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 the present 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-08M to 9.44E-07M. The association and disassociation of the hTR/pesticide complex revealed two 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 SPR assay. These results help to explain the differences of the TR activities of these tested pesticides.

Keywords: pesticides, thyroid receptor, ligand binding assay, surface plasmon resonance, molecular docking；

Introduction

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 non-persistent pesticides and their metabolites have been measured in human maternal and umbilical cord sera, and urine (Castorina et al. 2010, Barr 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 US 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 "non-persistent" 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 (Endocrine Disrupting Chemicals) 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 (MullurLiu and Brent 2014). Thyroid hormone (TH) is mainly mediated by multiple thyroid hormone receptor isoforms (α and β) derived from two distinct genes. Gauthier et al., suggested that specific isoforms mediated different functions while TRβ are the most potent negative feedback regulators by inhibiting the secretion of thyroid stimulating hormone (TSH) (Karine Gauthier et al. 1999). 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 nine 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 thyroid hormone 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 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 the present 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.

2. Materials and Methods

2.1. Materials and Reagents

3,3,5-Triiodo-L-thyronine (T3) was purchased from Sigma Aldrich (St. Louis, MO) and used as positive control; Pesticides(ten insecticides, five herbicides and six 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, MD, USA.) 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).

2.2. 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, UT, USA)) and 100IUpenicillin/ml and 100μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Every five days, they were separately passaged by trypsinization with 0.25% EDTA disodium salt solution (Gibco) in 75 cm^2 culture flasks (Corning, Schiphol-Rijk, The Netherlands) (Freitas et al. 2011).

2.3. Plasmid Construction and Transfection

A stable luciferase reporter gene assay was developed based on the thyroid hormone-responsive rat pituitary tumor GH3 cell line that constitutively expresses both thyroid hormone receptor isoforms as described by Freitas, J. et al.. (Freitas et al. 2011) The pGL4CP-SV40-2xtaDR4 plasmid was created by ligating a 2xtaDR4 adapter (2xtaDR4-Top: TCGAGTAAGGTCATTTAAGGTCATTTAAGGTCATTTAAGGTCAA and 2xtaDR4-Btm: CATTCCAGTAAATTCCAGTAAATTCCAGTAAATTCCAGTTTCGA that contains two 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, WI, USA). Cells were inoculated in 12-well plates (Corning) at a density of 1.6×10^5 cells per well in regular growth medium 24h 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 24h after transfection. 48h post-transfection, standard growth medium was replaced with selective medium containing 100 U/ml of hygromycin B (Gibco).

2.4. MTS Assay

Cytotoxicity induced by tested pesticides was performed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

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 three 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. 24h later, cells were treated with vehicle or various concentrations (10^{-9} to 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 24h. Cell proliferation was detected using the MTS kit (Cell Titer 96 Aqueous One Solution, Promega) according to the manufacturer's instruction.

2.5. 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 24h incubation. For agonistic activity tests, the cells were exposed to varying concentrations of T3 (10^{-12} to 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 from1.28×10^{-9} to 5×10^{-5} M). After 24h, activity was measured using Spectra Max i3 plate reader (Molecular Devices, Sunnyvale, CA, USA) using the Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. All tests were carried out at least three independent times, each with three to six replicates per test concentration. And each replicate was

sampled randomly from the 96-well microplates.

2.6. Protein Expression and Purification

Human TRβ (hTRβ) was produced in BL21 (DE3) cells at 22°C by using the pET22b plasmid and a six-histidine tag was engineered into the C-terminus. To induce protein expression, 0.5 mM isopropyl-D-thiogalactoside (IPTG) was added when an OD_{600} of 0.6 was reached, and the incubation continued for another 8h. The cells were harvested by centrifugation (5000 rpm, 5min), 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 (15000 rpm, 30min). 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.

2.7. Pesticides Binding

Direct bindings of T3 and pesticides with hTRβ were measured by surface Plasmon resonance (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 10min 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 10min at a rate of 10 μl/min. After immobilization of the protein, the remaining activated group on the surface was blocked by a 10min 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 μM to 5 μM (0.15625, 0.3125, 0.625, 1.25, 2.5, 5,10 μM) for T3 and 0.3125 to 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 120sec and on completion of injection, the buffer flow was continued to allow a dissociation time of 600sec. 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.

2.8 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.

