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Antiandrogenic activity of pyrethroid pesticides and their metabolite in reporter gene assay

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Abstract

Many pesticides possess hormonal activity and have thus been classified as endocrine disruptors. Pyrethroids are commonly used pesticides worldwide, but little has been done to characterize their antiandrogenic activity potential. We tested three frequently encountered pyrethroids (fenvalerate, cypermethrin, permethrin) and their metabolite 3-phenoxybenzoic acid (3-PBA) for antiandrogenic and androgenic activity using a human androgen receptor (AR) mediated luciferase reporter gene assay in CV-1 African green monkey kidney cell. The assay displayed appropriate response to the known AR agonist 5α-dihydrotestosterone and AR antagonist nilutamide and flutamide. At 0.1 mM, all the three tested pyrethroids significantly suppressed the luciferase expression. Further, their metabolite 3-PBA also showed antagonist activity. None of the test chemicals showed androgenic activity. Through the antiandrogenic pathways, exposure to certain pyrethroids may contribute to the damage of reproductive system. In conclusion, pyrethroid pesticides can act as antiandrogen *in vitro*, and metabolizing to 3-PBA cannot eliminate the antagonist activity. This result provides useful information for risk assessment of pyrethroid pesticides.

Keywords: Androgen receptor; Endocrine disruptor; Fenvalerate; Cypermethrin; Permethrin; 3-Phenoxybenzoic acid

1. Introduction

In recent years, public and researchers have expressed increasing concern that pesticides and many other environmental chemicals have hormonal activity, and thus modify the normal functioning of human and wildlife endocrine systems (Colborn et al., 1993; Colborn, 1995). A major mechanism of endocrine disruption is the action of chemicals as receptor agonists or antagonists through direct interaction with hormone receptors, thus altering endocrine function. In particular, chemicals mimicking endogenous estrogen via estrogen receptor (ER) have been the focus of research for the last 20 years (Kojima et al., 2004). Meanwhile, recent studies have shown that several chemicals may exert antiandrogenic effect by interfering with androgen receptor (AR) (Sohoni and Sumpter, 1998; Vinggaard et al., 1999). Some chemicals including the bioaccumulating DDT metabolite *p*,*p*′-DDE (dichlorodiphenyldichloroethylene), bisphenol A, octylphenol and nonylphenol have demonstrated AR-mediated antiandrogenic activities *in vitro* (Gray et al., 1999; Xu et al., 2005). However, we know comparatively little about the interference of pesticides with human AR.

Pesticides are commonly used for the control of agricultural and indoor pests (Garey and Wolff, 1998). Synthetic pyrethroid pesticide is one of the most popular pesticides used in China. They are known to exert their pesticidal actions by altering the sodium permeabilities of insect nerve membranes by modulating voltage-sensitive sodium channels (Bloomquist, 1996). Laboratory evidence suggested that pyrethroids were relatively safe to humans

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and wildlife (Miyamoto et al., 1995). However, Colborn et al. (1993) have already included pyrethroids in their lists of possible endocrine disruptor chemicals (EDCs). Recently, several *in vitro* studies have evaluated the potential hormonal activity of pyrethroids (Eil and Nisula, 1990; Miyamoto et al., 1995; Gaido et al., 1997; Saito et al., 2000). Most of these studies of pyrethroids are their ability to interact with estrogen receptors, yet little has been done to assess their interaction with human AR.

To study the interaction between chemicals and AR, we developed a transient reporter gene assay based on CV-1 African green monkey kidney cell. The receptor reporter gene assay was proposed by the United States EPA for inclusion in a Tier 1 screening battery (T1S) to detect EDCs acting as receptor agonists and antagonists (EDSTAC, 1998). Binding of androgen to AR in target cells results in the initiation of specific transcription activation events. Therefore, the introduction of artificial, AR-regulated reporter gene constructs into cells has become a useful method of measuring AR transcriptional activation. The present reporter assay utilized the human AR for transcriptional regulation of a luciferase reporter gene. The objective of the study was to evaluate the possible androgenic and antiandrogenic activity of pyrethroids and their metabolite.

