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The potential endocrine disruption of pesticide transformation products (TPs): The blind spot of pesticide risk assessment

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ABSTRACT

The ecological and health risk assessment of environmental pesticide residues have attracted ever-growing attention; however, their transformation products (TPs) have seldom been considered. Herein, we examined the endocrine-disrupting effects of 4 widely used pesticides as pyriproxyfen (Pyr), malathion (ML), benalaxyl (BX), and fenoxaprop-ethyl (FE), together with their 21 TPs through *in vitro* and *in silico* approaches, and found approximately 50% of the TPs exhibited stronger endocrine-disrupting effects than their corresponding parent compounds. Specifically, Pyr and 9 TPs (five TPs of Pyr, one of ML, one of BX, and two of FE) exhibited estrogen-disrupting effects, which were also confirmed by results of E-screen and *pS2* expression assays, and molecular docking showed that certain hydroxylated TPs could well mimic the binding mode of estrogen with ERα. Meanwhile, two TPs of Pyr, ML and its TP demonstrated weak glucocorticoid antagonistic activities partially contributed by hydrogen bonds. We also discovered that in H295R cells, all the endocrine disruptors increased hormone secretion and the related gene expression levels. Conclusively, since an increasing number of pesticide TPs have been being detected in various environmental media, a more comprehensive understanding of the ecological risk of pesticide TPs is imperative for risk assessments more extensively and regulatory policy-making on pesticide restriction in the future.

1. Introduction

Pesticide residues have now been widely detected in environmental, food, and biological samples (Botitsi et al., 2011; Carra et al., 2015; Félix et al., 2008; Gómez-Pérez et al., 2015; Miles, 1991; Reemtsma et al., 2013), as approximately 2.5 million tons of pesticides are released into the environment globally every year (Fenner et al., 2013; Liu et al., 2005; U.S. EPA, 2011; Wennberg, 2014). Through hydrolysis, photolysis, oxidation, and biodegradation processes, the pesticide residues are subsequently degraded into various transformation products (TPs) (Fenner et al., 2013), which have been detected in groundwater (0.62 μg/L, median total concentrations) and surface water (0.33 μg/L, median total concentrations) (Reemtsma et al., 2013; Segawa et al., 1991), and especially some TPs of organochlorine pesticides have been found even more frequently than their corresponding parent compounds in groundwater, placenta homogenate, cord blood, and human milk (Berton et al., 2014; Cerrillo et al., 2005; Félix et al., 2008). In addition, various physicochemical properties of the TPs such as their increasing environmental mobility and persistence, have been verified to be of more potent adverse impacts on the ecosystem and human health than their parent compounds (Lu et al., 2015; Sinclair and Boxall, 2003; Zhang et al., 2016b). However, the toxicity assessments of the pesticide TPs are still overlooked for the registration and use approval of pesticides. Therefore, the risk of environmental pesticide TPs is not only an emerging issue but also a scientific blind spot.

Endocrine-disrupting effects that are to threat the human and wildlife reproduction have become one of the most important evaluation criteria for pesticides, and many studies have already been conducted to screen pesticides for their endocrine-disrupting potentials (Xu et al., 2017; Kojima et al., 2004; Song et al., 2017; Zhang et al., 2017). A portion of pesticides that are used for agricultural and household pest control have been confirmed to be endocrine disruptors (Brander et al.,

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2016; Mann et al., 2009; McKinlay et al., 2008), based on which the pesticide TPs with similar molecular structures may also act as a potential endocrine-disrupting chemicals (EDCs), however very few studies have focused on these pesticide TPs. As a further matter, the variety of hormones also makes the risk assessment of pesticides an arduous task. Estrogen, the principal agent of the hypothalamic-pituitary-gonadal (HPG) axis, plays a fundamental role in growth, development, and reproduction (Cui et al., 2013; Ye and Liu, 2019). Glucocorticoids and mineralocorticoids can essentially impact on glucose metabolism and electrolyte homeostasis (Brem and Morris, 1993; Odermatt and Gumy, 2008; Zhang et al., 2016a), and links have been demonstrated between the perturbation of glucocorticoid and mineralocorticoid activities and neurological diseases, as well as metabolic disorders (Brem and Morris, 1993; Odermatt and Gumy, 2008). There are already several but still very limited studies having shown that the fipronil sulfone (a TP of fipronil) and 4-hydroxychlorothalonil (a TP of chlorothalonil) exerted more potent thyroid hormone-disrupting effects than their parent compounds (Lu et al., 2015; Zhang et al., 2016b), which poses assessing the endocrine-disrupting effects of TPs the current top priority to comprehensively understand the public health risk of pesticides.

Our study aimed to elucidate and evaluate the potential endocrine-disrupting effects of pesticide TPs, so that four widely used pesticides as pyriproxyfen (Pyr), malathion (ML), benalaxyl (BX), and fenoxaprop-ethyl (FE) together with their 21 TPs (Pyr-A ~ Pyr-K; malaoxon; DMDTP; ML-A; ML-B; BX-A; BX-B; BX-acid; EHPP; CDHB; HPPA) were selected to evaluate their endocrine-disrupting effects on the estrogen receptor α (ERα), the glucocorticoid receptor α (GRα), and the mineralocorticoid receptor (MR) by means of dual-luciferase reporter gene assays. Then, the mRNA levels of steroidogenic related genes, and the secretions of 17β-estradiol (E_2) and cortisol were measured in H295R cells. The MCF-7 cell proliferation assays and *pS2* mRNA expression level assays were also used to observe the estrogenic activities of the studied chemicals. Besides, the binding modes of chemicals with ERα and GRα were also explored by molecular docking simulation. Briefly speaking, our results showed that approximately 50% of the pesticide TPs exhibited stronger endocrine-disrupting effects than their parent compounds.

