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Research Article

Structure-Based Investigation on the Binding and Activation of Typical Pesticides With Thyroid Receptor

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ABSTRACT

A broad range of pesticides have been reported to interfere with the normal function of the thyroid endocrine system. However, the precise mechanism(s) of action has not yet been thoroughly elucidated. In this study, 21 pesticides were assessed for their binding interactions and the potential to disrupt thyroid homeostasis. In the GH3 luciferase reporter gene assays, 5 of the pesticides tested had agonistic effects in the order of procymidone > imidacloprid > mancozeb > fluroxypyr > atrazine. 11 pesticides inhibited luciferase activity of T3 to varying degrees, demonstrating their antagonistic activity. And there are 4 pesticides showed mixed effects when treated with different concentrations. Surface plasmon resonance (SPR) biosensor technique was used to directly measure the binding interactions of these pesticides to the human thyroid hormone receptor (hTR). 13 pesticides were observed to bind directly with TR, with a KD ranging from 4.80E-08 M to 9.44E-07 M. The association and disassociation of the hTR/pesticide complex revealed 2 distinctive binding modes between the agonists and antagonists. At the same time, a different binding mode was displayed by the pesticides showed mix agonist and antagonist activity. In addition, the molecular docking simulation analyses indicated that the interaction energy calculated by CDOCKER for the agonists and antagonists correlated well with the KD values measured by the surface plasmon resonance assay. These results help to explain the differences of the TR activities of these tested pesticides.

Key words: pesticides; thyroid receptor; ligand binding assay; surface plasmon resonance; molecular docking.

Pesticides have been identified as a large category of endocrine-disrupting chemicals (EDCs) based on a series of *in vivo* and *in vitro* studies (Orton *et al.*, 2009). Due to pesticides' extensive use, man-made chemicals are released into the environment and are detected in many food items (Ghisari and Bonefeld-Jorgensen, 2005). Consequently, humans are exposed to pesticides via a variety of ways, including environmental exposure (air, water, soil), food and through work. During the last several decades, use of the persistent pesticides, such as organochlorine insecticides, has been largely prohibited and replaced by pesticides do not persist in the environment, such as organophosphates, carbamates and pyrethroids (Ghisari *et al.*, 2015). However, residues of the nonpersistent pesticides and their metabolites have been measured in human maternal and umbilical cord sera, and urine (Barr *et al.*, 2010; Castorina *et al.*, 2010). Pyrethroid metabolite 3-phenoxybenzoic acid was detected in 67% of pregnant women with a geometric mean of 0.321 μg/l (0.316 μg/g) creatinine by the U.S. National Health and Nutrition Examination Survey (Castorina *et al.*, 2010). Similar urinary concentrations of organophosphate metabolites were tested in children (6–11-years old) in Spain by measuring contemporary pesticides and comparing to the U.S. population (geometric means ranged from 0.47 to 3.36 μg/g creatinine) (Roca *et al.*, 2014). Therefore, further investigation of the potential

impact on human health of contemporary "nonpersistent" pesticides is warranted.

Exposure to pesticides has been associated with adverse health effects like cancers, neurodegenerative and reproductive disorders (Sanborn *et al.*, 2002). Toxicological studies have also shown that pesticides have the potential to cause disruption to the endocrine system (Mostafalou and Abdollahi, 2013). Interaction of EDCs with nuclear receptors is one of the primary molecular events that initiate endocrine system disruption (Huet, 2000). Thyroid receptor (TR) is a member of nuclear receptor superfamily (Scognamiglio *et al.*, 2016). Thyroid hormone (TH) is essential for normal growth and development of mammals and also involved in many aspects of adult life (Zhang and Lazar, 2000), such as development and regulating metabolism. Thus, maintenance of normal thyroid function is very important for psychological and physiological wellbeing (Mullur *et al.*, 2014). TH is mainly mediated by multiple TH receptor isoforms (α and β) derived from 2 distinct genes. Karine Gauthier *et al.* (1999) suggested that specific isoforms mediated different functions while TRβ are the most potent negative feedback regulators by inhibiting the secretion of thyroid stimulating hormone. Recently, a great number of synthetic chemicals have been reported to exert thyroid effects through a variety of mechanisms of action, even chronic exposure to low level (Boas *et al.*, 2012). The organophosphate insecticide, chlorpyrifos, could induce TH-like stimulation of the GH3 cell proliferation, whereas the imidazole fungicides, iprodione and prochloraz, inhibited T3-induced proliferation of the cells (Ghisari and Bonefeld-Jorgensen, 2005). Subsequent studies also showed that gestational and postnatal exposure to chlorpyrifos caused a reduction of serum T4 in dams and F1 mice (De *et al.*, 2009). Rat studies have shown that pyrethroid insecticides are related to altered serum TH levels (Giray *et al.*, 2010). In another *in vitro* study 9 pyrethroids were evaluated for potential endocrine disrupting activities via nuclear hormone receptors including TRs using receptor-mediated reporter gene assays in CV-1 cell line, most of the tested compounds exhibited TR antagonistic effects (Du *et al.*, 2010).

The luciferase assay is one of the most popular *in vitro* models to evaluate endocrine effects of chemicals and has advantages of rapidity, sensitivity, and reproducibility (Wilson, 2004). Nevertheless, most studies employ transactivation reporter gene assays to characterize receptor-mediated TH activity and elucidate the mechanisms of action without assessing the binding affinity of compounds directly with the TR. Therefore, investigating TR-mediated transcription and direct TR binding affinity for the same compounds would address this data gap. Molecular docking, as a screening method, is an effective tool to interpret the potential interactions between ligands and receptors. The surface Plasmon resonance (SPR) technique makes it possible to measure interactions in real-time with high sensitivity and without the need of labels (Karlsson, 2004). We have assessed the interactions of 21 pesticides with TR using the SPR biosensor and demonstrated that it can be used to determine the affinity of ligand interactions and the kinetic constants. Hence, in this study, we employed the firefly luciferase assay to investigate these pesticides for their TH-disrupting effects. Additionally, SPR biosensor technique and molecular docking were combined to further investigate the interactions between TR and pesticides.

MATERIALS AND METHODS

Materials and reagents. 3,3,5-Triiodo-L-thyronine (T3) was purchased from Sigma Aldrich (St Louis, Missouri) and used as positive control; Pesticides(10 insecticides, 6 herbicides, and 6 fungicides) were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany), and their names, CAS numbers and purity are listed in Table 1. All test compounds were prepared as a stock solution in dimethylsulfoxide (DMSO, as a vehicle) at a concentration of 100 mM and stored in the dark at −4 °C. The stock solutions were diluted with 50/50% (v/v) Dulbecco's modified Eagle's medium plus Ham's F-12 nutrient mixture (DMEM/F-12; Gibco, Maryland) immediately before use. The final concentration of DMSO in the culture medium did not exceed 0.1% (v/v) and does not affect cell viability (Du *et al.*, 2010). BL21(DE3) Chemically Competent Cell were purchased from Transgene(Peking, China).

Table 1. Summary of Pesticides in the Reporter Gene Assays for TR

Chemicals Group	CAS No.	Purify (%)
1. Insecticides (n = 10)		
Imidacloprid	105827-78-9	99.7
Pymetrozine	123312-89-0	98
Theta-Cypermethrin	52315-07-8	99.7
Beta-Cypermethrin	65731-84-2	99.8
Bifenthrin	82657-04-3	99
Cyhalothrin	91465-08-6	99
Carbaryl	63-25-2	99.4
Metolcarb	1129-41-5	98
Fenobucarb	3766-81-2	99
Thiodicarb	59669-26-0	98.2
2. Herbicides (n = 5)		
Fluroxypyr	69377-81-7	97
Atrazine	102029-43-6	99.5
Pendimethalin	40487-42-1	99
Acetochlor	34256-82-1	99.2
Butachlor	23184-66-9	93
3. Fungicides (n = 6)		
Procymidone	32809-16-8	99.5
Mancozeb	8018-01-7	96.4
Azoxystrobin	131860-33-8	99
Kresoxim-methyl	143390-89-0	98
Pyraclostrobine	175013-18-0	99
Triadimefon	43121-43-3	98

Cell culture. GH3 cells kindly provided by Dr Bingsheng Zhou (State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, China) were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS; HyClone: Logan, Uttah) and 100IUpenicillin/ml and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Every 5 days, they were separately passaged by trypsinization with 0.25% EDTA disodium salt solution (Gibco, Maryland) in 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands) (Freitas *et al.*, 2011).

Plasmid construction and transfection. A stable luciferase reporter gene assay was developed based on the TH-responsive rat pituitary tumor GH3 cell line that constitutively expresses both TH receptor isoforms as described by Freitas *et al.* (2011) The pGL4CP-SV40-2xtaDR4 plasmid was created by ligating a 2xtaDR4 adapter (2xtaDR4-Top: TCGAGTAAGGTCATTTAAGGT CATTTAAGGTCATTTAAGGTCAA and 2xtaDR4-Btm: CATT CCAGTAAATTCCAGTAAATTCCAGTAAATTCCAGTTTCGA that contains 2 tandem consensus thyroid response elements (direct repeats of the consensus AGGTCA halfsite sequence, separated by the tetranucleotide sequence TTTA) between the XhoI and Hind III sites of pGL4.26 [luc2/minP/Hygro] (Promega, Madison, Wisconsin). Cells were inoculated in 12-well plates (Corning) at a density of 1.6×10^5 cells per well in regular growth medium 24 h

before transfection. Transient transfection was performed with pGL4CP-SV40-2xtaDR4 plasmid using Lipofectamine 2000 (Invitrogen, Paisley, Scotland) according to the manufacturer's protocol. To create the stable cell line GH3-TRE, cells were diluted 1:10 into fresh growth medium 24 h after transfection. 48 h post-transfection, standard growth medium was replaced with selective medium containing 100 U/ml of hygromycin B (Gibco).

MTS assay. Cytotoxicity induced by tested pesticides was performed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H- tetrazolium (MTS) assay on GH3-TRE cells. The vehicle control in the MTS assay was GH3-TRE cells treated with DMSO (0.1%) instead of these 3 kindspesticides. The toxicity of test pesticides was determined by the number of viable cells (indirectly from the absorbance of treated cells). Cells were collected from the culture flask and plated in 96-well plates (Corning) at a density of 1×10^4 cells in each well with DMEM/F-12 medium containing 10% FBS. 24 h later, cells were treated with vehicle or various concentrations (10^{-9}–10^{-5} M) of the test pesticides alone or with 5×10^{-9} M T3 and incubated at 37 °C with 5% CO_2 for 24 h. Cell proliferation was detected using the MTS kit (Cell Titer 96 Aqueous One Solution, Promega) according to the manufacturer's instruction.

TR reporter gene assay. GH3-TRE cells were plated in 96-well microplates at a density of 1.5×10^4 cells per well in DMEM/F-12 medium with penicillin/streptomycin and 100 U/ml hygromycin B followed by a 24 h incubation. For agonistic activity tests, the cells were exposed to varying concentrations of T3 (10^{-12}–10^{-6} M) (positive control), varying concentrations of tested pesticides or 0.1% DMSO (vehicle control). To determine antagonistic activity, the cells were treated with 5×10^{-9} M T3 and each of the 21 pesticides with varying concentrations (ranging from 1.28×10^{-9} to 5×10^{-5} M). After 24 h, activity was measured using Spectra Max i3 plate reader (Molecular Devices, Sunnyvale, California) using the Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. All tests were carried out at least 3 independent times, each with 3–6 replicates per test concentration. And each replicate was sampled randomly from the 96-well microplates.

Protein expression and purification. Human TRβ (hTRβ) was produced in BL21 (DE3) cells at 22 °C by using the pET22b plasmid and a 6-histidine tag was engineered into the C-terminus. To induce protein expression, 0.5 mM isopropyl-D-thiogalactoside was added when an OD_{600} of 0.6 was reached, and the incubation continued for another 8 h. The cells were harvested by centrifugation (5000 rpm, 5 min), re-suspended with 35 ml wash buffer (20 mM Na_3PO_4, 500 mM NaCl, pH 7.4) and subjected to ultrasonic treatment. The clear lysate was collected by centrifugation (15 000 rpm, 30 min). The expressed histidine-tagged recombinant protein was purified by commercially available Ni^+ columns (HisGraviTrap HP column [GE Healthcare]) following the manufacturer's instructions. The protein elution was performed under native conditions in an elution buffer (20 mM Na_3PO_4, 500 mM NaCl) and increasing imidazole concentration (60, 80, and 100 mM pH 7.4). The recombinant protein was eluted in 80 and 100 mM imidazole. The eluates were then analyzed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining and western blot analysis, and the protein concentration was determined using the Bradford method.

Pesticides binding. Direct bindings of T3 and pesticides with hTRβ were measured by SPR technique on a Biacore T200 analytical system (Biacore, Uppsala, Sweden) at 25 °C. The expressed recombinant hTRβ was immobilized on the CM7 sensor chip surface using the standard amine-coupling procedure and HBS-N buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) containing 0.05% surfactant P20 was used as the running buffer. Briefly, with this coupling method, the carboxymethylated dextran layer on the CM7 sensor chip surface was activated with a 10 min injection of an EDC/NHS mixture. The hTRβ (50 μg/ml in 10 mM sodium acetate [pH 5.0]) was injected onto the activated sensor surface for 10 min at a rate of 10 μl/min. After immobilization of the protein, the remaining activated group on the surface was blocked by a 10 min injection of 1 M ethanolamine hydrochloride (pH 8.5) at a flow rate of 10 μl/min. The stability of the sensor chip surface was demonstrated by the flat baseline achieved at the beginning (0–100 s) of each sensorgram.

T3 and all the 21 insecticides, herbicides and fungicides were dissolved in HBS-N (0.01 M HEPES, 0.15 M NaCl, pH 7.4) containing 5% DMSO and 0.05% surfactant P20 from 0.15625 to 5 μM (0.15625, 0.3125, 0.625, 1.25, 2.5, 5.10 μM) for T3 and 0.3125–2.5 μM (0.3125, 0.625, 1.25, 2.5 μM) for pesticides. The buffer samples containing 4.5–5.8% DMSO were injected to construct a DMSO calibration plot to correct for bulk refractive index shifts (Rich *et al.*, 2002). The assay solutions were injected at a flow rate of 30 μl/min using HBS-N containing 5% DMSO and 0.05% surfactant P20 as a running buffer. For each test chemical, the association phase of the SPR measurement was 120 s and on completion of injection, the buffer flow was continued to allow a dissociation time of 600 s. The kinetic constants were obtained by fitting the experimental data to a reversible 1:1 bimolecular interaction model included in the Biacore T200 evaluation software 3.0 from the instrument manufacturer.

Molecular docking. The crystal structure of hTRβ in complex with T3 (PDB code: 4ZOL) (Kojetin *et al.*, 2015) was employed as the template for molecular docking using the Discovery Studio Visualizer Pro, Dock ligands (CDOCKER) protocol. All crystallographic water molecules were removed, and the missing hydrogen atoms were adding using CHARMm forcefield with "Prepare Protein" module. The resulting target protein structure was subsequently utilized to define the docking site as a sphere, with a radius of 13 Å around the binding ligand T3. The minimum energy confirmation of each pesticide was generated by in situ ligand minimization using "Ligand minimization" module. Docking was later performed based on each of the final pose, and CDOKER interaction energy was calculated.

Data analysis. Datasets were tested for homogeneity of variance and normality. First, all datasets met these criteria. Data were analyzed using 1-way ANOVA followed by Duncan's multiple comparisons test when appropriate using SPSS 13.0 (SPSS, Inc.). The level of significance was set at $p < .05$. For hormone agonists, treatments were compared with the vehicle control groups, whereas for antagonists, treatments were compared with the 5×10^{-9} M T3 positive control groups. For SPR, all sensorgrams were processed using double referencing. First, the responses from the reference surface were subtracted from the binding responses collected over the reaction surfaces to correct for bulk refractive index changes. Second, the response from an average of the blank injections was subtracted to remove any systematic artifact observed between the reaction and reference flow cells.

RESULTS

Cytotoxicity of Tested Pesticides

The cytotoxic concentrations of tested pesticides were determined by MTS assay before performing the receptor assay. All

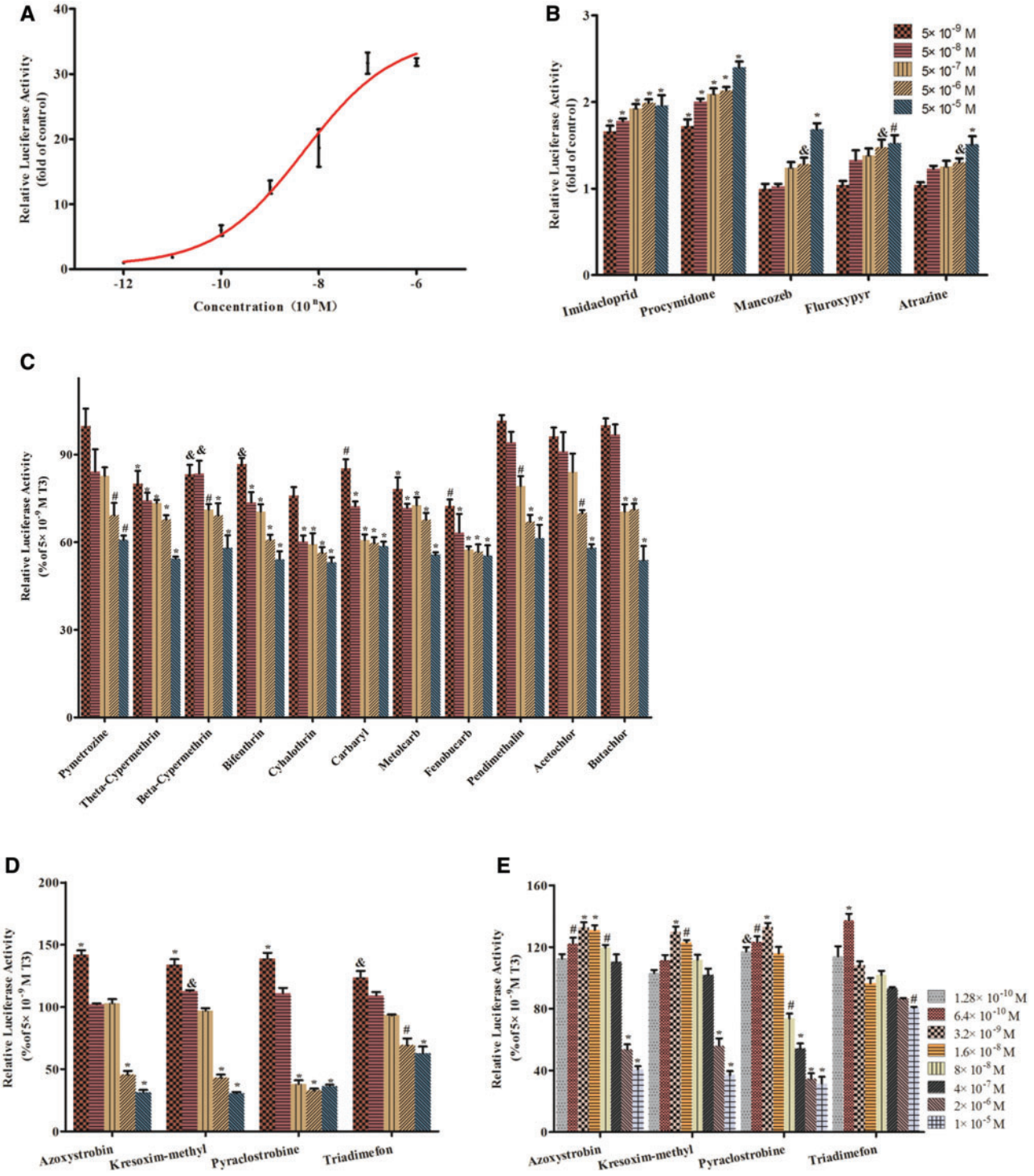

Figure 1. Agonistic and antagonistic activities of tested compounds in the TR reporter gene assay using stably transfected GH3-TRE cells. Values were mean ± SE of 3 independent experiments. Statistically significant $^{\&}p < .05$, $^{\#}p < .01$, $^{*}p < 0.001$relative to vehicle control. A, The TH activities of T3 TR-mediated gene assays. Cells were treated with increasing concentrations (10^{-12}–10^{-6} M) of T3 to detect the agonists' activities. B, Cells were treated with pesticides showed agonist activities in the concentrations from 5×10^{-9} to 5×10^{-5} M. Data were presented as n-fold induction compared with vehicle control. C, Cells were treated with tested compounds showed antagonist activities in the concentrations from 5×10^{-9} to 5×10^{-5} M with 5×10^{-9} M T3. D, Cells were treated with triadimefon and 3 strobilurin fungicides azoxystrobin, kresoxim-methyl, pyraclostrobine in the concentrations from 5×10^{-9} to 5×10^{-5} M with 5×10^{-9} M T3. E, Cells were treated with triadimefon and 3 strobilurin fungicides azoxystrobin, kresoxim-methyl, pyraclostrobine with additional concentrations. For (C–E), values were presented as percent induction, with 100% activity defined as the activity achieved with 5×10^{-9} M T3.

these pesticides tested ($\leq 5 \times 10^{-5}$ M) $\leq 5\times$ did not affect the viability and proliferation of GH3-TRE cells with or without T3. No cytotoxic effects were observed by microscopic examination throughout the entire assay.

