table 2).

3.4. Estrogenic activities of 4-phenylphenol and BPA in MCF-7 reporter assay

Phenylphenol and BPA have the similar chemical structure with alkylphenol compounds (Fig. 1). Because phenylphenol has another benzene-ring (six carbon atoms) at the 4-substituted of phenol-ring, we inferred that this chemical had the similar activity with hexylphenol (six carbons). The result indicated that the estrogenicity of 4-phenylphenol was just between the 4-pentylphenol (five carbons) and 4-octylphenol (eight carbons), with REC_{20} of 0.31 μM. And BPA, the widely used industrial material, showed relative high estrogenicity with REC_{20} of 0.10 μM (Table 2).

3.5. Estrogenic activities of 4-alkylphenols, phenylphenol and BPA in CV-1 reporter assay

CV-1 reporter assay was also developed to study the estrogenicity of test chemicals. In this system, CV-1 cells

Table 2
APs, phenylphenol and BPA showing estrogenic activity in MCF-7 cell assay and CV-1 cell assay

Chemicals	Carbon number[a]	MCF-7 cell assay (hER)			CV-1 cell assay (rER)		
		REC_{20} (μM)[b] mean $n = 3$	EC_{50} (μM)[c]	Maximum induction (%)[d]	REC_{20} (μM) mean $n = 3$	EC_{50} (μM)	Maximum induction (%)
17β-Estradiol(E2)	–	0.36×10^{-4}	1.66×10^{-4}	100	1×10^{-3}	4.7×10^{-3}	100
4-Propylphenol	3	9.18	1.19	21.1 ± 4.6	NA	1.32	13.4 ± 3.1
4-*t*-Butylphenol	4	7.25	4.22	33.7 ± 4.8	22.83	5.78	26.2 ± 3.4
4-Pentylphenol	5	1.45	1.68	43.8 ± 5.6	1.31	0.91	29.8 ± 1.4
4-Phenylphenol	6	0.31	0.61	55.0 ± 6.2	0.66	1.44	99 ± 3.8
4-Octylphenol	8	0.28	0.69	63.6 ± 2.9	0.52	0.64	48.9 ± 3.9
Bisphenol-A (BPA)	–	0.10	0.34	59.5 ± 4.4	0.13	0.63	142.8 ± 9.3

NA, not active.

[a] The carbon number in the 4-subtituent of phenol-ring.

[b] REC_{20}, which was defined as the concentration of test compound showing 20% of the activity of 10^{-9} M E2 in the MCF-7 assay and of 10^{-7} M E2 in the CV-1 assay.

[c] Concentration at which the induction of luciferase activity is 50% of the maximum.

[d] Percent luciferase activity induced by the test compound relative to the maximum luciferase activity induced by E2.

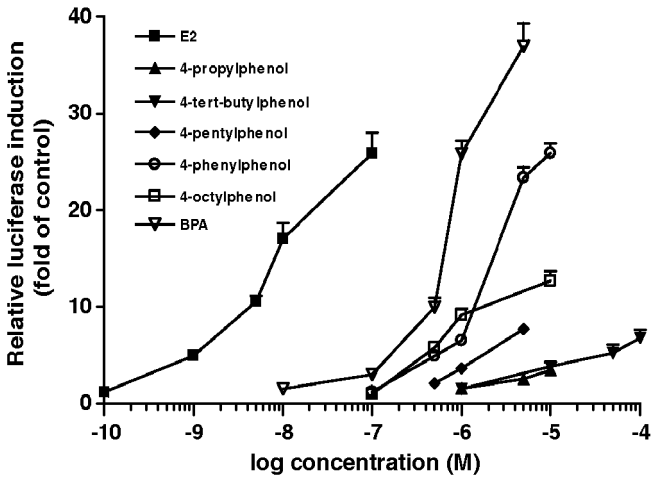

Fig. 4. The estrogenic activity of test chemicals in CV-1 cell assay. The CV-1 cells were transiently transfected with pERE-TATA-Luc+, rERα/pCI and phRL-tk, and then treated with various concentrations of APs, phenylphenol and BPA. Values were means ± SE of three independent experiments and the estrogenic activity were presented as fold of control.

were transiently transfected with rER and ERE-aug-LUC+, and phRL-tk was used as internal control. As a result, 10^{-7} M E2 could induce the expression of luciferase of 25.9-fold of control (Fig. 4). The alkylphenol compounds, phenylphenol and BPA also showed their ability of inducing luciferase expression. The related results are compared in Table 2. Briefly, the estrogenicity of test alkylphenol and phenylphenol was 4-propylphenol < 4-*tert*-butylphenol < 4-pentylphenol < 4-phenylphenol < 4-octylphenol < BPA. These results accorded with the rule getting from MCF-7 hERα mediated reporter assay. It was noticeable that phenylphenol and BPA showed surprising activity of inducing luciferase expression of 25.8-fold and 36.9-fold over solvent control, respectively (Fig. 4).