2.9. Data Analysis

Data sets were tested for homogeneity of variance and normality. Firstly, all data sets met these criteria. Data was analyzed using one-way ANOVA followed by Duncan's multiple comparisons test when appropriate using SPSS 13.0 (SPSS, Inc.). The level of significance was set at $p < 0.05$. 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.

3. Results

3.1. Cytotoxicity of Tested Pesticides

The cytotoxic concentrations of tested pesticides were determined by MTS assay before performing the receptor assay. All these pesticides tested ($\leq 5 \times 10^{-5}$M) 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.

3.2. 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 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 (Fig. 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. Fig. 1B shows that five 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 five 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. 11 of these 21 tested pesticides shown in Fig. 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 (Fig. 1D) among four chemicals, triadimefon and the three 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 four 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 (Fig. 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.

3.3. 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 Fig. 2A. The recombinant hTRβ was immobilized on the CM7 sensor chip by the standard amine-coupling procedure with an immobilization level approximately 25000RU 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 μM to 10 μM) across the hTRβ yielded the sensorgrams shown in Fig. 2B. The concentration-dependent responses rapidly reach equilibrium and return to baseline withinseconds, 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 (Fig. 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. 3 agonists

(procymidone, imidacloprid, fluroxypyr) showed the same trend as T3 with a fast association rate and dissociated from the receptor withinseconds (Fig. 3). 7 antagonists (metolcarb, carbaryl, fenobucarb, pymetrozine, pendimethalin, acetochlor, butachlor) displayed rapid dissociation from the hTRβ surface, although the association rates were slower (Fig. 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 two types. It is evident that these two ligands associate slower than the three 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 (Fig. 4H).

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

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

n=7, R^2=0.809

$\Delta G_{agonist} = -36.07 \times \log KD - 217.86$ (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 6A and 6B depict the close correlation of the seven antagonists and four 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. 5A and B) 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 two 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 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.

4. 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, RgensenAutrup and Hansen 1997, Raun Andersen et al. 2002), androgen receptors (Vinggaard et al. 2002, KjeldsenGhisari and Bonefeld-Jørgensen 2013, Luccio-Camelo and Prins 2011) and TRs (Du et al. 2010, Ren and Guo 2012, Ghisari et al. 2015, Zoeller 2005). The current 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 three 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 the present study, we employed these three 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 thyroid hormone 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 thyroid hormones were found in the freshwater catfish induced by carbaryl (SinhaLal and Singh 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 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 the present 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: (i) the receptor exists in different conformations when it is complexed with agonists or antagonists, (ii) the two 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 three 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 Table3, the association rate constants observed for the agonists (3.68×10^{4}-1.34×10^{5}M-1S-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-1S-1). The binding patterns for two “mixed” pesticides (pyraclostrobine and kresoxim-methyl) were different from former two modes,

showing slower association and disassociation rate. Combined with the luciferase gene assay results, we hypothesis that the patterns of the “mixed” are associated with thyroid hormone 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 (BourguetGermain and Gronemeyer 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 (HuangChandra and Rastinejad 2010). Taking the results and the structures of the tested pesticides together，our data indicate that two 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 C. W. Pike 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 the present 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, thyroid hormone production, interconversion of thyroid hormones, 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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Notes

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FIGURES

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 three independent experiments. Statistically significant $^{\&}$ $p < 0.05$, $^{\#}p < 0.01$, $^{*}p < 0.001$relative to vehicle control. (A) The thyroid hormone 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 three 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 three strobilurin fungicides azoxystrobin, kresoxim-methyl, pyraclostrobine with additional concentrations. For C, D, E, values were presented as percent induction, with 100% activity defined as the activity achieved with 5×10^{-9}M T3.

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 (lane1). 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, 5,10μM over hTRβ immobolized on the CM7 surface. (C) Sensorgram responses at equilibrium (t ≈100 s) were plotted against T3 concentration and fit to a simple binding isotherm to yield a equilibrium dissociation constant.

Figure 3. Representative data sets 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 600s. The rates of each interaction were fitted to a 1:1 bimolecular interaction model and the kinetic parameters obtained are reported in Table 3.

Figure 4. Representative data sets 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 600s. The dates of each interaction were fitted to a 1:1 bimolecular interaction model same as Fig.3 and the kinetic parameters obtained are also reported in Table 3.

Figure 5. Graphs for the docked complexes between hTR ligand binding domain. Procymidone and butachlor were used as two examples docked into the active pocket of hTRβ 4ZO1crystal structure. Pose comparison between agonist procymidone(A) or antagonist butachlor(B) with hTRβ 4ZO1crystal structure .

Figure 6. Correlations between SPR binding affinities and the CDOCKER interaction

energies. Seven antagonists(A) and four 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.

TABLES

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

Table2

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}	

Kinetic parameters of the binding of TR and the tested compounds and the interaction energies estimated by CDOCKER

	ka (1/Ms)	kd (1/s)	KD (M)	CDOCKER-Interaction energy(kcal/mol)
	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
	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
	6.79E+02	4.76E-04	7.01E-07	-33.03
	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

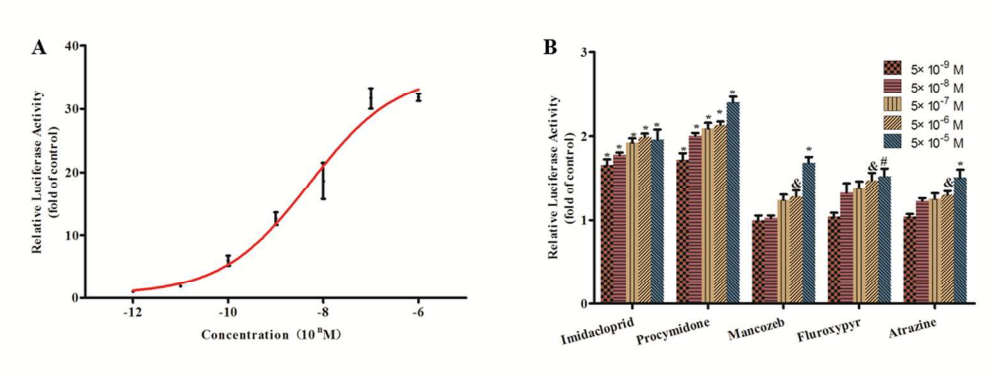

Figure 1. Agonistic and antagonistic activities of tested compounds in the TR reporter gene assay using stably transfected GH3-TRE cells.

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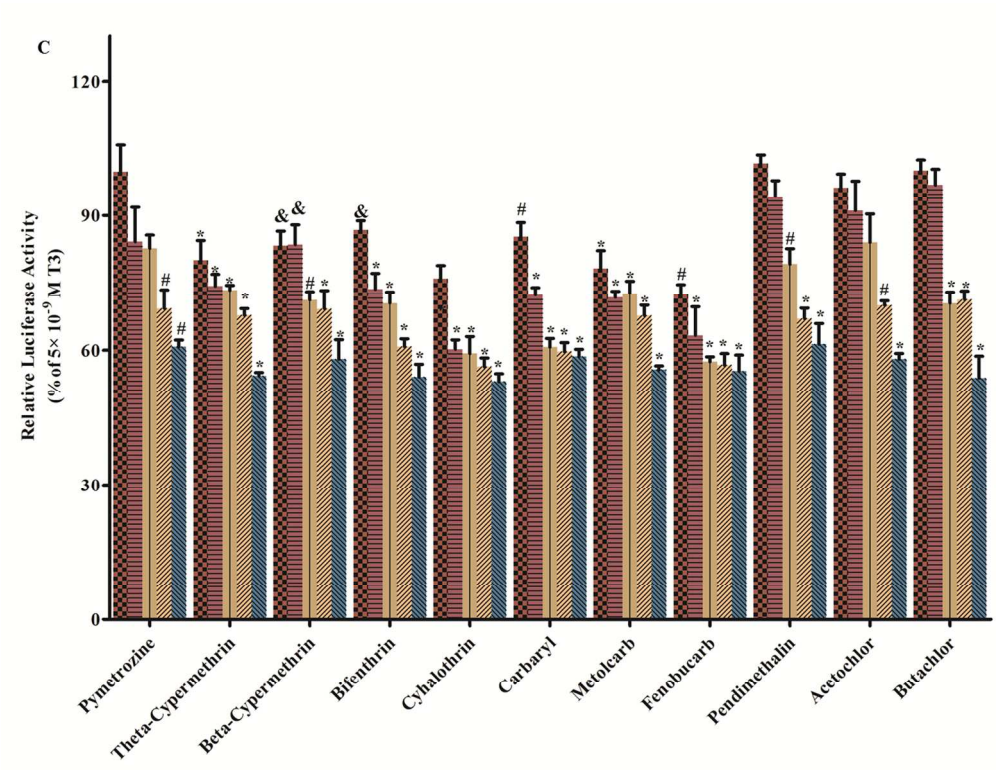
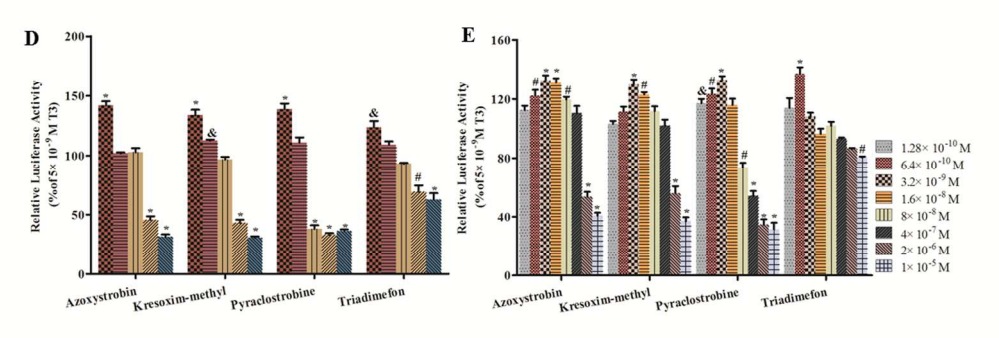


227x176mm (300 x 300 DPI)



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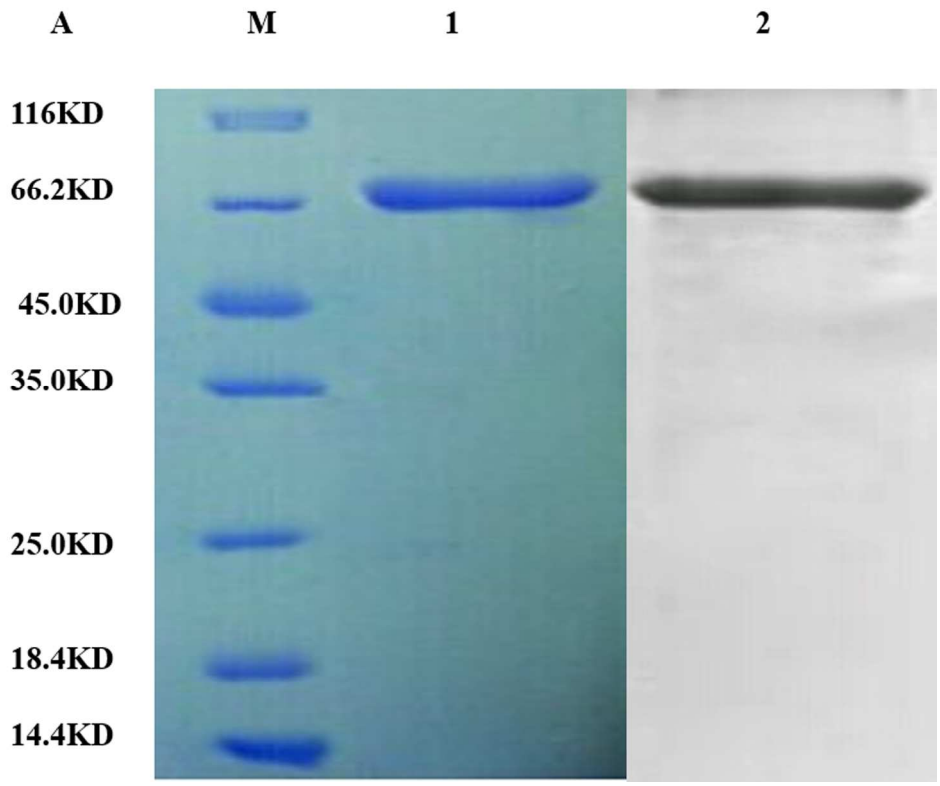

Figure 2. Interaction of T3 with recombinant hTRβ.

207x163mm (300 x 300 DPI)

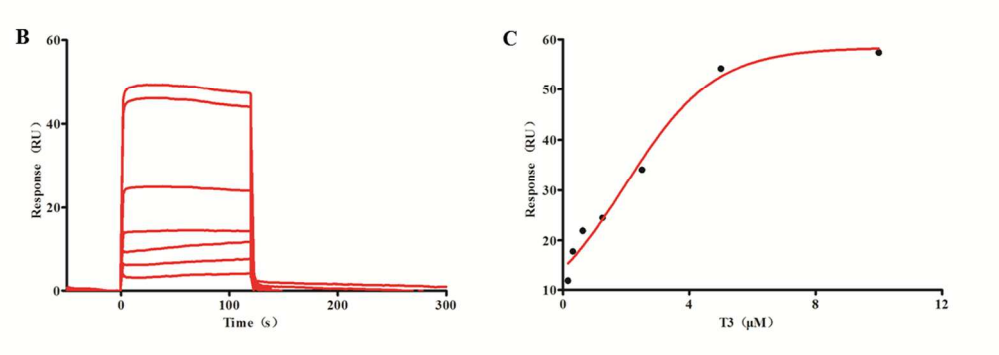

Figure 2. Interaction of T3 with recombinant hTRβ.

197x71mm (300 x 300 DPI)

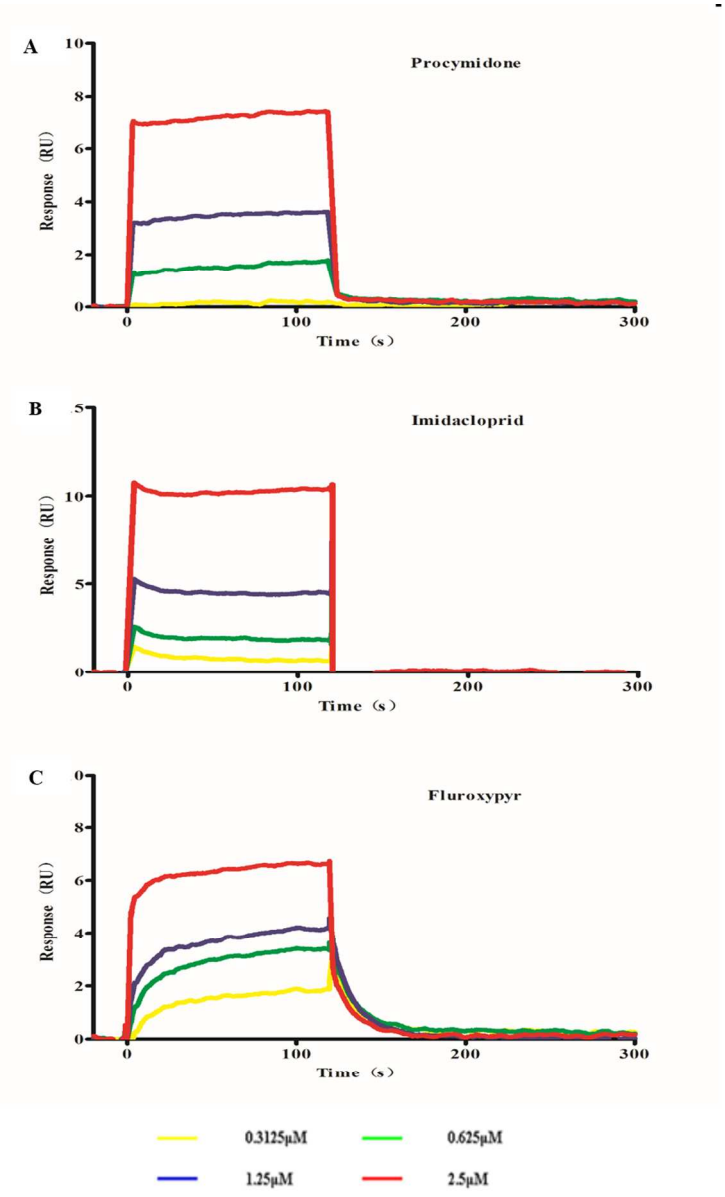

Figure 3. Representative data sets for kinetic analysis of agonists–hTRβ interactions.

