

Investigation on the interaction of pyrethroid pesticides to estrogen receptor alpha through computational and experimental methods

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ABSTRACT

Pyrethroid insecticides are a group of widely used bio-mimetic synthetic pesticides. However, recent studies reported that they could have an accumulation effect in human which may cause series of health problems. Estrogen receptors (ER) are a class of nuclear receptors that are vital in proper physiological behavior of estrogens. To investigate the reproductive toxicity of pyrethroids, homology modeling, molecular docking, molecular dynamic simulations (MDs) were conducted to explore the interaction between pyrethroids and ER α from atomic scale. The human ER α (2YJA) was selected as a template protein for homology modeling. Then eight typical pyrethroids and positive control estradiol were docked to the modeled protein. The highest scoring bifenthrin and the lowest scoring permethrin were chosen for in-depth analysis. MDs showed that the complex formed by permethrin with ER α had a lower RMSD value and binding free energies compared to bifenthrin. Based on these results from microscopic dimension, exposure experiments were implemented to validate the primary conclusions. VTG concentrations in male zebrafish's blood were significantly higher under permethrin exposure than bifenthrin, suggesting a stronger estrogenic activity and binding propensity. In this regard, the structural characteristics of molecules were analyzed, expecting to provide theoretical references for subsequent drug design and rational drug application.

1. Introduction

Pyrethroid insecticides are a kind of bio-mimetic synthetic pesticides synthesized based on natural pyrethrins. They are widely used in agricultural production because of their high efficiency, broad spectrum, low toxicity and easy degradation [1]. However, with the extensive use of pyrethroid pesticides, the population exposure risk level is also increasing gradually. Timothy [2] found that pyrethroids had an accumulation effect in nerve cells *in vitro*. There were also some studies revealed that pyrethroids could be detected in human breast milk [3] and urine [4]. These indicate that even if the pyrethroid residues detected in the environment are under maximum residue limit, they can accumulate in human body and eventually cause toxicity and side effects through various exposure approaches including dietary and non dietary ways, such as skin absorption system, respiratory system and digestive system [5]. As was shown in Ravula's research, although the dose was

far more lower than the residual level in the food, long-term exposure to pyrethroids would cause serious damage to the general physiological process and reproductive function [6]. Therefore, researches on the interaction of pyrethroid pesticides with important proteins in humans are significant for further understanding the toxicity of pyrethroid pesticides to humans and for scientific guidance on pesticide use.

In recent years, many studies have shown the reproductive toxicity of pyrethroids. Sun [7] reported that cyhalothrin, cypermethrin and deltamethrin exhibited some estrogenic activity at certain concentrations, while their metabolite 3-PAB had distinct anti-estrogenic activity. Ru [8] investigated the reproductive toxicity and estrogenic activity of pyrethroids by fish screening *in vivo* and found that metsulfenthrin, cyhalothrin, cypermethrin and deltamethrin had potential environmental estrogenic activity. Go [9] further demonstrated the estrogenic activity of pyrethroids on the basis of the phenomenon that pyrethroids could stimulate the proliferation of MCF-7 cells, implying its ability to

Abbreviations: ER, Estrogen receptors; MDs, Molecular dynamics simulations; MDM, Molecular docking method; GAFF, General Amber force field; PME, Particle Mesh Ewald; MM-PBSA, Molecular mechanics Poisson-Boltzmann surface area; Vtg, Vitellogenin; RMSD, Root Mean Square Deviation; RMSF, Root Mean Square Fluctuation.

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bond with the ER. As is broadly confirmed, women's reproductive health is closely related to estrogen, but research on the effects of pyrethroids on it is still limited. So it is necessary to conduct relevant studies.

Human nuclear receptor superfamily is one of the largest families of transcription factors with a total of 48 members [10] and they have been identified as potential.

pharmaceutical targets for treating several metabolic disorders [11]. Estrogen receptors (ER) are a group of nuclear receptors located in the nucleus of cells that mediate the genotypic effects of estrogen and are vital for the normal physiological effects of estrogen. In addition, studies have shown that ER is also involved in the regulation and maintenance of memory functions in the hippocampus of the central nervous system [12], the regulation of cardiovascular functions such as blood pressure and cardioprotection [13], the regulation of multifunctional haematopoietic stem cell differentiation [14], etc. It is evident that the ER plays an essential role in the metabolic activities of the human body and that the combination of pyrethroid pesticides with the ER will have an impact on the normal physiological activity of the ER.

As a model organism, zebrafish has been fully sequenced and its genome is highly homologous to the human genome, sharing similar neural and metabolic systems and many physiological traits with human. With these backgrounds mentioned above, we have used computational chemistry methods such as homology modeling, molecular docking and molecular dynamics simulations (MDs) to theoretically predict and analyse the interaction between pyrethroid pesticides and the zebrafish ER α . Then, on this basis we have carried out bio-assay experiments on zebrafish in order to further determine the binding of the molecules and the protein through the different VTG level of their blood, and to lay a foundation for further understanding of the estrogenic activity and the mechanism of action of pyrethroid pesticides.

2. Materials and methods

2.1. