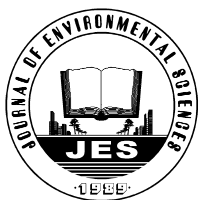

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Estrogenic activities of two synthetic pyrethroids and their metabolites

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Abstract

Synthetic pyrethroids (SPs) are among the most common pesticides in current use, and so far, several SPs have been assessed for their potential estrogenicities by various methods. Previous studies have shown that the estrogenicities partly come from their metabolites. Although considerable information is available with respect to the metabolism and environmental degradation of SPs, little is known about the estrogenicities of the metabolites. In this study, permethrin (PM) and β-cypermethrin (CP), as well as their metabolites (3-phenoxybenzoic alcohol (PBCOH), 3-phenoxybenzaldehyde (PBCHO) and 3-phenoxybenzoic acid (PBCOOH) were evaluated for their estrogenic activities in the MCF-7 human breast carcinoma cell line. In the MCF-7 cell proliferation assay, PM and CP exhibited significant estrogenic activities at 10^{-7} mol/L, comparable to 17β-estradiol (E_2) of 10^{-9} mol/L, with the relative proliferative effect ratios of 55.4% and 56.3%, respectively. The real-time quantitative polymerase chain reaction (qRT-PCR) results confirmed the estrogenicities of PM and CP with significant alteration of pS2 and ERα mRNA levels observed at 10^{-6} mol/L. For the three major metabolites, PBCOH and PBCOOH exhibited estrogenic activities in all assays, while no significant estrogenic responses was observed for PBCHO compared to the vehicle control. In particular, PBCOH had even slightly stronger estrogenic activity than its parent compounds, indicating that metabolism may be one of the reasons for the estrogenicities of the SPs. Given the widespread use of SPs, the toxicological effects of parent compounds and their metabolites should be taken into consideration in the risk assessment of SPs.

Key words: pyrethroids; metabolites; cell proliferation; gene expression; estrogenic activity

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Introduction

Over the past few decades, evidence that a large number of man-made chemicals discharged into the environment may have the ability to alter the normal function of the endocrine system has sparked an increasing concern in the public (Colborn et al., 1993). In particular, the most suspicious endocrine disruptors, often referred to as xenoestrogens, are a diverse group of substances that do not necessarily share any structural resemblance to the natural hormone 17β-estradiol (E_2), including organochlorines, organophosphorus pesticides and synthetic pyrethroids (SPs). However, they may exert estrogenic effects by mimicking or inhibiting the actions of endogenous estrogens, such as E_2, and can be recognized by their ability binding to the estrogen receptor, and therefore inducing or attenuating a response (Zacharewski, 1997; Kojima et al., 2004). Therefore, several *in vitro* assay, including competitive ligand binding, recombinant receptor/reporter gene, protein expression/enzyme activity, and cell proliferation assay, have been developed and repeatedly used to identify chemicals with estrogenic activities for the sake of simplicity, with more definitive end points utilized than *in vivo* assay (Korach et al., 1978; Markiewica et al., 1993; Miksicek, 1994; Soto et al., 1995; Zacharewski, 1997; Legler et al., 1999).

SPs, analogs of a natural chemical moiety, pyrethrin, derived from the chrysanthemum (Casida, 1980), are among the most common pesticides in current use, accounting for more than 30% of the world market of insecticides (Köprücü and Aydın, 2004). The widespread use of SPs for control of agricultural and indoor pests has resulted in an increased presence in the environment and extensive human exposure (Casida, 1980; Xu et al., 2008; Liu et al., 2008, 2009). However, SPs were designed to be relatively unstable to minimize their toxicological impact on both the environment and humans, and once released into the environment, they may undergo rapid degradation by abiotic and biotic processes (Tyler et al., 2000; McCarthy et al., 2006). Two common pyrethroids, permethrin (PM) and β-cypermethrin (CP), follow similar pathways of metabolism in insects, fish, birds and mammalian animals. The metabolite 3-phenoxybenzoic alcohol (PBCOH) is first produced via the cleavage of the central ester linkage,

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with hydrolysis of the parent compounds being the most important process in mammals. Subsequently, the primary alcohol may be further oxidized to 3-phenoxybenzoic acid (PBCOOH) via 3-phenoxybenzaldehyde (PBCHO) (McCarthy et al., 2006). The metabolites are widely found in the environment and usually detected in urine (Heudorf and Angerer, 2001).

Although the use of SPs has been encouraged by their supposed low toxicities in mammals and birds (Glickman and Lech, 1982; Köprücü and Aydın, 2004), possible adverse effects on the endocrine system have been observed with several SPs. Some publications have already included SPs in their lists of potential endocrine disruptors (Colborn, 1995; Zhao et al., 2009). For example, both fenvalerate and sumithrin were able to induce cell proliferation and pS2 expression in the MCF-7 human breast carcinoma cell line, while fenvalerate and *d-trans* allethrin significantly antagonized the action of progesterone in the T47D human breast carcinoma cell line (Garey and Wolff, 1998; Go et al., 1999). In our previous studies, both lambda-cyhalothrin and bifenthrin were found to exhibit estrogenic potential in *in vitro* human breast carcinoma MCF-7 cell proliferation assay (Wang et al., 2007; Zhao et al., 2008). However, little information is available regarding the estrogenic potentials of the metabolites of SPs due to their supposed lower toxicity to biota than parent compounds (Tyler et al., 2000; McCarthy et al., 2006; Sun et al., 2007). Nevertheless, the metabolites may be more toxic and pose a greater risk to the environment and humans (Sinclair and Boxall, 2003). For several chemicals, the products of their metabolism and/or environmental degradation, rather than the parent compound itself, are active as endocrine mimics (Tyler et al., 2000). With respect to the SPs, both PBCOH and PBCHO have shown significant estrogenic activities based on recombinant yeast assay (Tyler et al., 2000; McCarthy et al., 2006), and the potency of PBCOH was more than 100-fold greater than that of the parent compound, PM. 3-Phenoxybenzoic acid demonstrated anti-estrogenic activity in recombinant yeast expressing human estrogen receptors (Tyler et al., 2000), while inconsistent results were found using a human androgen receptor-mediated luciferase reporter gene assay and PBCOOH was shown to be anti-androgenic (Sun et al., 2007). Therefore, the knowledge of the relationship between the estrogenic activities of the parent compounds and that of the metabolites and whether SPs affect the endocrine system via these metabolites remains an issue to be further studied.

In the present study, the estrogenic activities of two SPs, PM and CP, were evaluated using real-time quantitative polymerase chain reaction (qRT-PCR) and cell proliferation assays based on the MCF-7 human breast carcinoma cell line (E-SCREEN), which have been widely used and proved to be simple and sensitive. In addition, PBCOH, PBCHO and PBCOOH, three major metabolites of PM and CP, were similarly examined to better understand the estrogenicities of SPs.

1 Materials and methods

1.1 Chemicals

Permethrin (PM; purity, 99%), 3-phenoxybenzoic alcohol (PBCOH; purity, 98%), 3-phenoxybenzaldehyde (PBCHO; purity, ⩾ 97%) and 3-phenoxybenzoic acid (PBCOOH; purity, 98%) were purchased from Sigma, USA, and cypermethrin (CP; purity, 98%) was obtained from Danyang Agrochemicals (Jiangsu, China). Structures of all chemicals tested in this study are given in Fig. 1. 17β-estradiol (E_2; purity, > 98%) was also obtained from Sigma, USA and used as the positive control. All tested compounds were initially prepared in ethanol, with the final ethanol concentrations not exceeding 0.1% (*V/V*), which did not affect cell yields. Charcoal/dextran-treated fetal bovine serum (CDFBS) and fetal bovine serum (FBS) were purchased from HyClone, USA. Other chemicals or solvents used in this study were of cell culture or analytical grade.

1.2 Cell lines and cell culture conditions

MCF-7 cells, purchased from the cell bank of the Chinese Academy of Sciences, Shanghai, China (the original source is American type culture collection (ATCC), USA), were grown in Eagle's minimum essential medium (MEM) with phenol red, supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mmol/L nonessential amino acids, 4 mmol/L L-glutamine, 0.1 mmol/L sodium pyruvate, 10 µg/mL insulin, and 10% FBS. Cells were maintained at 37°C with 5% CO_2, 95% air in a humidified incubator (Thermo Electron, Marietta, OH, USA). The culture media was refreshed every 3 days, and subculture at a ratio of 1:3 (*V/V*) was performed with routine trypsinization every 6 days. Before the treatment, the medium was removed and replaced with the experimental medium

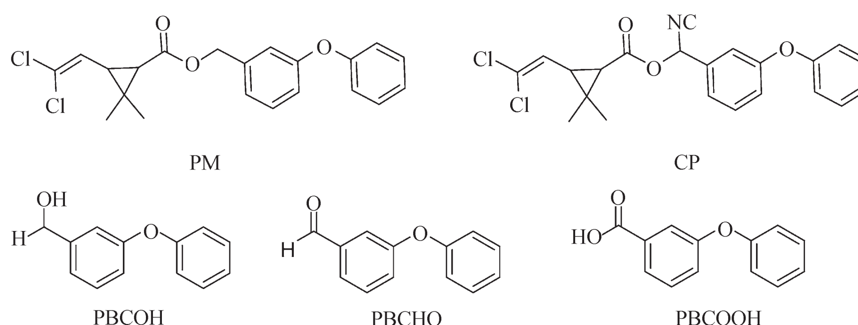

Fig. 1 Chemical structures of permethrin (PM), cypermethrin (CP) and their metabolites.

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(phenol red-free MEM containing 5% CDFBS) for 2 days to minimize its estrogenic activity and improve the sensitivity of MCF-7 cells to estrogen and xenoestrogens.

1.3 Cell proliferation assay

This assay was carried out according to previously described methods (Wang et al., 2007; Zhao et al., 2008). MCF-7 cells (1000 cells per well) were seeded on 96-well plates (Costar, Cambridge, USA) and allowed to attach for 24 hr. The medium was then replaced with the experimental medium containing five concentrations of the chemicals tested (0.001–10 μmol/L). On day 6, cell proliferation was determined by measuring the absorbance at 490 nm with a Bio-Rad Model 680 micro plate reader (Bio-Rad Laboratories, USA). Results were presented as the mean ± standard deviation (SD) of five independent measurements performed in four replicates. The proliferative effect (PE) and the relative proliferative effect (RPE) (the definition shown in Table 2) were used for assessment of the estrogenicity of the chemicals. E_2 (10^{-9} mol/L) and ethanol (0.1%, *V/V*) were used as the positive and negative control, respectively. The statistical analyses of the data were performed in Origin 6.0 (OriginLab, USA) and the *t*-test was used to evaluate differences between the treatment groups, where values were considered statistically significant when *P* was less than 0.05.

1.4 RNA isolation and real-time quantitative PCR

The assay was performed as described by Wang et al. (2008). After incubation in the experimental medium for 2 days, fresh phenol red-free MEM containing 5% CDFBS was added to the cells along with the chemicals to be tested. We used ethanol (0.1%, *V/V*) and E_2 (10^{-9} mol/L) as the negative and positive control, respectively. The cells were incubated for 2 days, and total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The OD_{260}/OD_{280} ratio as well as the banding patterns on a 2% agarose gel was routinely checked for the purity and integrity of the RNA sample.

Reverse transcription (RT) was carried out using an M-MLV reverse transcriptase kit (Takara Biochemicals, Dalian, China). First-strand complementary DNA synthesis was performed by priming with 1 μL of total RNA from each sample and 100 pmol oligo (dT)15 primer in a final reaction volume of 20 μL containing 0.5 mmol/L each of dNTP mixture, 1 U/μL of RNase inhibitor, 10 U/μL M-MLV reverse transcriptase, and 1× M-MLV buffer. The reaction mixture was maintained at 42°C for 50 min, followed by 15 min of heating at 70°C to stop the reaction.

A portion of 1 μL RT products was used directly for the real-time quantitative PCR on a 7300 Real Time PCR System (Applied Biosystems, USA) in a 20.0-μL SYBR reaction mixture with specific primers (Biotechnology, Shanghai, China) designed based on the cDNA sequences from the NCBI database (Table 1). The SYBR reaction mixture consisted of modified polymerase (including SYBR Green, optimized PCR buffer, $MgCl_2$, and dNTP mix), ROX reference dye, and RNase-free water (Takara Biochemicals, Dalian, China). Thermal cycling conditions were as follows: denaturation (95°C for 10 sec), and 40 cycles of amplification and extension (95°C for 5 sec, and then 60°C for 31 sec). Following PCR, analysis of a melting curve was performed to demonstrate the specificity of the PCR product, as displayed by a single peak. The C_T value, the cycle number at which a fluorescent signal rises statistically above the background, was determined for each transcript, and the relative quantification of target gene expression among the treatment groups normalized to GAPDH was calculated with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Data were presented as the mean ± SD of three independent measurements.

Table 1 Sequences of primers used in the real-time quantitative PCR reactions

Gene name	Sequence of primer	PCR product size (bp)
pS2	Forward 5'-CATCGACGTCCCTCCAGA AGAG-3' Reverse 5'-CTCTGGGACTAATCACCGTGCTG-3'	105
ERα	Forward 5'-CCACCAACCAGTGCACCATT-3' Reverse 5'-GGTCTTTTCGTATCCCACCTTTC-3'	108
GAPDH	Forward 5'-AAATCAAGTGGGGCGATGCTG-3' Reverse 5'-GCAGAGATGATGACCCTTTTG-3'	118

2 Results

2.1 E-SCREEN assay

The potentials of the test compounds to induce MCF-7 cell proliferation were estimated as a hallmark of estrogenic activity. The cells were treated with SPs and their metabolites or with E_2/ethanol as the positive/negative control, and cell proliferation was determined on day 6 during the logarithmic growth phase. The solvent ethanol was tested for its ability to affect MCF-7 cell proliferation, and was found to have neither estrogenic nor inhibitory activity (data not shown). As expected, E_2 induced cell proliferation at a concentration of 10^{-9} mol/L, and PE value was 2.12 (Table 2). With the exception of PBCHO,

Table 2 Estrogenic effects of permethrin, β-cypermethrin and their metabolites as measured by the different assays

Compound	Conc.[a] (mol/L)	E-SCREEN PE[b]	E-SCREEN RPE (%)[c]	pS2 expression RIE[d]	ERα expression RIE[e]
E_2	10^{-9}	2.12	100	100	100
CP	10^{-7}	1.63	56.3	38.4	68.7
PM	10^{-7}	1.62	55.4	53.1	69.3
PBCOH	10^{-7}	1.7	62.5	81.8	88. 7
PBCHO	10^{-7}	1.17	15.2	12.5	7.3
PBCOOH	10^{-7}	1.32	28.6	28.5	24

[a] The lowest concentration needed for maximal cell proliferation.
[b] The proliferative effect (PE) calculated as the ratio of the maximal cell yield of the test compound to the cell yield of the negative control.
[c] The relative proliferative effect (RPE) calculated as the ratio (PE-1) of the test chemical over (PE-1) of E_2 (× 100).
[d] The relative inductive efficiency (RIE) is the ratio between the maximal up-regulation of pS2 expression level by the test compound to that of E_2 (× 100).
[e] The relative inhibitory efficiency (RIE) is the ratio between the maximal down-regulation of ERα expression level by the test compound to that of E_2 (× 100).

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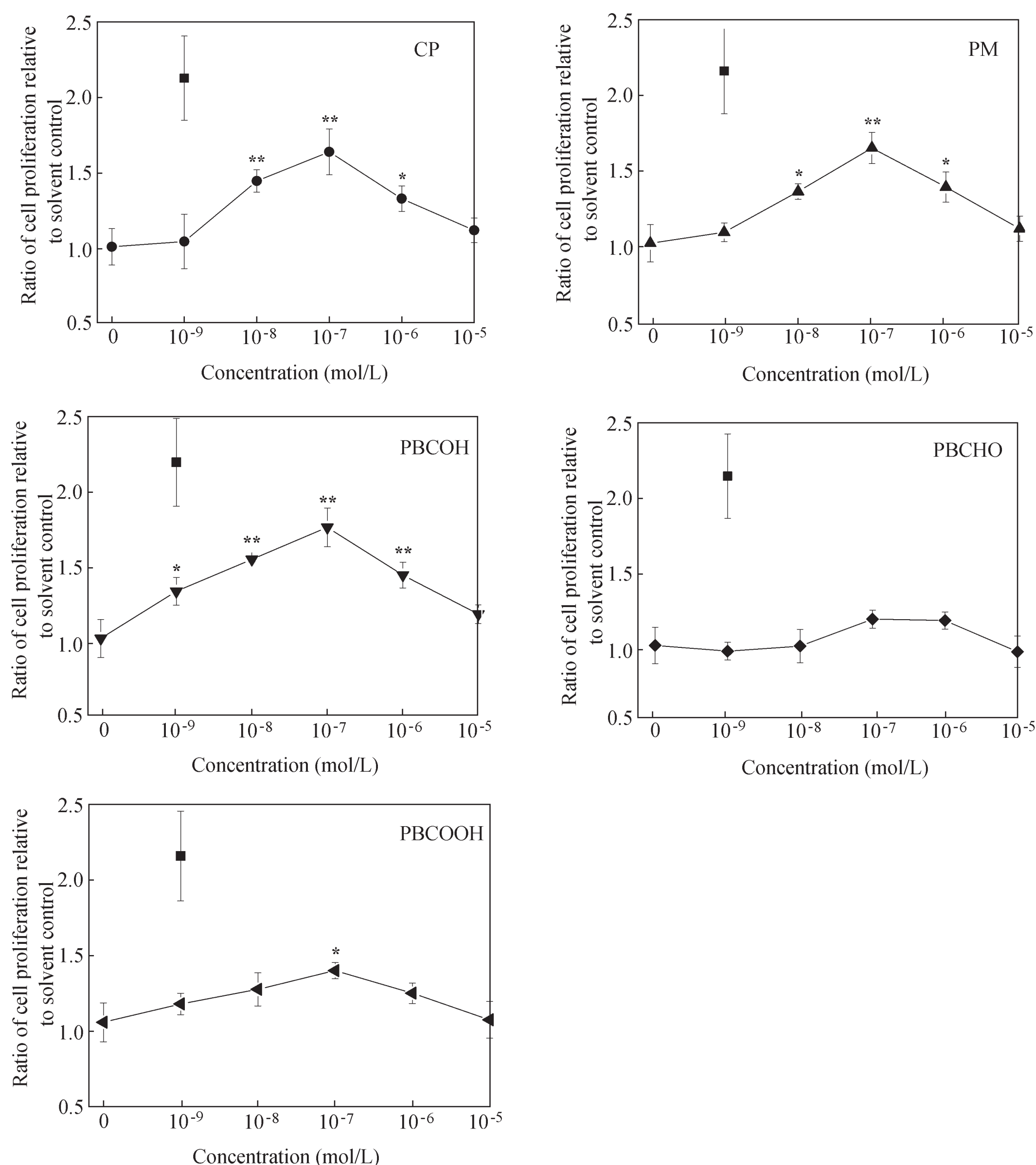

Fig. 2 Proliferation of MCF-7 cells grown in 5% CDFBS-supplemented medium exposed to various concentrations of CP, PM, PBCOH, PBCHO and PBCOOH. Results are presented as mean ± SD (n = 3, * P < 0.05; ** P < 0.01).

the other four compounds examined induced significant proliferation of MCF-7 cells (Fig. 2) in a concentration-related manner. The nonlinear response seemed to follow an inverted U-shaped dose-response pattern (Fig. 2) with the most significant effects observed at a concentration of 10^{-7} mol/L. The two parent compounds, CP and PM, exhibited proliferative effects comparable to 10^{-9} mol/L E_2, and RPE values were determined to be 56.3% and 55.4%, while the three metabolites induced cell proliferation to various degrees with RPE values for PBCOH, PBCHO and PBCOOH being 62.5%, 15.2%, and 28.6%, respectively (Table 2).

2.2 Expression of pS2 and ERα mRNA

Activation of pS2 gene transcription is a primary response to estrogen or estrogen-like chemicals in the human breast cell line MCF-7, and is widely used as a biomarker for the assessment of xenoestrogen (Go et al., 1999). Based on preliminary experiments, the pS2 mRNA levels of the test compounds at 10^{-6} mol/L concentration under which the maximal induction was achieved were measured in MCF-7 cells by qRT-PCR. The data in Fig. 3a indicated a significant increase in pS2 mRNA levels by E_2 (10^{-9} mol/L) compared with the vehicle control. All tested chemicals up-regulated the transcription of pS2 to various degrees. CP and PM induced pS2 expression 2.15 and 2.59 folds, respectively, as compared to 10^{-9} mol/L E_2 positive control, which induced pS2 expression approximately 4-fold. PBCOH induced 3.45-fold pS2 expression, while the effects of the other two metabolites were much less (1.37- and 1.85-fold).