2. Materials and methods

2.1. Chemicals

To evaluate the endocrine-disrupting effects of pesticide TPs, 21 pesticide TPs listed in Table S1 were synthesized by China Agricultural University (College of Science, Beijing, China). Analytical standards of the 4 parent pesticides (purity 97.5–99.5%) listed in Table S1 were purchased from Dr. Ehrenstorfer (Germany). E_2 (purity > 97%) and dimethyl sulfoxide (DMSO, as a vehicle) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrocortisone (HC) and aldosterone (AD) were purchased from J&K Scientific Limited (Beijing, China). All chemicals were dissolved in DMSO. The final DMSO concentration in the culture medium was no more than 0.1%.

2.2. Cell cultures and plasmids

Chinese hamster ovary (CHO) cells, HepG2 cells, and human breast adenocarcinoma (MCF-7) cells were routinely cultured in a 25-cm^2 flask (Corning, NY, USA) with 5 mL of complete medium containing 90% RPMI-1640 medium (with L-glucose) (HyClone, Logan, UT, USA) and 10% FBS (Hyclone) (Ji et al., 2019a). Human adrenocortical carcinoma (H295R) cells were cultured following the methods in a previous study (Brem and Morris, 1993).

ERα and estrogen receptor element (*ERE*) were generously provided by Dr. M. Takeyoshi (Chemicals Assessment Center, Chemicals Evaluation and Research Institute, Oita, Japan) (Kojima et al., 2003). The human GRα expression plasmid *pF25GFP-hGRα* and the response element containing the reporter plasmid *pMMTV-luc* were generously provided by Dr. Evangelia Charmandari (Biomedical Research Foundation of the Academy of Athens, Greece) (Charmandari et al., 2005). The human MR expression plasmid *EGFP-C1-hMR* was generously provided by Dr. Claudia Gromann (Julius Bernstein Institute for Physiology, Martin Luther University, Germany) (Govindan and Warriar, 1998). The plasmid *phRL-tk* purchased from Promega (Madison, WI, USA), was used as an internal control for transfection efficiency.

2.3. MTS assay

The cytotoxicity of the 25 chemicals to CHO cells, HepG2 cells, MCF-7 cells and H295R cells were measured by MTS assays. Briefly, cells were seeded in 96-opaque-walled plates (Corning) at the density of 5000 cells/well. After cell adherence, cells were treated with various concentrations (from 10^{-8} to 10^{-4} M) of the tested chemicals or 0.1% DMSO (as vehicle control) for 24 h or 48 h (for H295R cells). Cell viability was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) per by the manufacturing instructions and descriptions presented previously (Song et al., 2017; Zhang et al., 2016c).

2.4. CTG assay

The Cytotoxicity CellTiter-Glo® (CTG) luminescent cell viability assay (Promega) was used to further assess cytotoxic concentrations for the tested chemicals. CHO cells were seeded in 96-opaque-walled plates (Corning) at a density of 5000 cells/well. After cell adherence, cells were treated with various concentrations (from 10^{-8} to 10^{-4} M) of the tested chemicals or 0.1% DMSO (as vehicle control) for 24 h. Finally, the number of cells was detected by CTG according to the manufacturing instructions.

2.5. Dual-luciferase reporter gene assays for ERα, GRα, and MR

CHO cells were plated in 96-well microtiter plates with a density of 15,000 cells/well for 24 h with each well containing 95 μL of phenol red-free RPMI-1640 and 5 μL of charcoal-dextran-treated FBS (CD-FBS; HyClone). For the detection of ERα, GRα, and MR activities, the CHO cell transfection process followed the methods of previous studies (Brem and Morris,1993; Kojima et al., 2003; Song et al., 2017). After four hours of transfection, transfection medium was replaced with fresh culture medium overnight. To measure the agonistic activity, we used the concentrations ranging from 10^{-10} to 10^{-6} M of Pyr-C and Pyr-H, 10^{-13} to 10^{-9} M of ML-A, 10^{-11} to 10^{-7} M of BX-B, and 10^{-9} to 10^{-5} M of the other tested chemicals to treat cells. For the measurement of antagonistic activity, the cells were exposed to the 25 tested chemicals in the presence of 10^{-10} M E_2/5 × 10^{-8} M HC/10^{-10} M AD. After 24 h, *firefly* and *renilla* luciferase activity were measured by a fluorescence spectrophotometer (Tecan) using the Dual-Luciferase Reporter Assay Kit (Promega) as previously described (Song et al., 2017; Zhang et al., 2016a).

2.6. Molecular docking

Docking studies were completed by the CDOCKER module of Discovery Studio 2.5 (Accelrys, Inc., San Diego, CA, USA). The crystal structures of ERα and GR (PDB entry code: 1ERE, 1ERR, and 3H52) were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) using E_2 (ER agonist), raloxifene (ER antagonist) and RU486 (GR antagonist) as reference. Before docking, the ligands were optimized by minimization with the CHARMM force field using the simulation module of Discovery Studio 2.5. The default values for CDOCKER parameters were used, and 100 poses for each ligand were saved. The negative CDOCKER interaction energy between the ligand and receptor

was calculated, and the conformation with the greatest negative CDOCKER interaction energy for each ligand was selected as the most likely bioactive conformation.

2.7. E-screen assays

MCF-7 cells (2500 cells/well) were plated in 96-well plates with experimental media for 24 h. To measure the estrogen agonistic activities with the studied chemicals, cells were treated with concentrations ranging from 10^{-7} to 10^{-5} M of the tested chemicals and 0.1% DMSO (vehicle control), while for the measurement of antagonistic activity, the cells were exposed to 10^{-7} to 10^{-5} M of the chemicals in the presence of 10^{-9} M E_2 (with 10^{-9} M E_2 as the control group). After five days of incubation, cell proliferation was detected by the MTS kit (Cell Titer 96 Aqueous One Solution) following manufacturing instructions and previous research (Zhang et al., 2014).