231x386mm (300 x 300 DPI)

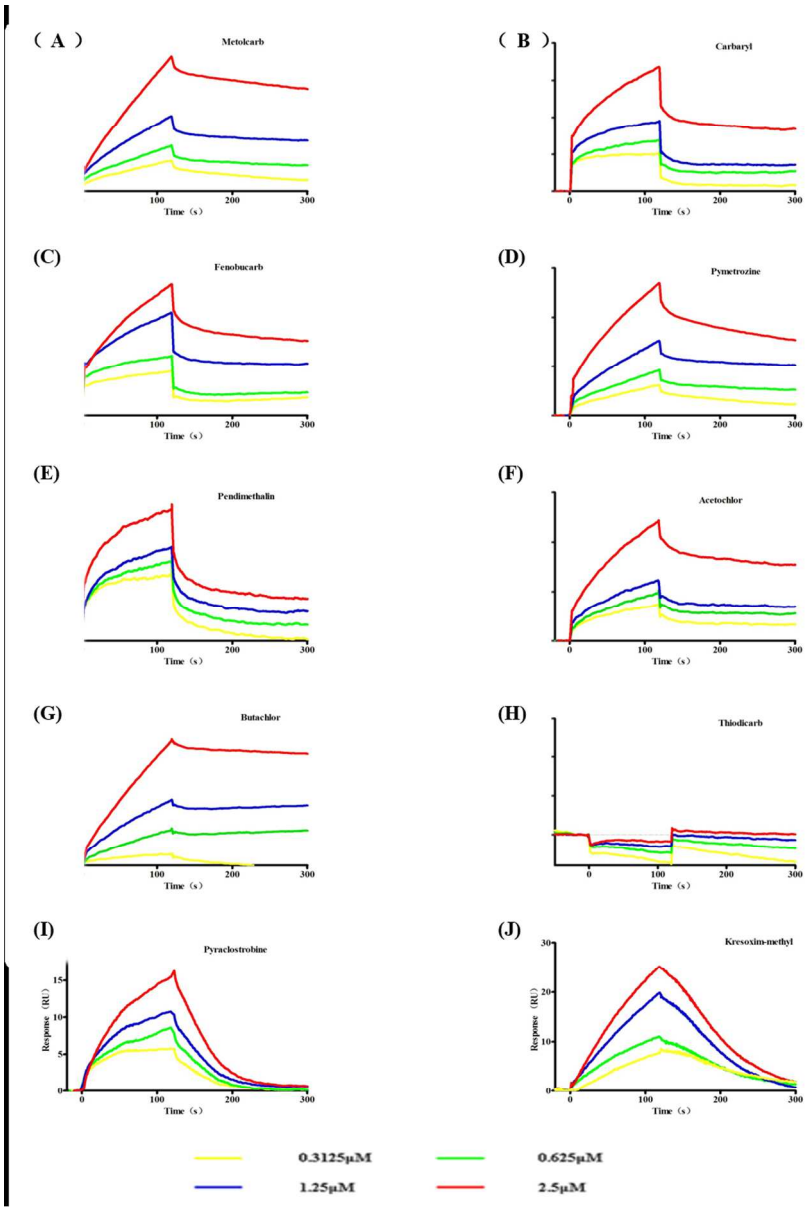

Figure 4. Representative data sets for kinetic analysis of antagonists–hTRβ interactions.

283x396mm (300 x 300 DPI)