Agonistic and Antagonistic Effects of Pesticides by Luciferase Reporter Gene Assay

Agonistic and antagonistic effects of pesticides mediated via TR were estimated by luciferase reporter gene assay. GH3-TRE cell

Table 2. Agonistic and Antagonistic Effects of Pesticides Measured in GH3-TRE Cells

Pesticides	REC20 (M)	RIC20 (M)
1. Insecticides		
Imidacloprid	2.36×10^{-10}	
Pymetrozine		3.06×10^{-8}
Theta-Cypermethrin		5.98×10^{-10}
Beta-Cypermethrin		5.79×10^{-12}
Bifenthrin		1.06×10^{-9}
Cyhalothrin		5.59×10^{-10}
Carbaryl		1.57×10^{-9}
Metolcarb		7.23×10^{-8}
Fenobucarb		1.01×10^{-10}
2. Herbicides		
Fluroxypyr	6.49×10^{-9}	
Atrazine	5.97×10^{-9}	
Pendimethalin		6.69×10^{-8}
Acetochlor		1.59×10^{-7}
Butachlor		2.86×10^{-12}
3. Fungicides		
Procymidone	1.79×10^{-11}	
Mancozeb	8.57×10^{-8}	

reporter system showed an appropriate response to the natural TR ligand T3. T3-induced luciferase activity in a concentration-dependent manner ranging from 10^{-12} to 10^{-6} M (Figure 1A). From the concentration-response curve, the EC50 of T3 was 5.10×10^{-9} M and the maximal induction was 32.15-fold at 10^{-6}M compared with vehicle control. For evaluation of agonistic effect of the pesticides, GH3 cells were treated with pesticides in the range of 5×10^{-9} to 5×10^{-5} M in the absence of T3. Figure 1B shows that 5 of the pesticides tested had agonistic effect and these compounds induced agonistic activity at least 1.23-fold compared with vehicle control. The 20% relative effective concentration (REC20) (Du *et al.*, 2010) was defined as the concentration of pesticide where 20% of 5×10^{-9} M T3 is observed relative to TR (Table 2). The relative agonistic activities of tested chemicals are in following order: procymidone > imidacloprid > atrazine > fluroxypyr > mancozeb, according to the REC20 we got. The most potent pesticide among the 5 compounds is a fungicide, procymidone, with a TR agonistic activity being 2.40-fold higher than that of solvent control at 5×10^{-5} M and with an REC_{20} value of 1.79×10^{-11} M. And at the same time the imidacloprid, a classical neonicotinoid insecticides, also could induce an average 1.99-fold agonistic effect with 5×10^{-6} concentration.

The antagonistic activity was tested by incubation with 5×10^{-9} M T3. In total 11 of these 21 tested pesticides shown in Figure 1C induced antagonistic effects with 5×10^{-9} M T3 in the medium. The 20% relative inhibitory concentration (RIC_{20}) values of the compounds showing TR antagonistic activity are also given in Table 2. Among these compounds, butachlor and beta-cypermethrin exhibited stronger antagonist activity, with RIC_{20} values 2.86×10^{-12} M and 5.79×10^{-12} M. The relative decreasing potency of the tested chemicals is in the following order: butachlor, beta-cypermethrin, fenobucarb, cyhalothrin, theta-cypermethrin, bifenthrin, carbaryl, pymetrozine, pendimethalin, metolcarb, and acetochlor. Mixed agonist and antagonist activity were found (Figure 1D) among 4 chemicals, triadimefon and the 3 strobilurin fungicides; azoxystrobin, kresoxim-methyl, pyraclostrobine. They displayed antagonistic effects at higher concentrations in the reporter gene assay and showed agonistic activities when the concentrations get lower in the presence of 5×10^{-9} M T3. To verify this mix agonist/antagonist effect, we have conducted a series of additional concentrations for these 4 pesticides. The concentration are 1.28×10^{-10}, 6.4×10^{-10}, 3.2×10^{-9}, 1.6×10^{-8}, 8×10^{-8}, 4×10^{-7}, 2×10^{-6}, 1×10^{-5} M. The results (Figure 1E) we got this time for these pesticides confirmed our former result once again. They displayed mixed agonist and antagonist activity depending on their concentration. At high concentration, they showed antagonistic effects and at low concentration they showed agonistic activity in the luciferase reporter gene assay.

Binding Kinetics of Tested Chemicals to hTRβ

The *Escherichia coli*-derived purified recombinant hTRβ was confirmed by SDS/PAGE analysis and Western blotting. Same as the predicted molecular weight, the protein has a molecular weight of 67 kDa with a ≥90% purity level, as shown in Figure 2A. The recombinant hTRβ was immobilized on the CM7 sensor chip by the standard amine-coupling procedure with an immobilization level approximately 25 000RU for the SPR study. Kinetic analysis for the agonists and antagonists was performed to determine the rate constants associated with TR. 13 out of 21 pesticides showed a direct binding reaction with hTRβ. Injecting a concentration series of T3 (0.15625–10 μM) across the hTRβ yielded the sensorgrams shown in Figure 2B. The concentration-dependent responses rapidly reach equilibrium and return to baseline within seconds, indicating a fast association and a fast disassociation between T3 and the receptor. To extract a binding constant for this interaction, the responses at equilibrium were plotted against T3 concentration and fit to a simple binding isotherm (Figure 2C). The differences in binding responses observed for a concentration series of tested agonists and antagonists injected across the hTRβ surface are dramatic. The association and dissociation rates of these ligands varied tremendously. Three agonists (procymidone, imidacloprid, fluroxypyr) showed the same trend as T3 with a fast association rate and dissociated from the receptor with in seconds (Figure 3). Seven antagonists (metolcarb, carbaryl, fenobucarb, pymetrozine, pendimethalin, acetochlor, butachlor) displayed rapid dissociation from the hTRβ surface, although the association rates were slower (Figure 4). However, at the same time pyraclostrobine and kresoxim-methyl displayed antagonistic effects at high concentration and showed agonistic activity at low concentration in the reporter gene assay, their association and dissociation trends are totally different from the former 2 types. It is evident that these 2 ligands associate slower than the 3 agonists (Figs. 4I and 4J). The association (ka) and dissociation (kd) rate constants and the equilibrium dissociation constants (KD) estimated by the 1:1 binding model are listed in Table 3. Thiodicarb was also tested by the SPR, and as expected, its SPR response remained close to the baseline (Figure 4H).

Correlation of Interaction Energies with Binding Affinities

To further understand the potential interaction pattern between pesticides and TR, we implemented a structure-based computational approach. T3 and the pesticides which showed direct binding with hTRβ were docked into the active pocket of hTRβ 4ZO1crystal structure using the CDOCKER program to analyze the correlation between the binding free energy and the SPR binding affinities.

The interaction energy could be regarded as an index of binding affinity of these tested compounds. Table 3 lists the interaction energies of the thirteen compounds to hTRβ 4ZO1 calculated by CDOCKER. By means of a linear regression analysis, the regression equations for binding affinity (KD) were established by using the predicted interaction energy as the sole descriptor. It was demonstrated that the interaction energy

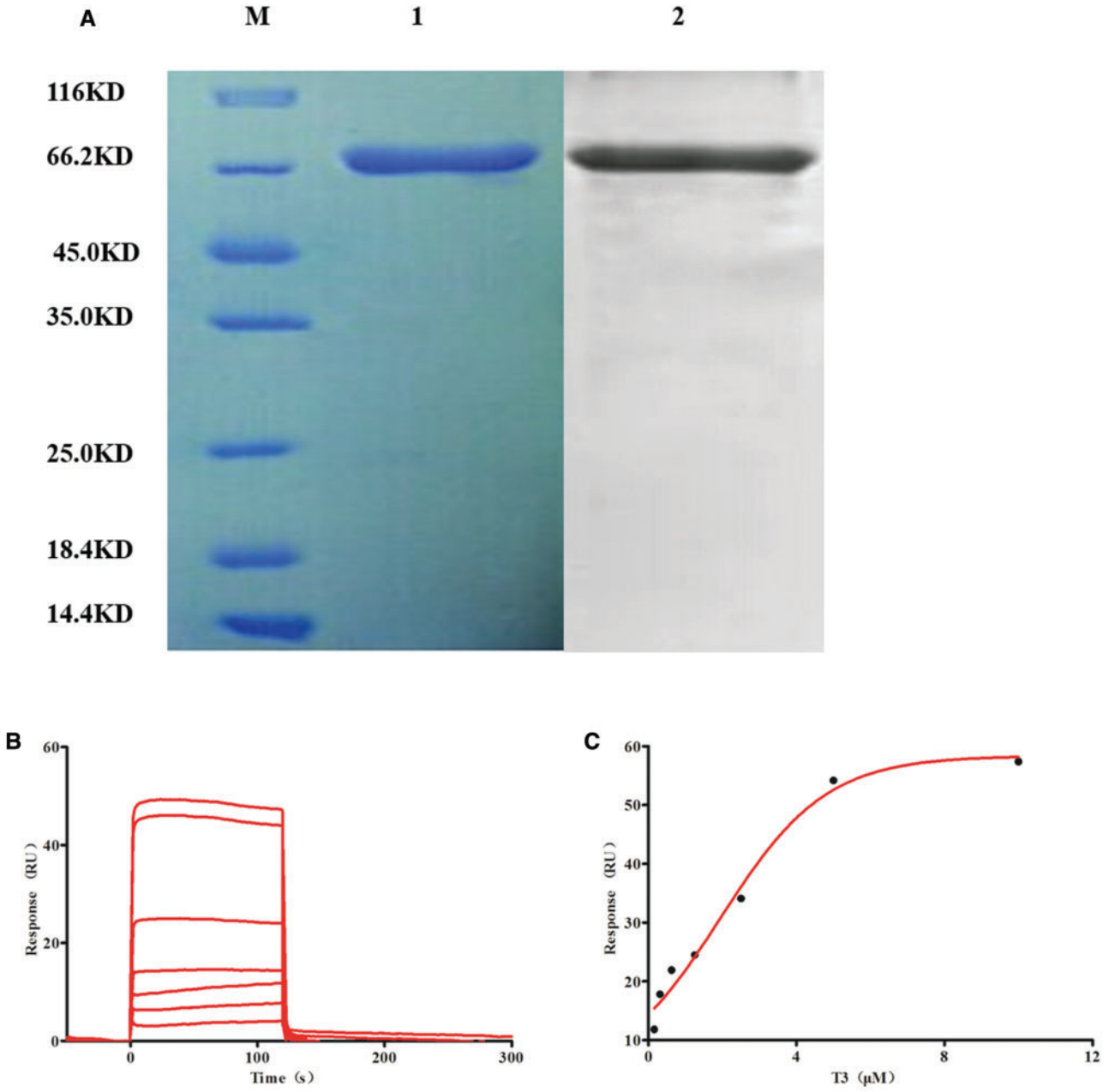

Figure 2. Interaction of T3 with recombinant hTRβ. A, Identification of the purified recombinant hTRβ. The hTRβ was purified and electrophoresed on an SDS-12% polyacrylamide gel. The gel was stained with Coomassie Blue (lane 1). A protein band in lane 2 of 67 kD was detected by western blot analysis of a gel prepared as in lane1 incubated with anti-His-tag antibody and then was detected by binding to goat anti(mouse IgG) antibody. B, Representative sensorgrams obtained from injections of T3 at concentrations of 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, and 10 μM over hTRβ immobolized on the CM7 surface. C, Sensorgram responses at equilibrium ($t \approx 100$ s) were plotted against T3 concentration and fit to a simple binding isotherm to yield a equilibrium dissociation constant.

exhibited correlation with the SPR-determined KD shown by the following equations (Equations 1 and 2)

$$\Delta G_{antagonist} = -10.65 \times \log\ KD - 32.58 \quad (1)$$

$n = 7, R^2 = 0.809$

$$\Delta G_{agonist} = -36.07 \times \log\ KD - 217.86 \quad (2)$$

$n = 4, R^2 = 0.980,$

where $\Delta G_{antagonist}$ and $\Delta G_{agonist}$ are the interaction energies of antagonists and agonists, n is the number of antagonists or agonists, and R^2 is the correlation coefficient. Figures 5A and 5B depict the close correlation of the 7 antagonists and 4 agonists interaction energies with –logKD, which further indicates the reliability of the binding conformations and binding modes derived from the docking simulation.

Agonist procymidone and antagonist butachlor (Figs. 6A and 6B) were used as examples to elucidate the structural basis for different TR activity between agonist and antagonist. It showed that the hydrophobic interactions between the carbon chain of the 2 pesticides and amino acid residues in the binding pocket of hTRβ contribute significantly to the stabilization of the pesticide/receptor complex. However, a key distinction was

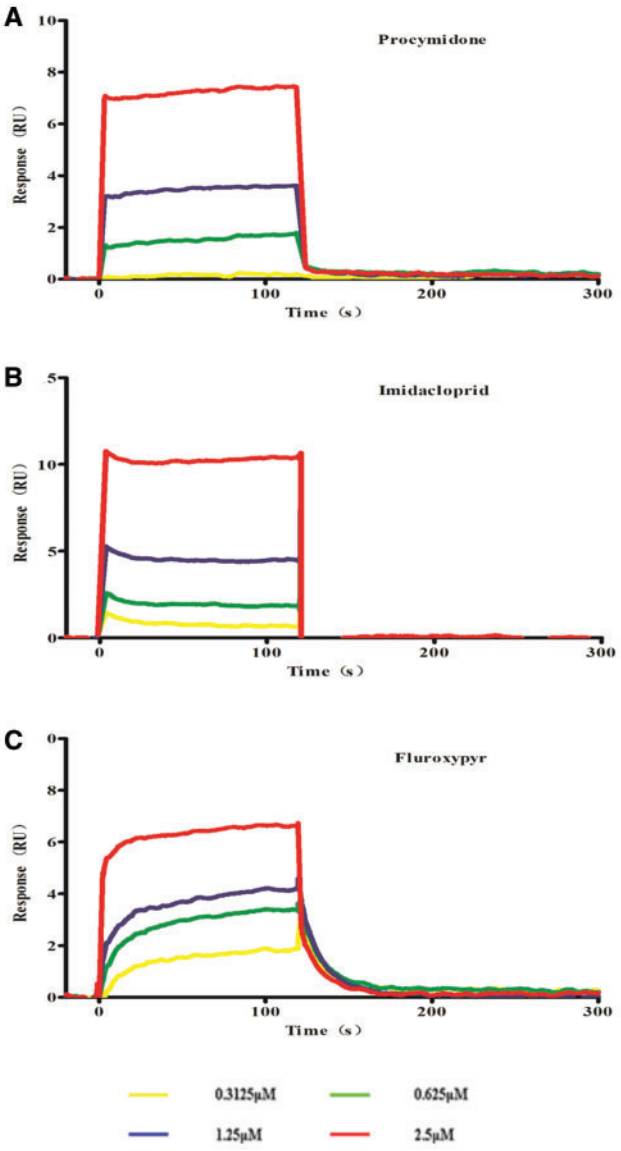

Figure 3. Representative datasets for kinetic analysis of agonists–hTRβ interactions. The agonists procymidone (A), imidacloprid (B), and fluroxypyr (C) were injected at concentrations of 0.3125, 0.625, 1.25, and 2.5 μM for 120 s, and dissociation was monitored for 600 s. The rates of each interaction were fitted to a 1:1 bimolecular interaction model and the kinetic parameters obtained are reported in Table 3.

found between the docked complexes, procymidone was completely inside the binding pocket, while antagonist presents an additional extension that would clash with the active conformation of H12.

DISCUSSION

An increasing number of studies have shown that pesticides and their metabolites may act as EDCs by interfering with hormone receptors as hormone agonists or antagonists (Zhang *et al.*, 2016), such as estrogen receptors (Grünfeld and Bonefeld-Jorgensen, 2004, Rgensen *et al.*, 1997; Raun Andersen *et al.*, 2002), androgen receptors (Vinggaard *et al.*, 2002; Kjeldsen *et al.*, 2013, Luccio-Camelo and Prins, 2011) and TRs (Du *et al.*, 2010 ; Ghisari *et al.*, 2015; Ren and Guo, 2012; Zoeller, 2005). This study presents a qualitative and quantitative analysis of the agonistic and antagonistic disrupting effects of 21 pesticides for TR. The disrupting effects caused by pesticides should be of great concern due to the crucial role of TH playing in vertebrate development. We investigated both TR-mediated transcription activity and direct TR-binding affinity for the same compounds by using the combination of transactivation reporter gene assays, SPR ligand binding assays and molecular docking. Although these 3 techniques referred above are very popular and have led to high-profile discoveries and better understanding in receptor-ligand interaction, they have their own limitations respectively. For reporter gene assay, high basal level was found due to the detection of nonspecific signals. What is more, chemicals used in high-throughput screening may affect other mechanisms further along the signal-transduction pathway, leading to false positives (Hill *et al.*, 2001). For molecular docking, the disadvantages have always been widely mooted: the scoring functions are inaccurate, the sampling of conformational states is crude, and many solvent-related terms are typically ignored (Doman *et al.*, 2002). With regard to SPR, often, signal-to-noise ratio in analysis of small molecules makes it very difficult to differentiate between true signal and noise (Pattnaik, 2005). In this study, we employed these 3 methods together to make the dates more persuasive.

Effects on TH function of TR against the 21 pesticides were all tested by treating with GH3-TRE cells and measuring luciferase activity, using T3 as positive control. T3-induced luciferase activity and reached maximum activity at approximately 10^{-7} M, with an EC50 of 5.10×10^{-9} M. This confirms previously reported value (EC50 of T3 = 2.94×10^{-9} M) (Du *et al.*, 2010). 5 of the 21 tested pesticides showed agonistic effects in the reporter gene assay with the relative agonistic activities and 11 of them showed apparent antagonistic effect. The results we got are consistent with former *in vivo* reports. For example, pyrethroid insecticides could induce thyroid endocrine disruption in fish and significantly elevate the TRα and TRβ genes transcription (Tu *et al.*, 2016). T3 can induce metamorphosis of Xenopus laevis, and the metamorphosis process can be accelerated by acetochlor, causing forelimb emergence and increase mRNA expression of TH β receptors in ranid tadpoles (Crump *et al.*, 2002; Giray *et al.*, 2010). Both imidacloprid and mancozeb could lead to an impairment of HPT axis in preparatory and breeding phases and lead to distinct alterations in weight, volume, and histopathology of thyroid gland (Pandey and Mohanty, 2015). Thyroid dysfunction and changes in circulating THs were found in the freshwater catfish induced by carbaryl (Sinha *et al.*, 1991). Surprisingly, among these tested pesticides, triadimefon and strobilurin fungicides azoxystrobin, kresoxim-methyl, pyraclostrobine displayed antagonistic effects at high concentration and showed agonistic activity at low concentration in the luciferase reporter gene assay. This phenomenon was also found in a previous study when testing the ER-mediated reporter gene assay against permethrin (Du *et al.*, 2010). Except pesticides, raloxifene, a selective estrogen receptor modulator, is a mixed estrogen agonist/antagonist that has been shown to prevent osteoporosis and breast cancer in women (Kim *et al.*, 2002). Resveratrol, a phytoalexin present in grapes and grape products, was reported to exert mixed estrogen agonist/antagonist activities in some mammary cancer cell lines in the absence of E2 (Bhat *et al.*, 2001). As reported, some compounds displayed this kind of activity depending on their concentration, ligand binding affinity, and existence of natural ligands (Wilson *et al.*, 2002). Although mixed agonist/antagonist is mechanistically distinct from conventional agonists and antagonists, we postulated that these pesticides might have mixed ligand dimers, that is, an agonist-bound receptor dimerized with an antagonist

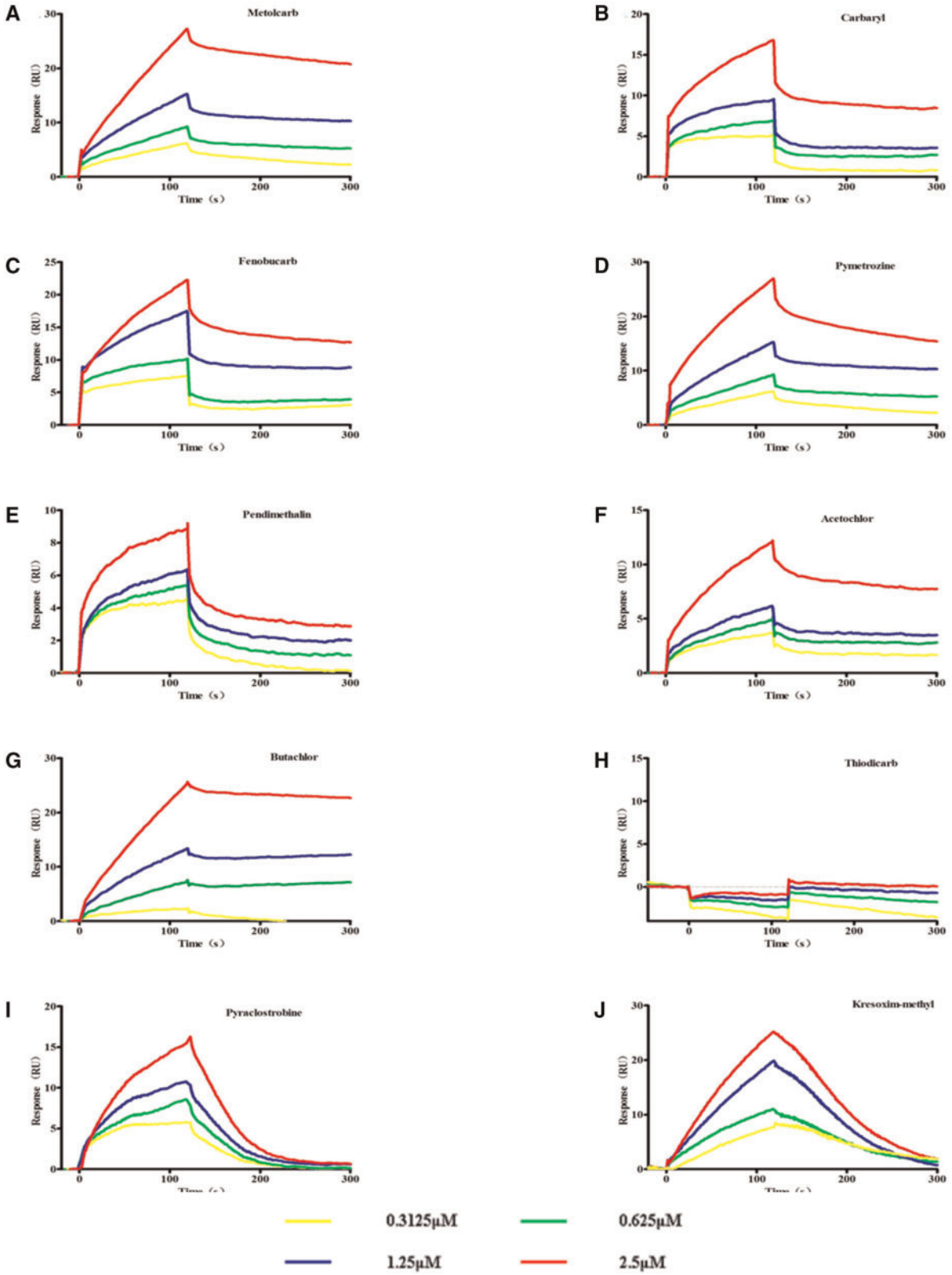

Figure 4. Representative datasets for kinetic analysis of antagonists–hTRβ interactions. The antagonists Metolcarb (A), Carbaryl (B), Fenobucarb (C), Pymetrozine (D), Pendimethalin (E), Acetochlor (F), Butachlor (G), Thiodicarb (H), Pyraclostrobine (I), and Kresoxim-methyl (J) were injected at concentrations of 0.3125, 0.625, 1.25, and 2.5 μM for 120 s, and dissociation was monitored for 600 s. The dates of each interaction were fitted to a 1:1 bimolecular interaction model same as Figure 3 and the kinetic parameters obtained are also reported in Table 3.

Table 3. Kinetic Parameters of the Binding of TR and the Tested Compounds and the Interaction Energies Estimated by CDOCKE

Interaction	ka (1/Ms)	kd (1/s)	KD (M)	CDOCKER-Interaction Energy(kcal/mol)
T3	1.34E+05	4.18E-04	3.12E-09	–89.23
Procymidone	8.59E+04	7.19E-03	8.37E-08	–39.33
Imidacloprid	8.67E+04	6.53E-03	7.52E-08	–34.74
Fluroxypyr	3.68E+04	3.71E-03	1.01E-07	–36.70
Butachlor	1.64E+03	7.90E-05	4.80E-08	–43.65
Acetochlor	4.31E+03	9.09E-04	2.11E-07	–38.73
Pendimethalin	5.41E+03	1.57E-03	2.91E-07	–40.13
Pymetrozine	4.08E+03	1.29E-03	3.16E-07	–37.24
Fenobucarb	1.08E+03	5.12E-04	4.74E-07	–35.73
Carbaryl	6.79E+02	4.76E-04	7.01E-07	–33.03
Metolcarb	8.77E+02	8.29E-04	9.44E-07	–28.53
Pyraclostrobine	3.07E+04	2.05E-02	6.66E-07	–47.11
Kresoxim-methyl	2.08E+04	1.54E-02	7.42E-07	–50.29

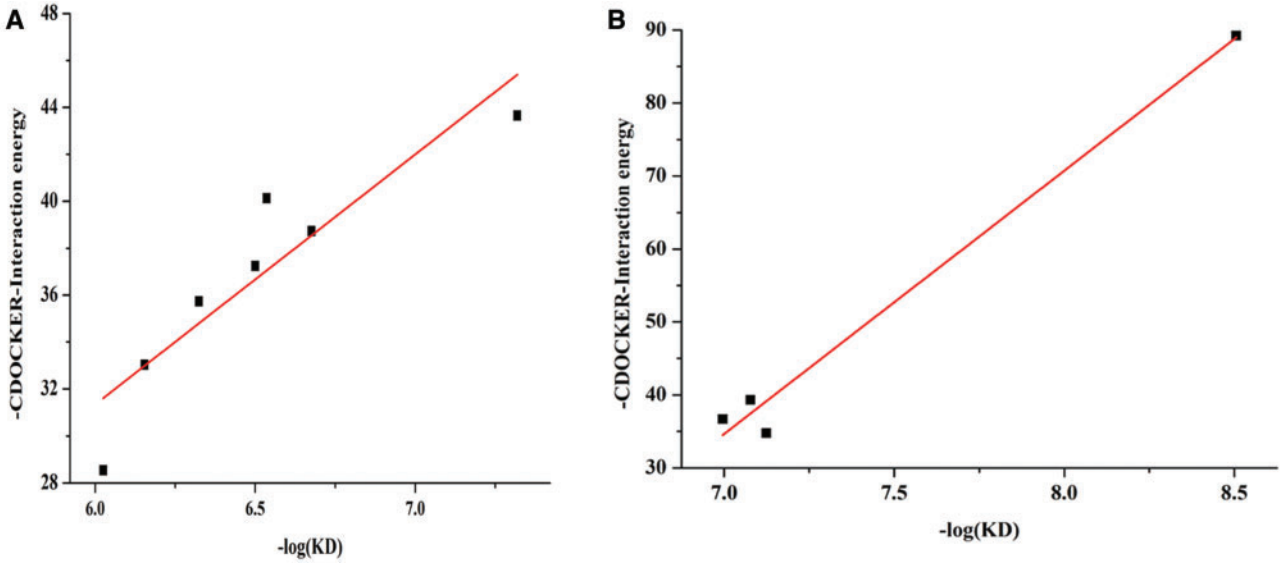

Figure 5. Correlations between SPR-binding affinities and the CDOCKER interaction energies. Seven antagonists (A) and 4 agonists (B) were fitted with linear regression analysis respectively. The negative CDOCKER interaction energies was plotted against the negative logarithm of KD. The data were analyzed by linear fitting method using OriginPro8.5.1.

bound receptor; this may be required for antagonism. As a result, the gene activation may be promoted by same-ligand dimers (Wong *et al.*, 1995).

Overall, we demonstrate *in vitro* that these pesticides impact the endocrine system via TRs. Although the luciferase reporter assay is able to monitor transactivation, luciferase activity may not fully reflect direct binding of the ligand to the receptor since significant transactivation by ligand metabolites cannot be ruled out (Fujino *et al.*, 2004). In this study, we determined that the binding potency of the pesticides showed agonist and antagonist effects in the luciferase assay with hTRβ using SPR biosensor technology-based direct binding assay.

Prior functional and structural analyses of ligand-bound receptor provided the groundwork for understanding TR regulation by agonist and antagonist ligands: (1) the receptor exists in different conformations when it is complexed with agonists or antagonists, (2) the 2 ligand types bind to TR at the same site, and with similar affinities (Schapira *et al.*, 2003). Therefore, SPR technology and molecular docking were further applied to study the binding features of the pesticides to hTRβ. As previously mentioned, SPR technology has been recognized as a powerful tool for investigating the interactions between molecules with the advantages of permitting real-time measurement without requiring labeling (Karlsson, 2004; Nguyen *et al.*, 2015). In SPR study, each of the agonists and antagonists binding responses were well-described by a 1:1 interaction model. Upon review, there are no differences found in the KD values between agonists and antagonists (4.80×10^{-8}–9.44×10^{-9}), but a clear trend of binding potency emerged and differences in their association rates are apparent. The binding trend of all selected pesticides could be divided into 3 categories (agonist, antagonist, and mixed), which is highly consistent with our luciferase gene assay results. The agonist pesticides characterized by fast association and dissociation phases, whereas the antagonists showed slower association rates and rapid dissociation from the hTRβ surface. As shown in Table 3, the association rate constants observed for the agonists (3.68×10^4–1.34×10^5 M^{-1} S^{-1}), all displayed rapid association to hTR and were on average 40-fold faster than the antagonist (6.79×10^2–5.41×10^3 M^{-1} S^{-1}). The binding patterns for 2 "mixed" pesticides (pyraclostrobine and kresoxim-methyl) were different from former 2 modes, showing slower association and disassociation rate. Combined with the

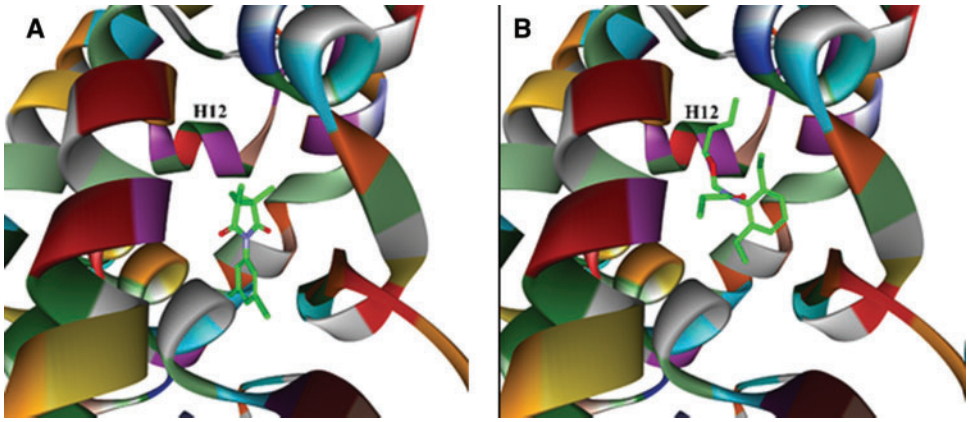

Figure 6. Graphs for the docked complexes between hTR ligand binding domain. Procymidone and butachlor were used as 2 examples docked into the active pocket of hTRβ 4ZO1crystal structure. Pose comparison between agonist procymidone (A) or antagonist butachlor (B) with hTRβ 4ZO1crystal structure.

luciferase gene assay results, we hypothesis that the patterns of the "mixed" are associated with TH disruption characters. For the thiodicarb, it showed neither agonistic nor antagonistic effect in the luciferase gene assay. Consistently, The SPR result showed that there is no direct binding between thiodicarb and hTR which confirmed our conjecture.

To explore the binding characteristics of the agonists and antagonists to TR at the molecular level, molecular docking was also performed between pesticides and hTRβ to construct the ligand/hTR-binding model. The fact that ligand binding can induce a conformational change in nuclear receptors is well known. Agonists and antagonists would trigger distinct structural alterations of nuclear receptor LBDs (Bourguet *et al.*, 2000). For agonist, it can be considered as a molecule that enhances the interactions of LBDs with one or more coactivator LXXLL motifs and therefore leads to transcriptional activation in a cell-based assay. By contrast, antagonists position H12 to physically compete with and block the site of coactivators, or otherwise do not support this binding (Huang *et al.*, 2010). Taking the results and the structures of the tested pesticides together, our data indicate that 2 distinct binding modes exist for agonists and antagonists. As an agonist, procymidone was completely inside the binding pocket; however, for antagonists, they present an additional extension clash with the active conformation of H12. H12 is the most C-terminal helix and rather dynamic even though most of the ligand binding domain of TR remains static. It can undergo dramatic shifts in position in response to the molecule in the pocket. H12 will fold like a lid onto the agonist and form a hydrophobic cavity at the surface of the receptor (Ashley *et al.*, 2001). When it comes to antagonists, H12 will be prevented from folding on the ligand-binding pocket. As a result, this extension clash is believed to prevent the fold-back of TR H12 which would seal the binding pocket and therefore coactivator binding is not possible.

Because there are no reports on the KD values for binding of the tested compounds to hTR, we compared the KD values obtained from the SPR assay and interaction energy calculated by CDOCKER. The interaction energies calculated by CDOCKER are in line with the KD values obtained in SPR experiments, and the correlation coefficient (R^2) is 0.980 for agonists and 0.809 for antagonists.

The relationship between pesticide structure and hormonal activity was found in this study. Most of the pesticides tested had TR binding activity, they can add to the overall "thyroid load" of the whole body. It should be emphasized the fact that humans and wildlife are exposed to complex EDCs continuously and there may have a great likelihood that many EDCs act together to increase their individual effects. Maintenance of normal thyroid function is essential for psychological and physiological wellbeing. Thyroid physiology can be affected by thyroid disruptors in many phases. The complex system, from iodine uptake, TH production, interconversion of THs, cellular uptake, cell receptor activation to hormone degradation and elimination could be altered directly or indirectly by the thyroid disruptors (Kabir *et al.*, 2015). Serum hormone levels disruption may be caused by thyroid-disrupting chemicals exposure, which in turn may have significant consequences for public health. These findings show that integrated screening TR antagonists/agonists of EDCs, including pesticides, need to be undertaken to better understand the potential risk to human and animal health.

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