2.8. Real-time quantitative PCR (RT-qPCR)

MCF-7 and H295R cells were treated with the tested chemicals from 10^{-7} to 10^{-5} M for 24 h and 48 h, respectively, and then collected for RNA isolation. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then cDNA was synthesized using the ReverTra Ace qPCR RT Kit (TOYOBO, Japan). RT-PCR was performed with an SYBR Green Real-time PCR Master Mix Kit (TOYOBO) on an ABI-7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to methods described in a previous study (Ji et al., 2019b; Zhong et al., 2019). The primer sequences for the genes of concern are listed in Table S2. Relative mRNA levels were normalized to the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and calculated by means of the $2^{-\Delta\Delta Ct}$ method (Ji et al., 2019c).

2.9. Hormone measurements

H295R (10^6 cells/well) cells were plated in six-well plates for 24 h and then treated with 10^{-5} M of the tested chemicals and 0.1% DMSO for 48 h. The media were collected to detect the levels of E_2 and cortisol using the radioimmunoassay kit (Shanghai Yubo Biological Technology Company, Shanghai, China) according to the manufacturer's instructions. The detection limits were 10 pg/mL for E_2 and 10 ng/mL for cortisol.

2.10. Statistical analysis

The statistical analysis was performed in OriginPro 2016 (OriginLab Corporation, Northampton, MA, USA). The variables studied for all of the experiments are presented as the mean ± SD (standard deviation) of at least three independent assays with 3 to 5 repetitions for each. In concentration-response relationship estimation, the concentrations of E_2, HC, and AD were modeled as continuous variables, and logistic-shaped curve-fitting regression was applied. When comparing different treatment sample groups with the control group, the parametric test that one-way ANOVA with a Tukey post-hoc analysis was applied in IBM® SPSS Statistics Software 24.0 (IBM Corp., Armonk, NY, USA), setting the level of significance as $p < 0.05$.

3. Results and discussions

3.1. Cytotoxicity

In order to distinguish the specific activity of a chemical from nonspecific effects, we used two different assays and four different cell lines to evaluate the cytotoxicity of the target chemicals.

In the MTS assay, Pyr-C and Pyr-H exhibited CHO cytotoxicity at the concentration of 10^{-5} M, while BX-B and ML-A showed CHO cytotoxicity over the concentrations of 10^{-6} and 10^{-8} M, respectively. Pyr-G induced HepG2 cytotoxicity when the exposure concentration was over 10^{-4} M. No cytotoxic effects were observed at concentrations ranging from 10^{-7} to 10^{-5} M in the MCF-7 and H295R cells (Fig. S1, Table S3).

However, in the CTG assay, only ML-A showed CHO cytotoxicity at the concentration of 10^{-4} M. The other chemicals showed no cytotoxicity at our tested concentrations (Table S3).

Subsequently, the noncytotoxic concentration region of chemicals was used in the dual-luciferase reporter gene assays, hormone measurements, and RT-PCR.

3.2. Reporter gene assays mediated by ER

According to previous studies (Brem and Morris, 1993; Kojima et al., 2004; Zhang et al., 2016c), the concentration-response curve of E_2 was fitted by the logistic model to calculate the EC_{20} (Fig. S2). The concentration approaching the upper plateau from the curve of E_2 was used as a positive control in agonistic activity, whereas the approximate EC_{80} value was used as a positive control in antagonistic activity. Chemicals that induced greater than 20% activity of positive control

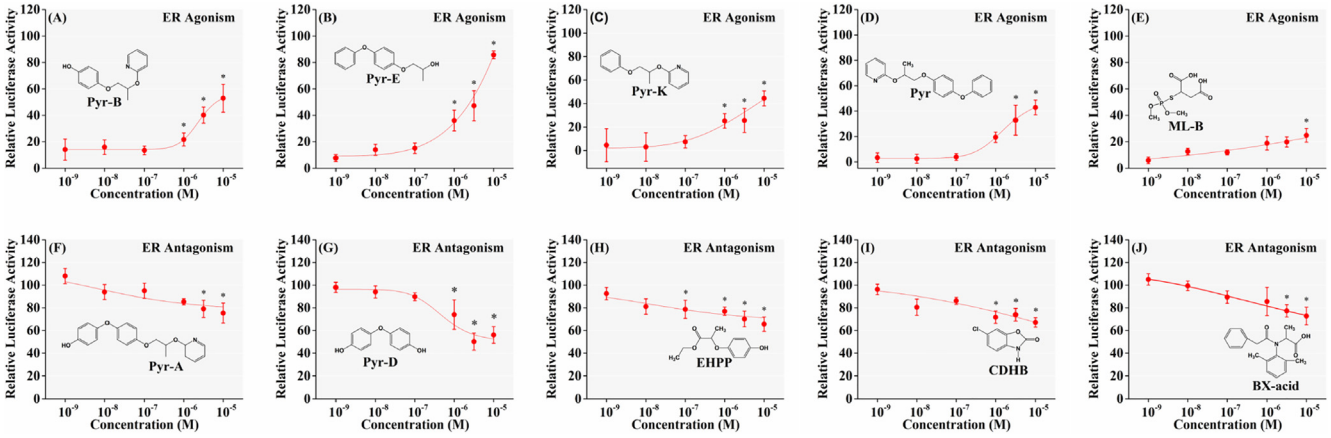

Fig. 1. The agonistic and antagonistic activities of test chemicals in ERα-mediated reporter gene assays. A-E: CHO cells transfected with *rERα/pCI*, *pERE-AUG-Luc*, and *phRL-TK* were then incubated with different concentrations of the tested chemicals. F-J: CHO cells transfected with *rERα/pCI*, *pERE-AUG-Luc*, and *phRL-tk* were then incubated with different concentrations of test chemicals as well as 10^{-10} M E_2. *Statistically speaking, the data are significantly different ($p < 0.05$) from 10^{-10} M E_2 and shown with the mean ± SD (standard deviation) of at least 3 independent experiments using one-way ANOVA with a Tukey post-hoc analysis.