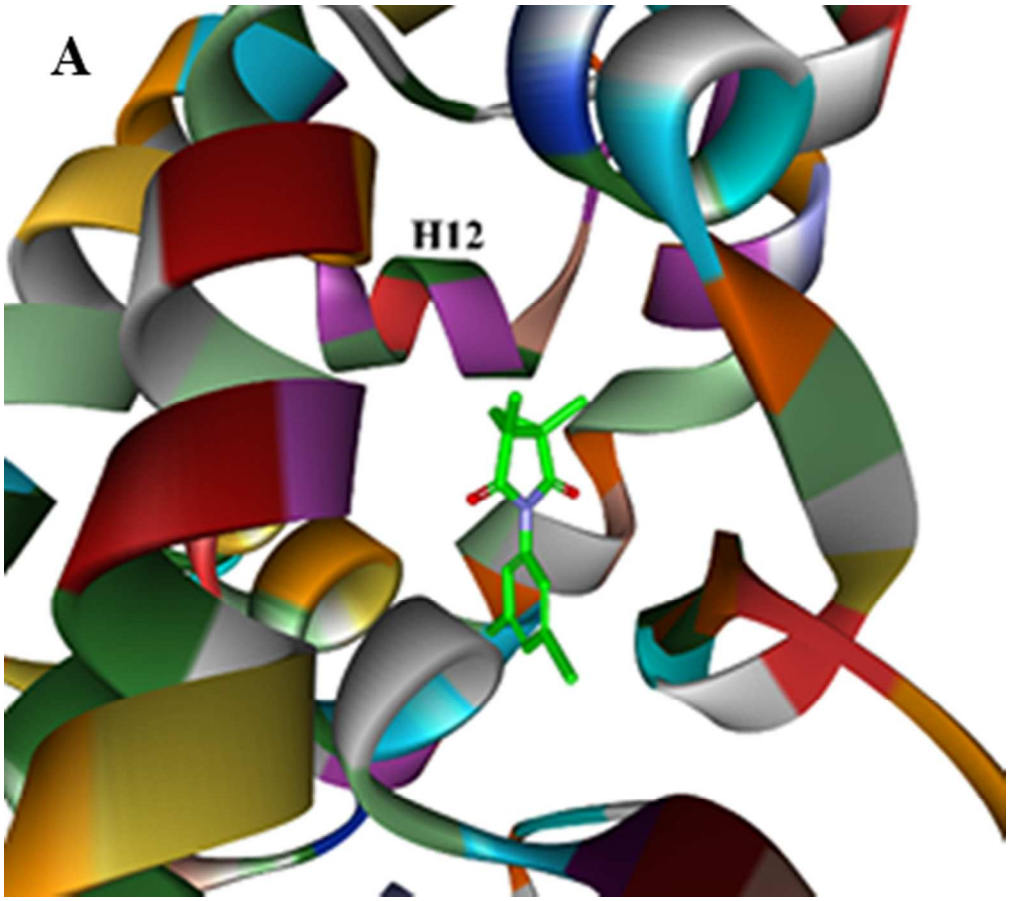

Figure 5A. Graphs for the docked complexes between hTR ligand binding domain.

75x67mm (300 x 300 DPI)

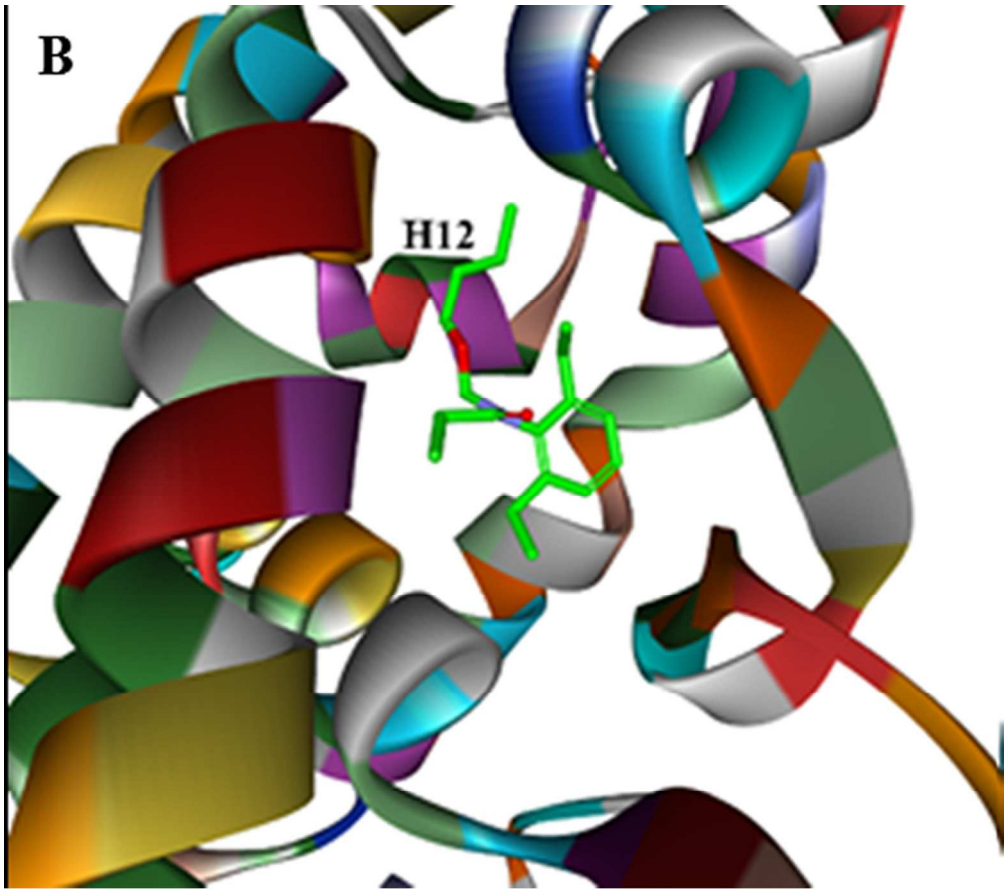

Figure 5B. Graphs for the docked complexes between hTR ligand binding domain.