The effects of the two SPs and their metabolites on ERα mRNA expression were also evaluated in respect that xenoestrogen may exert interference on the cells through the alteration of estrogen-responsive ER gene expression initiated by interaction with ERs. As shown in Fig. 3b, 10^{-9}

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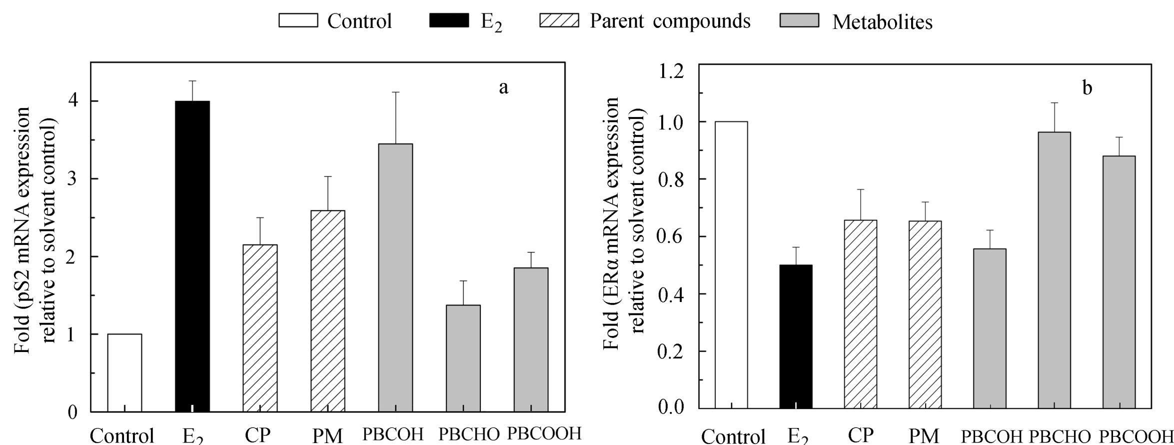

Fig. 3 Expressions of pS2 (a) and ERα (b) exposed to 10^{-6} mol/L of two synthetic pyrethroids and their metabolites. Values were normalized against GAPDH and the results are presented as mean ± SD (n = 3) relative to the control.

mol/L E_2 down-regulated the transcription level of ERα significantly compared with the control culture (50% of the control). Similarly, ERα mRNA expression was decreased significantly by 10^{-6} mol/L of CP, PM and PBCOH, while PBCHO and PBCOOH only down-regulated the expression levels by 3.7% and 12.0%, respectively.

The results of qRT-PCR were consistent with what was obtained in the cell proliferation assays. Two parent compounds, CP and PM, as well as two major metabolites, PBCOH and PBCOOH, demonstrated significant estrogenic activities, while no significant effect was observed for PBCHO.

3 Discussion

Chemicals with estrogenic properties can elicit primary effects normally regulated by endogenous estrogens, such as expression of specific genes, the production of certain hormones, and diverse processes like cell proliferation through pleiotropic actions (Fang et al., 2000). In this study, two SPs and three of their major metabolites were tested for their estrogenic potencies using *in vitro* combination assays, an MCF-7 proliferation assay, as well as pS2 and ERα mRNA expression assay.

In the MCF-7 cell proliferation assay, both CP and PM showed estrogenic activities comparable to the effect of E_2. The results were in good agreement with the previous study in which certain SPs displayed different abilities to induce proliferation of MCF-7 cells (Go et al., 1999). However, it seemed the maximal responses of the same compound from different laboratories were achieved at different concentrations indicating variability in E-SCREEN assay methods, and the various origins of the MCF-7 cell lines, the passage number or the serum used may be contributing factors (Zhao et al., 2008).

In addition to the proliferation effect of MCF-7 cells, we measured pS2 mRNA levels in MCF-7 cells as an endpoint. Previous studies have shown that the induction of pS2 gene expression was not necessarily correlated with cell proliferation (Go et al., 1999). However, in our study, CP and PM were found to be able to induce both pS2 gene expression and MCF-7 cell proliferation. To our knowledge, evaluation of the classic ER-mediated activation pathways are the first priority in understanding biological actions of environmental estrogens, and ERα is reported to be the predominant estrogen receptor isoform in MCF-7 cells (Saito et al., 2000). Therefore, in the present study, we focused on the alteration of ERα gene expression by the tested compounds. The data from Fig. 3b showed that ERα gene transcription levels were significantly down-regulated following treatment with CP and PM for 2 days similar to E_2.