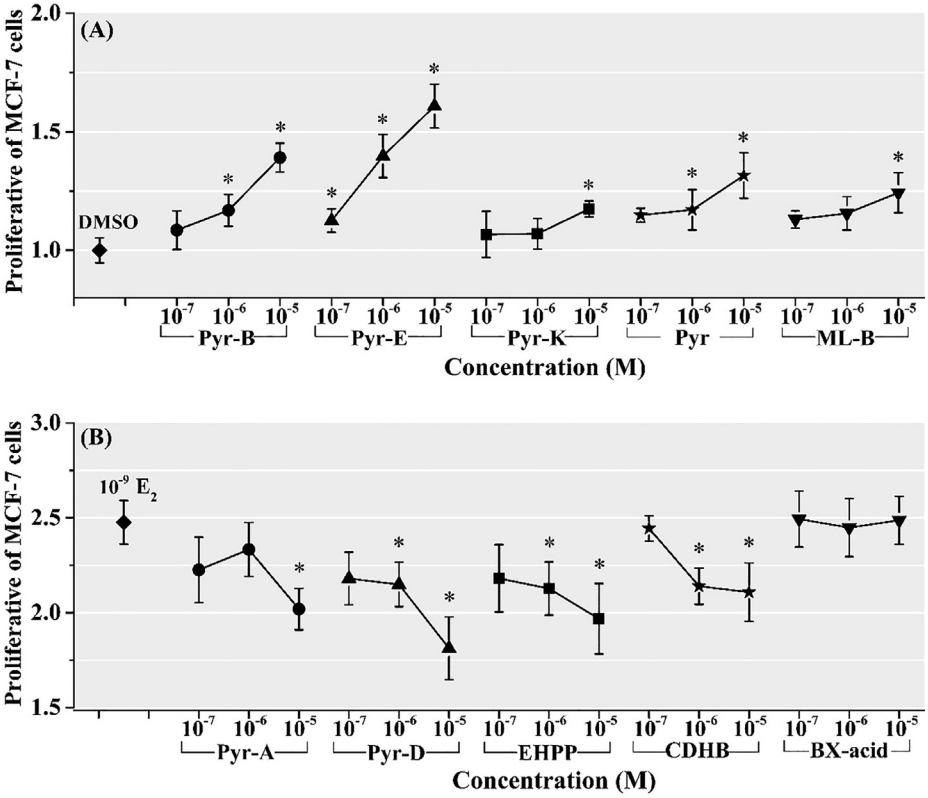

Fig. 2. The estrogenic activities induced by the tested chemicals in MCF-7 cells. A: Proliferation effects of MCF-7 cells induced by Pyr-B, Pyr-E, Pyr-K, Pyr, and ML-B. *The data are statistically significantly different from 0.1% DMSO ($p < 0.05$). B: Inhibiting estrogen-induced MCF-7 cell proliferation by Pyr-A, Pyr-D, EHPP, CHDB, and BX-acid. *The data are statistically significantly different from 10^{-9} M E_2 ($p < 0.05$) using one-way ANOVA with a Tukey post-hoc analysis.

were regarded as agonists, while chemicals that inhibited over 20% of the positive control were defined as antagonists.

In the ER reporter gene assays, there were 10 chemicals exhibiting estrogenic endocrine-disrupting effects (Fig. 1). Among these ERα agonists, Pyr-B, Pyr-E, and Pyr-K showed greater estrogen agonistic activities than the parent compound Pyr, with a lower REC_{20} value of 8.47×10^{-7} M, 2.50×10^{-7} M, and 7.68×10^{-7} M, respectively (Table S4). In antagonistic effects, Pyr-A, Pyr-D, EHPP, CDHB, and BX-acid attenuated E_2-induced ERα transactivation with RIC_{20} values of 4.49×10^{-6} M, 3.51×10^{-7} M, 1.77×10^{-7} M, 3.66×10^{-7} M, and 2.04×10^{-6} M, respectively (Table S4). No estrogenic disrupting effects were observed from other chemicals (Figure S3).

3.3. E-screen assays and pS2 expression levels in MCF-7 cells

E-screen assays were carried out to verify the estrogenic effects of the target chemicals. The five ER agonists (Pyr, Pyr-B, Pyr-E, Pyr-K, and ML-B) induced the MCF-7 cell proliferation by 1.2- to 1.6-fold (Fig. 2A). The five ER antagonists except for BX-acid (thus, Pyr-A, Pyr-D, EHPP, and CDHB), inhibited the proliferation of MCF-7 cells induced by E_2 (Fig. 2B).

The estrogen-responsive gene *pS2* is an important biomarker for the evaluation of the estrogenic activities of chemicals (Kangaspeska et al., 2008; Zhao et al., 2008). Thus, we determined the mRNA levels of the *pS2* gene in MCF-7 cells after exposure to the target chemicals. E_2 (10^{-9} M) significantly induced the *pS2* expression level by 2.21-fold compared to the 0.1% DMSO ($p < 0.05$, ANOVA). The five ERα agonists (Pyr, Pyr-B, Pyr-E, Pyr-K, and ML-B) significantly upregulated *pS2* mRNA expression from 1.57- to 2.04-fold at the concentration of 10^{-5} M ($p < 0.05$, ANOVA) (Fig. 3). Among the five ER antagonists, Pyr-A, Pyr-D, and BX-acid significantly downregulated *pS2* gene expression levels by 0.80-, 0.76-, and 0.60-fold at the concentration of 10^{-5} M ($p < 0.05$, ANOVA), respectively. However, EHPP and CDHB did not significantly alter the gene expression level of *pS2* based on our observations (Fig. 3, Table S5).