In this study, we tested three most commonly used pyrethroids including fenvalerate, cypermethrin and permethrin which were suspected to possess endocrine disruptor activity. Fenvalerate and cypermethrin are both type II pyrethroid insecticides, meaning they have an α-cyano group at the α-carbon position of the alcohol moiety. Our previous study showed that fenvalerate could induce significant reduction in testis weight, epididymal sperm count, sperm motility and marker testicular enzymes for testosterone biosynthesis (Bian et al., 2004; Xia et al., 2004, 2005; Xu et al., 2004). Ingestion of cypermethrin at 18.93 and 39.66 mg d^{-1} resulted in a significant decrease in the perimeter and number of cell layers of the seminiferous tubules. Epididymal and testicular sperm counts as well as daily sperm production were significantly decreased in cypermethrin exposed males (Elbetieha et al., 2001). These results demonstrated the adverse effects of fenvalerate and cypermethrin on fertility and reproduction in male rats. And permethrin, a type I pyrethroid, also showed antiandrogenic activity in Hershberger assay (Kim et al., 2005). In the present study, we detected significant antiandrogenic activity of the three pesticides and their major metabolite 3-phenoxybenzoic acid (3-PBA) in the AR-mediated reporter gene assay.

2. Materials and methods

2.1. Chemicals

Test chemicals were the highest grade available for the environmental analysis. The source, purity, CAS and abbreviation of chemical were listed in Table 1. Cypermethrin and permethrin were obtained from Dr. Haiyan Chen (Nanjing Medical University, Nanjing, China). Dexamethasone and 5α-dihydrotestosterone (5α-DHT, purity >99%) were purchased from Sigma Chemical CO. (St. Louis, MO, USA). Chemical structures of the pyrethroid pesticides tested in this study were shown in Fig. 1. Stock solutions of the chemicals were prepared in absolute ethanol at a concentration of 10^{-1} M, stored at −20 °C, and diluted to desired concentrations in phenol red-free RPMI1640 medium (Sigma Chemical CO.) immediately before use. The final ethanol concentrations in the culture medium did not exceed 0.2% (v/v) that did not affect cell yields.

2.2. Plasmids and cell line

We constructed a reporter plasmid pMMTV-LUC based on the pGl3-Basic vector (Promega, Madison, WI, USA). The oligonucleotides of mouse mammary tumor virus (MMTV) containing four androgen response element (ARE) sequences and TATA promoter were synthesized and inserted into the *Kpn* I and *Bgl* II sites of pGl3-Basic

Table 1
Data on test chemicals

Chemicals and abbreviation	Supplier	CAS	Purity (%)
Cypermethrin	Gift	52315-07-8	>99
Flutamide	Sigma	13311-84-7	>99
Nilutamide	Sigma	63612-50-0	>98
Permethrin	Gift	52645-53-1	>99
3-Phenoxybenzoic acid (3-PBA)	Accustandard[a]	3739-38-6	>99

[a] Accu Standard Chemicals (New Haven, CT, USA).

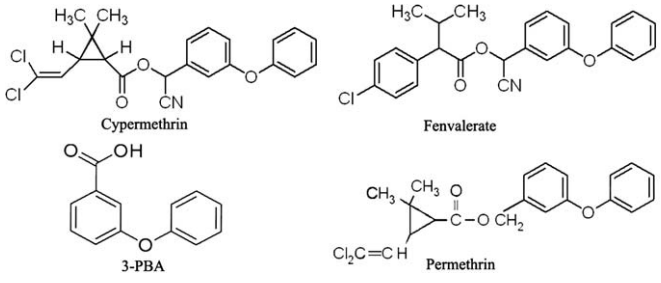

Fig. 1. Structures of three synthetic pyrethroid pesticides and their metabolite 3-PBA.

to construct the plasmid pMMTV-LUC. The plasmid pRL-SV40 (used as internal control for transfection efficiency and the cytotoxicity of test chemicals), containing *Renilla* luciferase gene, was purchased from Promega (Promega, Madison, WI, USA). The human AR expression plasmid AR/pcDNA3.1 containing the full open reading frame of human AR cDNA was a kindly gift from Dr. Takeyoshi M (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan). The plasmid was constructed as previously described (Takeyoshi et al., 2003). The CV-1 cell line was obtained from Institute of Biochemistry and Cell Biology in Shanghai, Chinese Academy of Science.

2.3. Cell culture and transfection

The CV-1 cells were maintained in phenol red-free RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg ml^{-1} streptomycin at 37 °C in an atmosphere containing 5% CO_2. The host cells were plated in 24-well microplate (Nunc, Denmark) at a density of 1.0×10^5 cells per well in the RPMI1640 medium containing 10% charcoal-dextran-stripped FBS (CDS-FBS). Following 12 h incubation, each well was transfected with 1.5 μg of pMMTV-LUC, 0.1 μg of AR/pcDNA3.1 and 0.02 μg of pRL-SV40 using 7.5 μg Sofast™ (Sunma Company, Xiamen, China) transfection reagent which is a sort of new generation cationic polymer gene transfection reagent with high transfection efficiency. After an incubation period of 24 h, the transfection medium was removed before various concentrations of DHT and test chemicals dissolved in medium were added for measurement of agonistic activity. In order to measure the AR antagonistic activity, 1 nM DHT was added along with the test chemicals. Medium with 0.2% ethanol was used as a vehicle control respectively.