76x67mm (300 x 300 DPI)

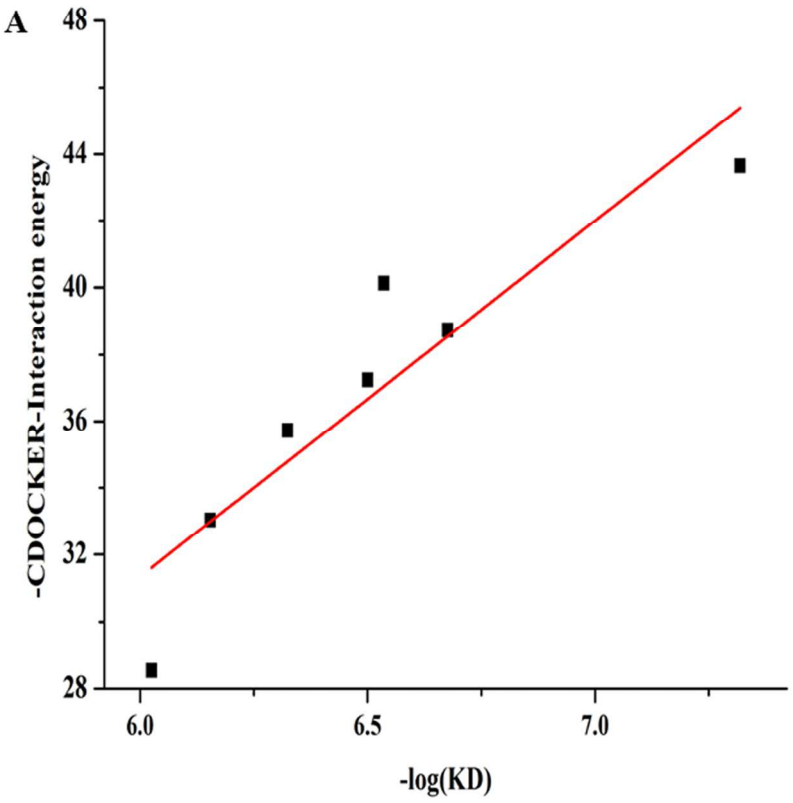

Figure 6A. Correlations between SPR binding affinities and the CDOCKER interaction energies.

129x120mm (300 x 300 DPI)

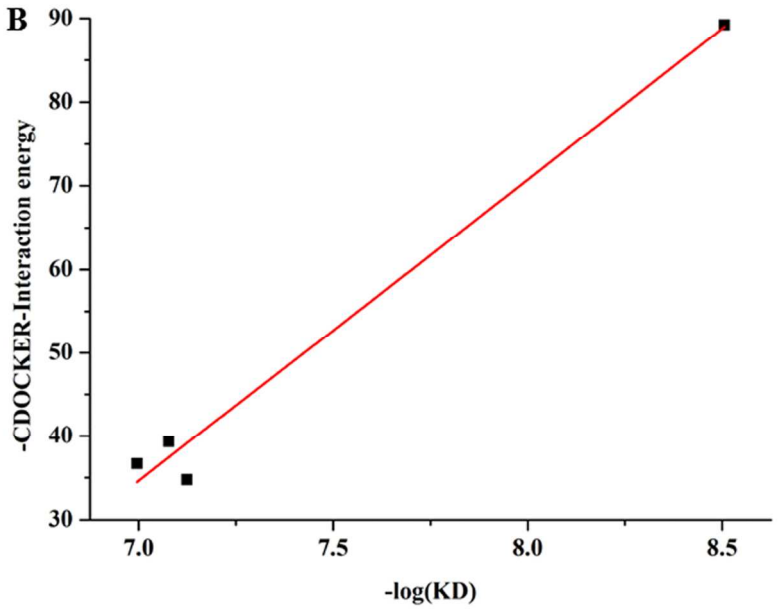

Figure 6B. Correlations between SPR binding affinities and the CDOCKER interaction energies.

129x90mm (300 x 300 DPI)