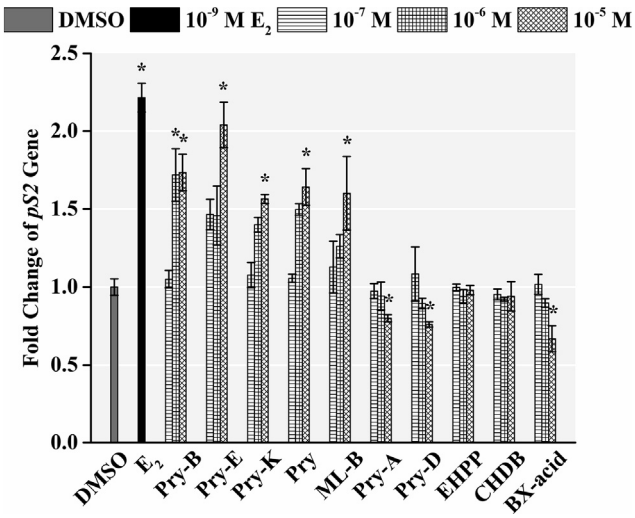

Fig. 3. The relative mRNA expression levels of *pS2* were altered by the 10 tested chemicals. *The data are statistically significantly different from 0.1% DMSO ($p < 0.05$) and shown as the mean ± SD (standard deviation) of at least 3 independent experiments using one-way ANOVA with a Tukey post-hoc analysis.

3.4. ER docking model

Molecular docking was used to analyze the different receptor binding interactions between ERα and the target chemicals. The calculated-cdocker_interaction_energy was used to evaluate the affinity between target chemicals and receptors. In comparison with the -cdocker_interaction_energy of the inactive chemicals, most ERα agonistic/antagonistic chemicals had an increased -cdocker_interaction_energy (Table S6). For the ERα docking model, three hydrogen bonds with three amino acids (GLU353, ARG394, and HIS524) were observed between E_2 (reference ligand) and the ER

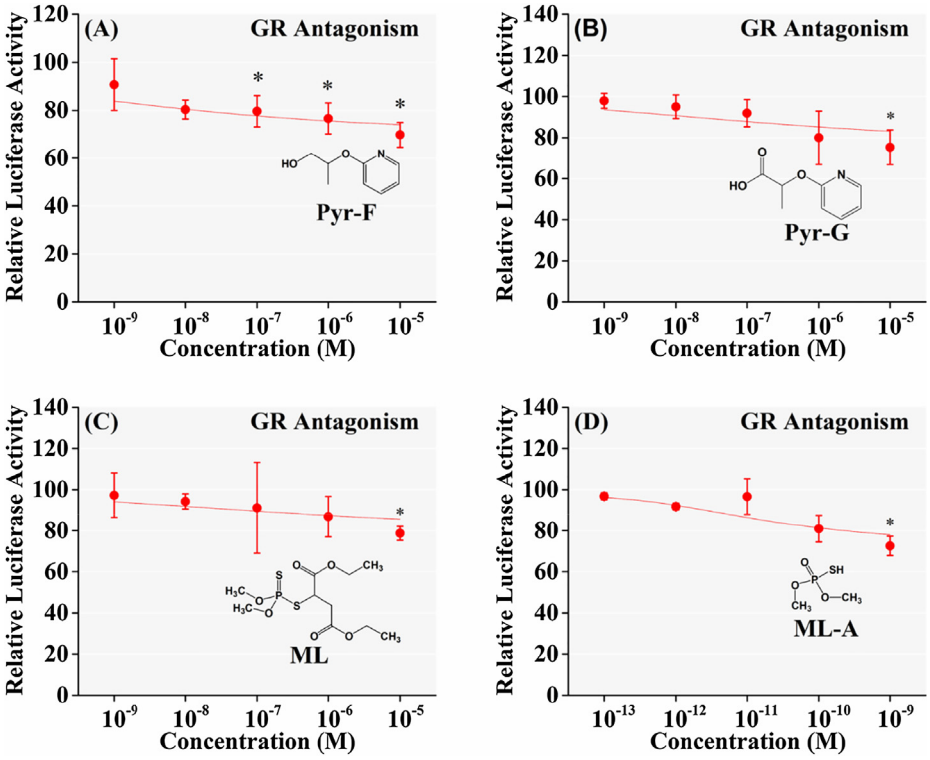

Fig. 4. The antagonistic activities of test chemicals in GRα-mediated reporter gene assays. A-D: CHO cells were transfected with *pF25GFP-hGR*, *pMMTV-luc*, and *phRL-TK* were then incubated with different concentrations of test chemicals as well as 5×10^{-8} M HC. *Statistically, the data are significantly different ($p < 0.05$) from 10^{-10} M E_2 and shown with the mean ± SD (standard deviation) of at least 3 independent experiments using one-way ANOVA with a Tukey post-hoc analysis.

Table 1
The production of E_2 and cortisol in H295R cells treated with target chemical concentrations of 10^{-5} M for 48 h.