2.4. 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, Madison, WI, USA). 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, Madison, WI, USA) following the manufacturer's instructions. The value of luciferase for each lysate was normalized to the *Renilla* luciferase activity. The relative transcriptional activity was converted to fold induction above the vehicle control value (*n*-fold).

2.5. Statistical analysis

The values shown were mean ± SD from three independent experiments with triplicate wells for each treatment. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons test when appropriate. The level of significance was set at $P < 0.05$. For agonists, treatments were compared to the vehicles control group; while for androgen antagonists, treatments were compared to the 1 nM DHT positive control groups.

3. Results

3.1. Cytotoxicity of the tested chemicals

Test chemicals did not affect the viability and proliferation of CV-1 cell, alone or with 1 nM DHT in MTT assay (data not shown). And no cytotoxic effect could be observed by microscopic examination throughout the transfection assay. Furthermore, the cytotoxicity of the chemicals was assessed by transfected the cells with the plasmid pRL-SV40. There was no significant difference between the tested groups and the vehicles control group in the expression of *Renilla* luciferase (data not shown). It was suggested that no cytotoxic effect existed in the tested concentration range.

3.2. Response to a known AR agonist DHT and a known GR agonist dexamethasone

Our reporter assay system showed appropriate response to DHT which is a known AR agonist. The chemical induced luciferase activity in a concentration-dependent manner in the concentrations ranging from 10^{-11} M to 10^{-5} M (Fig. 2). The maximal induction of 61.83-fold of vehicle control was achieved at 10^{-7} M and greater. From the dose–response curve, the median effective concentration (EC_{50}) value of DHT was 3.65 nM. In addition, in

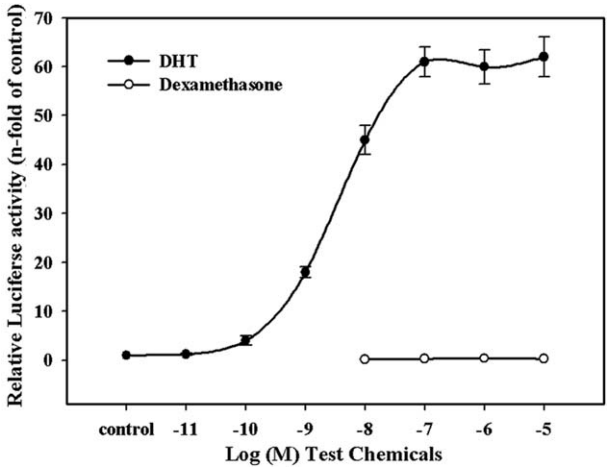

Fig. 2. Androgenic activity of DHT and dexamethasone in AR mediated reporter gene assay with CV-1 cells. CV-1 cells were transiently transfected with pMMTV-LUC, AR/pcDNA3.1 and pRL-SV40 as described in "Section 2". Then the cells were treated with increasing concentrations of chemicals. Androgenic activity was expressed as a relative activity (*n*-fold) with respect to the solvent control. Values were mean ± SD of three independent experiments.

CV-1 cells without transfection of AR expression plasmid, the luciferase expression did not significantly change after being treated with DHT. This result indicated that DHT was not able to induce luciferase transactivation in the absence of AR. Analysis of responses of six replicates showed that 10^{-7} M DHT induced luciferase activity with an intra-assay (within plate) coefficient of variability (CV) of 8.9%, and the interassay (across all plates) CV of 13.1%.

Since AR and GR (glucocorticoid receptor) shared a common hormone response element in the MMTV promoter, dexamethasone was tested to assess assay specificity. The luciferase activity could not be induced by dexamethasone at the tested dosages from 10^{-8} M to 10^{-5} M (Fig. 2). It indicated that the assay system was highly specific without cross-talk to GR agonist.