For each *in vitro* assay, the response to a chemical was rated as fully estrogenic if the response was > 75%, partially estrogenic if the response was 25% to 75%, weakly estrogenic if the response was 10% to 25%, and negative if the response was below 10% of the response induced by E_2 according to a previous study (Andersen et al., 1999). Results shown in Table 2 indicated that both CP and PM exhibited partially estrogenic responses in our three different assays. However, responses observed for the three metabolites varied among the assays. For example, PBCOH was fully estrogenic in both pS2 and ERα mRNA expression assays, but partially estrogenic in the E-SCREEN assay. It seemed that, for the weaker estrogenic chemicals, e.g., PBCOOH, the responses in E-SCREEN assay were stronger than the other two assays, while in ERα mRNA expression assays, the responses were much stronger for those with high potency estrogenic chemicals such as CP and PM. Binding to other receptors (e.g., androgen receptor) expressed in the MCF-7 cells may antagonize E_2-induced cell proliferation (Zacharewski, 1997), and a degree of cellular toxicity resulting from SPs at high concentrations may have also complicated the outcome of the assays (Fang et al., 2000).

Previous studies have suggested that chemicals with estrogen-like activities may share similar structure properties to natural estrogen such as E_2 (Fang et al., 2000). Go et al. (1999) proposed that certain SPs partially composed of biphenyl ether moieties may signal through protein kinase C to affect pS2 mRNA expression and cell proliferation. However, this cannot explain the results in our studies as all compounds tested share biphenyl ether moieties, while the estrogen responses varied. Four of the five tested

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compounds exhibited estrogenic potentials with a decreasing potency order of PBCOH, PM, CP and PBCOOH. For the three metabolites of CP and PM, both PBCOH and PBCOOH had estrogenic activities, while PBCHO exhibited no significant estrogenic response compared to the vehicle control in all three assays. It seemed PBCOH was more potent than its parent compounds in causing endocrine disrupting effects in agreement with results from a previous study performed in recombinant yeast expressing human estrogen receptors (Tyler et al., 2000). PBCOOH was found to have anti-estrogenic activity, but no estrogenicity in prior yeast recombinant assays (Tyler et al., 2000; McCarthy et al., 2006). However, in our study, PBCOOH showed weak estrogenic activity. The discrepancy may reflect differences in sensitivities among the various assays. Much is known concerning SPs metabolism and their degradation in the environment, and the pathways have been well characterized for several SPs, including fenpropathrin and fenvalerate, similar to CP and PM, resulting in PBCOOH and PBCOH as two of their major derivatives (Tyler et al., 2000). Thus, for many SPs, rather than inactivating or detoxifying the parent compound, metabolism may generate metabolites with different estrogenic activities, and the parent compounds may act as estrogenic precursors if their metabolites have a much higher estrogenic potential. There are several potential explanations for the increasing toxicity of these metabolites. First, the metabolism process may keep the active component reserved or even result in chemical structures favoring receptor binding (McCarthy et al., 2006; Sinclair and Boxall, 2003). Secondly, a biotransformation step may activate those exerting estrogenicity via their metabolites, i.e., proestrogen (Sumida et al., 2001). However, the further oxidation of PBCOH via PBCHO to PBCOOH seemed to reduce the metabolites' estrogenicities by producing two less estrogenic compounds. Nevertheless, even with less estrogenic activities, the metabolites may still have an adverse impact on the environment due to the difference in environmental behaviors compared to the parent compounds, e.g., increased mobility and persistency, contributing to the bioavailability for the site of action (Escher and Hermens, 2002).

4 Conclusions

In summary, all tested compounds except PBCHO have been shown to be estrogenic in our MCF-7 cell proliferation and pS2 and ERα mRNA expression assays. In particular, PBCOH was even more estrogenic than its parent compounds to various degrees in different assays due to varying sensitivity of the three assays used here. The results in our study indicated that the metabolism may increase the toxicities of the parent compounds, instead of being a detoxification process. For SPs, the degradates as well as the parent compound are of great concern with respect to the assessment of their ability to induce estrogenic responses, and further research in both *in vitro* and *in vivo* systems are required for mechanism(s) elucidation.

Acknowledgments

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