4. Discussion

The value of reporter gene assays, both as a mechanistic tool to characterize receptor mediated endocrine activity but also as an important screening assay for endocrine disruptors has been well recognized (Freyberger and Schmuck, 2005). In this study, we developed two different reporter gene assays to investigate the effect of various size of alkyl group on the estrogenicity of APs and their derivatives. The results showed that test APs possessed potent activity of inducing the expression of estrogen response reporter gene, and their activity increased with the increase of substituent size in both assays.

In the present study, MCF-7 cells were transfected with chimeric Gal4 fused ligand binding domain (LBD) of hERα and Gal4-response reporter gene to study the interaction of hERα and chemicals. This assay showed acceptable induction of luciferase under the stimulating of E2, with the 17.9-fold induction over control (Fig. 3). Additionally, the known ER agonist DES was examined and showed similar activity with E2 in MCF-7 assay, this result accorded with many others (Olsen et al., 2005). Furthermore, 10 μM ICI 182,780 completely blocked the action of E2, which indicated that the expression of reporter gene reflected a special ER-mediated activity. All these preliminary results meant that this reporter assay had the ability to detect the estrogenic activity of unknown candidate chemicals. Then, a series of APs with various size of alkyl groups were examined. As a result, we found that there was a general trend of increase in estrogenicity with increased substituent size. When we tried to explain this rule, we believed that the addition of each carbon in alkyl group increased the hydrophobicity of the APs. And these changes in structure facilitated their binding with the LBD of hERα. This structure–activity relationship was also reported by the study using recombinant yeast screen (Routledge and Sumpter, 1997).

Although MCF-7 cell line has been widely used to screen estrogenic activity of environmental chemicals, data from a single cell line may contain both false negative and false positive results which were related to certain cross-talk pathways in the cell (Kitamura et al., 2005). Then another mammalian cell line CV-1 was utilized to develop a traditional rERα mediated transcriptional activation assay. This CV-1 assay was less sensitive than MCF-7 assay with EC_{50} of 4.7 nM. However, tested APs showed the same REC_{20} order in the two assays (Table 2). That was to say, the estrogenic activity of APs increased with the addition of carbon number of substituent in the two different assays. These results suggested that the types of cell lines and the sources of ER showed little effect on the estrogenic activity of APs. And the 96% homology in rat and human estrogen receptor LBD (Koike et al., 1987) could partly explain this similar response. This consistency helped to extrapolate rodent data from rER binding assay and uterotrophic assay to humans being.

Both phenylphenol and BPA showed relative strong ability of inducing luciferase expression. And the estrogenicity of 4-phenylphenol was just between the 4-pentylphenol (five carbons) and 4-octylphenol (eight carbons) in both hERα and rERα assay. It was noteworthy that ERE-dependent transcriptional activation (CV-1 assay) of BPA reached 142.8% of the maximal E2 effect (Table 2). Such "supramaximal" effects had been reported on genistein and other phytoestrogens, although the mechanism involved was not clear (Kuiper et al., 1998), and this phenomenon caused by BPA was also observed by the other researchers (Kitamura et al., 2005). However, the 3-day uterotrophic assays did not get the same outcome even 80,000-fold higher than E2 in dose (Laws et al., 2000). This difference in results would be attributed to the feedback of hypothalamus–pituitary–gonad axis *in vivo* animal experiment.

The relationship between substituent size of APs and their estrogenicity were also confirmed by the animal experiments. Kwack et al. (2002) studied the uterotrophic effects of APs in ovariectomized rats. In their study, a series of APs (including 4-propylphenol, 4-*tert*-butylphe-

nol, 4-pentylphenol, 4-octylphenol, and 4-phenylphenol) were examined for estrogenic activity. Their results showed APs with bulky alkyl groups possessed higher estrogenic capacity. Our data from rERα mediated reporter gene assay accorded with their *in vivo* assay. And this consistency in conclusion indicated that the reporter gene assay was a useful *in vitro* tool to screen xenoestrogens.

In conclusion, we evaluated and compared the estrogenic activity of 4-APs, phenylphenol and BPA using two *in vitro* reporter gene assays. As a result, all test chemicals could exert estrogenic activity, and the activity increased with the increase of substituent size. This structure–activity relationship helped to infer the activity of chemicals with similar feature. The most important was that the agonist potency of APs based on hERα was in accordance with rERα; this consistency improved the reliability of extrapolating rat data to human when performing risk assessment of endocrine disruptors. The impact of APs and their derivatives would be expected to be small because of lower in activity than E2 (Table 2), but they still added to the overall "estrogen load" of bodies. Fortunately, there's no adequate evidence that low levels of exposure, either to single chemicals or to mixtures with potentially similar actions, cause adverse effects in humans. It was these lower levels of exposure that raise the most important public health questions, because even small shifts in population distributions of adverse health outcomes, such as infertility, could potentially impact on the overall health of large populations (ICCVAM, 2002). Our laboratory has been working on the relationships between endocrine disruptors and male infertility (Wu et al., 2007). And it seemed there was still a long way to go after screening endocrine disruptors using limited *in vitro* and *in vivo* assays which were mainly based on cell lines or animals.

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