3.3. Response to a known AR antagonist nilutamide and flutamide

To characterize the response of the assay system to antiandrogen, some known AR antagonists including nilutamide and flutamide were co-administered with 1 nM of DHT in the medium. Nilutamide and flutamide were potent antiandrogen that significantly inhibited the luciferase activity induced by 1 nM of DHT at concentration of 10^{-6} M and greater (Fig. 3). The median inhibitory concentration (IC_{50}) value of nilutamide and flutamide was 4.05 μM and 3.49 μM, respectively.

3.4. Antiandrogenic and androgenic activities of pyrethroid pesticides and their metabolite

When the test pyrethroid pesticides were co-administered with 1 nM DHT, each of them suppressed the luciferase expression (Fig. 4). The RIC_{20} (20% relative inhibitory concentration) value of fenvalerate cypermethrin and permethrin was 0.37 mM, 0.42 mM and 0.43 mM, respectively. 3-PBA also showed antiandrogenic activity with RIC_{20} of 1.21 mM. There is no significant difference between original pyrethroid and 3-PBA on the antiandrogenic activity.

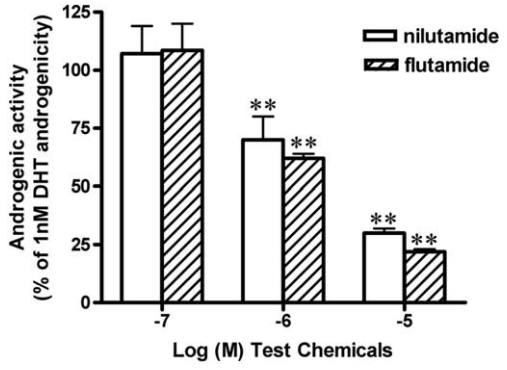

Fig. 3. Antiandrogenic activity of nilutamide and flutamide in AR-mediated reporter assay using CV-1 cells. CV-1 cells were transiently transfected with pMMTV-LUC, AR/pcDNA3.1 and pRL-SV40 as described in "Section 2". Then test chemicals were added to the cell medium along with 1 nM of DHT. Values were mean ± SD of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with 1 nM of DHT. $^{**}P < 0.01$ compared with the value of 1 nM of DHT (100%).

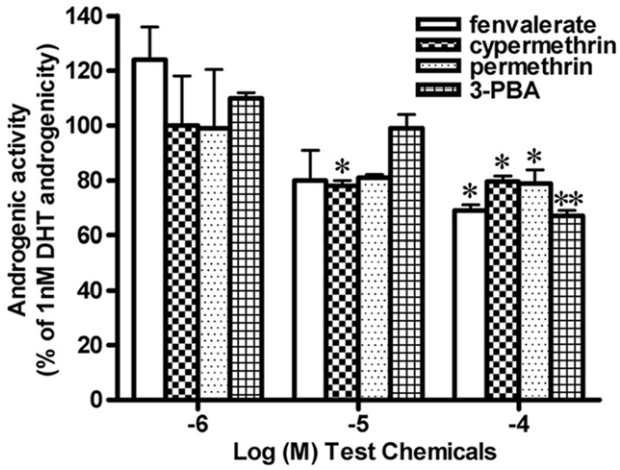

Fig. 4. Antiandrogenic activity of fenvalerate, cypermethrin, permethrin and 3-PBA in AR-mediated reporter assay using CV-1 cells. Test chemicals were added along with 1 nM of DHT. Values were mean ± SD of three independent experiments and were presented as percent induction, with 100% activity defined as the activity achieved with 1 nM of DHT. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the value of 1 nM of DHT (100%).

For agonist activity, none of the four test chemicals could induce more luciferase expression than ethanol control (data not shown).

4. Discussion

Previously, we reported that some pyrethroids, including fenvalerate permethrin and cypermethrin, were able to produce an ER-specific agonist response based on the induction MCF-7 cell proliferation and PS2 mRNA expression in MCF-7 cells, and inhibition of binding [^{3}H] estradiol to ER (Chen et al., 2002). In this experiment, we further examined the antiandrogenic activity of three pyrethroids and one of their major metabolites 3-PBA by an *in vitro* AR-mediated reporter gene assay in CV-1 cell. Our study indicated that pyrethroids suppressed AR-mediated gene transcription induced by DHT. Furthermore, we found that their metabolite 3-PBA could also reduce the reporter gene expression.