Chemicals	E2 (pg/mL)	Chemicals	Cortisol (ng/mL)
Control (DMSO)	345.67 ± 26.69	Control (DMSO)	12.60 ± 1.05
Pyr-B	753.67 ± 52.62* (↑)	Pyr-F	15.19 ± 0.22* (↑)
Pyr-E	872.33 ± 48.95* (↑)	Pyr-G	15.75 ± 0.43* (↑)
Pyr-K	673.67 ± 36.67* (↑)	Malathion	15.84 ± 0.49* (↑)
Pyr	692.00 ± 40.73* (↑)	ML-A	15.24 ± 0.43* (↑)
ML-B	616.33 ± 43.25* (↑)	/	/
Pyr-A	385.00 ± 19.08	/	/
Pyr-D	469.33 ± 40.87* (↑)	/	/
EHPP	440.00 ± 36.86* (↑)	/	/
CDHB	349.33 ± 14.36	/	/
BX-acid	336.67 ± 31.50	/	/

The values are represented as the mean ± SD (standard deviation) from three replicated samples. * indicates $p < 0.05$ vs control (0.1% DMSO) using one-way ANOVA with a Tukey post-hoc analysis. ↑: The production of E_2 and cortisol was upregulated vs control (0.1% DMSO).

(1ERE), and four hydrogen bonds with three amino acids (GLU353, ARG394, and HIS524) and one π-σ bond with an amino acid (PHE404) were observed between raloxifene (reference ligand) and the ER (1ERR). Meanwhile, we also found that Pyr-A, Pyr-B, and EHPP formed two hydrogen bonds with amino acids (GLU353 and ARG394), Pyr-E had hydrogen bond with HIS524; Pyr-K had a π-π bond with HIS524, Pyr had a π-σ bond with LEU346, ML-B had two hydrogen bonds with ALA350 and HIS524, Pyr-D had three hydrogen bonds with amino acids (GLU353, ARG394, and THR347), and CDHB had hydrogen bond with THR347 (Fig. S4A–J).

3.5. Reporter gene assays mediated by GR and MR

The concentration-response curves of HC and AD were also fitted by the logistic model (Fig. S2). In the GRα reporter gene assays, Pry-F, Pry-G, ML, and ML-A revealed GR antagonistic activities with a RIC_{20} valued equal to 3.10×10^{-8} M, 1.57×10^{-6} M, 7.19×10^{-6} M, and 1.09×10^{-9} M, respectively (Fig. 4, Table S7), while the other target chemicals showed no GRα agonist effects (Fig. S5). What's more, Pry, Pry-B, and Pry-E induced maximal effects, but the underlying mechanism remains unknown (Fig. S5).

In the MR reporter gene assays, no target chemicals showed MR agonistic or antagonistic activities (Fig. S6).

3.6. GR docking model

The results of molecular docking showed that Pyr-F, Pyr-G, and ML-A had hydrogen bonds with LEU563, GLN570, and GLN570, respectively. However, for RU486 and ML, there were no bonds between the amino acid residues (Fig. S7A–D).

3.7. Hormone secretion and related gene expression levels in H295R cells

The changes in hormone secretion levels caused by the target chemicals were analyzed in H295R cells. The results showed that Pyr-B, Pyr-K, Pyr-E, ML-B, Pyr, Pyr-D, and EHPP at 10^{-5} M significantly promoted the production of E_2 by 2.18-, 2.52-, 1.95-, 2.00-, 1.78-, 1.36-, and 1.27-fold, respectively at 10^{-5} M ($p < 0.05$, ANOVA). However, 10^{-5} M of Pyr-A, CDHB, and BX-acid did not alter E_2 secretion levels (Table 1). H295R cells expressed key enzymes involved in steroid hormone synthesis; thus, the expression of steroidogenic related genes (*HMGR*, *StAR*, *CYP11A*, *3βHSD2*, *CYP17*, *17βHSD*, *CYP11B1*, *CYP11B2*, *CYP21*, and *CYP19*) was detected after exposure to the target chemicals. Potential ER agonists (Pyr-B, Pyr-E, Pyr-K, and Pyr) significantly upregulated the gene expression of *HMGR*, *StAR*, *CYP11A*, *3βHSD2*, *17βHSD*, *CYP17*, and *CYP19* ($p < 0.05$, ANOVA), and ML-B also upregulated the expression of these genes except for *HMGR*. Pyr-D, EHPP, CDHB, and BX-acid (potential ER antagonists) significantly increased the expression levels of *CYP11A*, *3βHSD2*, *17βHSD*, *CYP17*, and *CYP19* at concentrations of 10^{-5} or 10^{-6} M ($p < 0.05$, ANOVA), but slightly downregulated the gene expression of *HMGR* and *StAR*. Only *CYP11A*, *CYP17*, and *CYP19* were significantly upregulated by Pyr-A ($p < 0.05$, ANOVA). Among these gene expression changes, the largest was an upregulation of 44.08-, 45.56-, 56.66-, 161.16-, and 366.93-fold of the

CYP19 gene after exposure to five ER agonists (ML-B, Pyr-B, Pyr-K, Pyr-E, and Pyr) at 10^{-5} M, respectively (Fig. S8A–J and Table S8).

Hormone measurement assays also showed that Pyr-F, Pyr-G, ML, and ML-A significantly promoted the secretion of cortisol in H295R cells by 1.21-, 1.19-, 1.19-, and 1.11-fold ($p < 0.05$, ANOVA), respectively (Table 1). Pry-F, Pry-G, ML, and ML-A also significantly changed the expression levels of genes related to steroid hormone synthesis ($p < 0.05$, ANOVA). Pry-F induced the gene expression of *StAR*, *CYP11B1*, and *CYP11B2* at the concentration of 10^{-6} M. The mRNA levels of *StAR*, *3βHSD2*, *CYP17*, *CYP11A1*, and *CYP11B2* were induced by Pry-G at a concentration of 10^{-5} M. ML significantly increased the expression levels of *StAR*, *HMGR*, *3βHSD2*, *CYP11A1*, *CYP11B2*, and *CYP21* by 1.32–, 1.35-, 2.97-, 4.79-, 6.03-, and 2.22-fold compared to the control group ($p < 0.05$, ANOVA) (Fig. S8K–N and Table S9).

The risks assessment of pesticide TPs is a current blind spot but thus also represents an emerging opportunity in pesticide evaluation. Herein, our study innovatively revealed that three of the four detected pesticides could generate more poisonous TPs and that nearly 50% of the pesticide TPs exhibited more potent endocrine-disrupting effects than their parent compounds.

Acute toxicity is the basic evaluation standard of pesticides and is seldom considered for pesticide TPs. The results of the MTS assay reflected that the noncytotoxic concentrations of Pry-C, Pry-H, ML-A, and BX-B were lower than those of their parent chemicals for CHO cells. Recently, some studies have shown that some pesticide TPs exhibit more potent toxicity than their parent chemicals. For example, fipronil and chlorothalonil could generate more poisonous TPs (Boxall et al., 2004; Zhang et al., 2016b). Molecules of *p,p′*-DDE could exhibit comparative toxicity to DDT (Wang et al., 2014). Thus, it is desirable to focus more on ecological risk assessments for the TPs than for their parent compounds.

Although the risk assessment of pesticides is getting ever-growing attention, most previous studies still focused on the environmental fate and safety of the parent compounds (Chen et al., 2019). Due to the fact that an increasing number of pesticide TPs have been detected in the environment, the potential endocrine disruption effects or chronic toxicity of pesticide TPs have become a major issue in pesticide risk assessment. In the present study, nearly 50% of pesticide TPs exhibited endocrine-disrupting effects mediated by ERα and GRα. Some pesticide TPs possessed stronger endocrine-disrupting effects than their parent compounds (Table S4).

Pyr is considered a nontoxic pesticide categorized in EPA toxicity class III and is the only pesticide approved by the World Health Organization (WHO) for controlling mosquitoes in drinking water containers, so it is widely consumed around the world. After oral administration for rats, Pyr could be degraded into 7 major TPs in the blood, kidneys, and liver, including but not limited to Pyr-A, Pyr-D, and Pyr-E (Matsunaga et al., 1995). In our conducted reporter gene assays, Pyr-B, Pyr-E, and Pyr-K exhibited stronger ERα agonist effects than Pyr. As ERα antagonist, Pyr-A and Pyr-D showed stronger endocrine-disrupting activities, while Pyr did not induce any effect. The ER docking model revealed that the hydroxylated TPs as Pyr-A, Pyr-B, Pyr-D, and Pyr-E, formed hydrogen bonds with ER by treating the hydroxyl group as both a hydrogen bond donor and acceptor in the ER-binding site. Previous studies also reported that hydroxylated TPs of PCBs and PBDEs could exert estrogenic effects (Mercado-Feliciano and Bigsby, 2008; Pan et al., 2019).

Moreover, the OH-PBDEs showed much higher binding potency than their parent PBDEs (Cao et al., 2018a). Cao et al. reported that 11 of the OH-PBDEs, but none of the PBDEs, could bound to GPER directly (Cao et al., 2018b). Our results also followed the same trend in which hydroxyl groups in pesticides can help promote estrogen-disrupting activities.

Historically, the majority of studies have focused on estrogen-disrupting effects. Recently, glucocorticoids and mineralocorticoids have attracted public attention to their fundamental roles (Ji et al., 2019d). Zhang et al. screened the GR disrupting effects of 34 pesticides (Zhang et al., 2016b), but the effects of pesticide TPs continue to be a blind spot (Miles, 1991). In this study, Pyr-F and Pyr-G showed stronger GR antagonist effects than Pyr, formed hydrogen bonds with nearby amino acids, and simulated the optimal conformation of RU486. These acting forces may provide strong evidence for their antagonistic effects on GR.

Besides, hormones secretion and expression levels of the H295R cell line and related genes could also be used to screen the endocrine-disrupting effects of pesticide TPs (Zhang et al., 2016a). In the H295R steroid hormone synthesis pathways, genes such as *StAR*, *HMGR*, *CYP11A1*, *3βHSD2*, *CYP17*, *17βHSD*, and *CYP19* are involved in the synthesis of E_2, while *CYP21* and *CYP11B1* are associated with the synthesis of cortisol. *CYP11A1*, *CYP11B1*, *CYP17*, *CYP19*, and *CYP21* encode members of the cytochrome P450 superfamily of enzymes (Zhang et al., 2005). Three genes were involved in the last step of E_2 synthesis: *3βHSD2* converts pregnenolone to progesterone and dehydroepiandrosterone (DHEA) to androstenedione, *17βHSD* catalyzes the reduction of 17-ketosteroids and the dehydrogenation of 17β-hydroxysteroids, and *CYP19* is responsible for converting androgens into estrogens. It was suggested that the ability of chemicals to alter *CYP19* expression levels represents a potential influence on the mechanism of estrogen secretion (Hilscherova et al., 2004; Nicol et al., 2009). In the present study, Pyr-B, Pyr-E, Pyr-K, and Pyr (ER agonists) induced E_2 secretion by significantly upregulating the expression levels of *3βHSD2*, *17βHSD*, and *CYP19* (n > 3.7-fold) at 10^{-5} M ($p < 0.05$, ANOVA). *CYP19* had the largest expression change (from 44- to 367-fold). The ER antagonists (Pyr-A and Pyr-D) also significantly upregulated *CYP19* expression levels (from 3- to 12-fold) with Pyr-D having a greater effect than Pyr-A. Pyr-D promoted E_2 secretion at the concentration of 10^{-5} M ($p < 0.05$, ANOVA), while Pyr-A did not induce the secretion of E_2. Two potential GR antagonists (Pyr-F and Pyr-K) displayed different effects on *StAR*, *HMGR*, *3βHSD2*, *CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP17*, and *CYP21* expression and promoted cortisol secretion at the concentration of 10^{-5} M.

ML kills insects by inhibiting the acetylcholinesterase (AChE) activities in the nervous tissue, with only nonspecific inhibition of AChE being valid, environmental dispersion of malathion has become a severe threat to wildlife as well as to public health (Bavcon Kralj et al., 2007). ML is not stable in water and could be metabolized by aquatic organisms making it highly toxic to amphibians at larval stages and aquatic invertebrates, though the potential mechanism remains unknown (Cook et al., 1976). Reporter gene assays showed that ML-B and ML-A displayed stronger estrogen agonistic and glucocorticoid antagonistic activities than ML, respectively. ML-B was also being able to form hydrogen bond between the hydroxyl and the amino acids of ER inducing E_2 secretion by significantly upregulating the expression levels of *3βHSD2*, *17βHSD*, and *CYP19* (n > 3.7-fold) at 10^{-5} M ($p < 0.05$, ANOVA). Potential GR antagonists (ML-B and ML) displayed different effects on *StAR*, *HMGR*, *3βHSD2*, *CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP17*, and *CYP21* expression, and promoted cortisol secretion at 10^{-5} M.

FE is highly toxic to aquatic organisms (*Daphnia magna* and *Scenedesmus subspicatus*) (Lin et al, 2007, 2008; Song et al., 2005) and can rapidly be degraded into 2 major TPs (EHPP and CDHB) by hydrolysis or photolysis in soil and sediment (Lin et al, 2007, 2008). Lin et al showed that FE was most toxic to *D. magna* with a 48 h EC_{50} of 14.3 μM, followed by CDHB (49.8 μM) and EHPP (333.1 μM) (Lin et al, 2007). However, reporter gene assays showed that EHPP and CDHB have greater ERα antagonist activities than FE. CDHB and EHPP also significantly upregulated *CYP19* expression levels by 5.8- and 12.0-fold ($p < 0.05$, ANOVA), respectively.

BX-acid, which is the primary TP of benalaxyl in rat, dog and human hepatocytes (Nallani et al., 2017), is of higher water solubility and persistence than the parent benalaxyl in the environment (Liu et al., 2015). Our data showed that BX-acid possessed significant ERα

antagonist activities ($p < 0.05$, ANOVA), whereas its parent compound did not. BX-acid also significantly upregulated *CYP19* expression levels by 3-fold ($p < 0.05$, ANOVA). However, the relationship between steroid hormone secretion and steroidogenic gene expression was complicated. Thus, the underlying mechanism for this result should be further examined.

The registration of pesticide use should prioritize those with low toxicity and low environment persistence (Fenner et al., 2013). Nonetheless, pesticide residues are ubiquitously found in the environment, in addition to an even more striking situation where approximately 50% of the detected pesticide residues have long been phased out, and 10–20% are stable pesticide TPs (Huntscha et al., 2008). For pesticides, a large body of data is acquired from laboratory testing for market authorization and use registration (Fenner et al., 2013). However, pesticide TPs are still a blind point of pesticide risk assessment. Recent studies have revealed that identification of particularly prevalent or toxicologically relevant pesticide TPs usually emerged 20–30 years after commercial availability (Fjelsted et al., 2011). Pesticides with typically lowered original effects could generate highly relevant TPs (Boxall et al., 2004). Pesticide TPs with intact active moiety would pose additive effects together with their parent compound (Choung et al., 2011; Pesce et al., 2010). However, a more critical concern was that specific TPs generated more potent structures in the environment, which could result in potentially increased endocrine disruption or other chronic effects than their corresponding parent compounds (Zhang et al., 2018; Jin et al., 2010).

The enormous annual consumption of pesticides would undoubtedly lead to high concentrations of environmental residues of pesticides TPs in soil, air, water system, and even crops *via* spray-drift, volatilization (Xu et al. 2018, Kitsiou et al. 2009). Previous studies have reported that various pesticide TPs have been detected in surface water and groundwater, where some TPs were detected even more frequently than their parent compounds (Reemtsma et al., 2013; Segawa et al., 1991). What's worse, pesticides TPs can undergo the bioaccumulation process to reach higher concentrations in the terrestrial food-chain and thus pose tremendous ecological and public health risks, which are still of places for considerable improvement in pesticide regulation and related policy-making directed by future relevant scientific researches.

4. Conclusions

We compared the endocrine-disrupting effects of 4 widely used pesticides and their 21 TPs through *in vitro* and *in silico* approaches, and our results showed that approximately 50% of the pesticide TPs exhibited stronger endocrine-disrupting effects by altering the hormone secretion and the related gene expression level than their corresponding parent compounds. Therefore, it is urgent to pay more attention to the TPs in the process of environmental risk assessment of pesticides, and the profound findings of the endocrine-disrupting effects from pesticide TPs provided in this current study would be beneficial to further risk assessment and regulatory improvement of pesticide use.

CRediT authorship contribution statement

Chenyang Ji: Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Qin Song:** Software, Formal analysis, Investigation, Data curation, Writing - original draft. **Yuanchen Chen:** Formal analysis, Writing - review & editing. **Zhiqiang Zhou:** Resources, Project administration, Funding acquisition. **Peng Wang:** Resources, Project administration, Funding acquisition. **Jing Liu:** Methodology, Resources, Writing - review & editing. **Zhe Sun:** Formal analysis, Writing - review & editing. **Meirong Zhao:** Methodology, Conceptualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105490.

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