Reporter gene assay has been regarded as a powerful tool in screening endocrinal activity chemicals (Kojima et al., 2004). In this test, we used luciferase and *Renilla* luciferase as reporter gene and utilized the Dual-luciferase assay kit to detect the luciferase activity. In the present assay, 10^{-7} M DHT stimulated as high as 61.83-fold expression of reporter gene with an intra-assay CV of 8.9%. In addition, the assay showed no response to GR agonist dexamethasone which meant there was no GR in the CV-1. It was reported that there is 84 ± 4 ng DHT in 100 ml serum of the man from

20 to 40 years old (Lewis et al., 1976). This concentration of serum DHT was equal to 1 nM and could induce 17.46-fold luciferase expression in our test. Then it was chosen to detect the antiandrogenic ability of test chemicals. In order to characterize the response to antiandrogen, some known AR antagonists including flutamide and nilutamide were tested. As a result, two known AR antagonists could all significantly inhibit the luciferase expression which meant that the assay had the ability to detect antiandrogen. To date, no AR agonists have been found among environmental chemicals, and in this study, we also failed to detect the agonist ability of all the four test chemicals.

Recently, Kojima et al. (2004) tested 200 pesticides including some pyrethroids for agonism and antagonism to two human estrogen receptor subtypes and human AR by reporter gene assay using Chinese hamster ovary cells. As a result, they found that fenvalerate acted as an antiandrogen with RIC_{20} of 6.9×10^{-6} M while cypermethrin and permethrin did not show the ability at concentrations $\leqslant 10^{-5}$ M. But in this study, all the three pesticides could inhibit the normal activity of DHT when its concentration increased to 10^{-4} M. This concentration would be expected to be high, but we did not observe the cytotoxicity caused by 10^{-4} M of test chemicals. The difference between results was common in reporter gene assay. An interlaboratory comparison of three AR reporter gene assays indicated that many factors including the plasmids and cell line must be taken into account (Korner et al., 2004). Kunimatsu et al. (2002) reported that fenvalerate and permethrin did not exhibit any potential to cause adverse antiandrogenic activity at dose levels below that of those causing excessive systemic toxicity in the Hershberger assay. However, the animal study results of Kim et al. (2005) suggested that permethrin had antiandrogenic effects on male rats. The conflict of results from *in vitro* and *in vivo* study stressed the need for the international standard experiment protocols.

3-PBA was the major metabolite of pyrethroid including cypermethrin and permethrin. Tyler et al. (2000) identified 3-PBA had antiestrogenic activity in genetically modified yeast cells. McCarthy et al. (2006) studied the estrogenicity of pyrethroid metabolites and found 3-PBA did not show activity in their yeast assay. However, to our knowledge, no study was carried out to test the antiandrogen activity of 3-PBA. In the present test, 3-PBA showed weak antagonist activity with RIC_{20} of 1.21 mM. This result indicated that metabolizing to 3-PBA could not eliminate the antiandrogenic activity of pyrethroid.

Tyler et al. (2000) showed that pyrethroid insecticides had AR receptor-binding activity, and in the present study, we found that pyrethroid and 3-PBA could not induce androgen response gene expression. These results indicated that the binding of pyrethroid could not exert androgen like activity, and with the increasing of concentration, test pyrethroids could compete with the low concentration of natural androgen and affect the expression of androgen receptor mediated reporter gene.

Many clinic and animal studies showed that pyrethroid pesticides possess endocrine disruptor activity. Lifeng et al. (2006) reported that occupational exposure to fenvalerate could affect the semen quality of the workers by computer-assisted sperm motility analysis. Our laboratory found that fenvalerate may cause sperm motility changes and testicular lesions in male rats (Xu et al., 2004). Results from animal experiment with male New Zealand White rabbits showed that treatment with cypermethrin caused a significant decrease in ejaculate volume, sperm concentration, total sperm output, sperm motility, total motile sperm per ejaculate, packed sperm volume, semen initial fructose and plasma testosterone (Yousef et al., 2003). All these damages caused by pyrethroid pesticides connected with male reproductive system and especially with the function of androgen. We focused on the relationship between the pyrethroid pesticides and natural androgen at the molecular level in the present study. Our results suggested that all the three chemicals at concentrations of 0.1 mM could suppress the normal gene expression regulated by androgen. And because androgen and AR play important roles in the development of reproductive system, the results from our study can partly explain the findings from above clinic and animal studies. All the three pyrethroids show weak antagonist activity. The impact would be expected to be small, but still adds to the overall environmental antiandrogen load. Though endocrine disrupter activity is only part of the pyrethroid toxicology, still we should pay attention to their bad effects on development and reproductive system. Considering the wide use of these pyrethroids, more comprehensive study including Tier 2 Testing battery (EDSTAC, 1998) and epidemic investigation should be carried out to provide more information for the risk assessment.

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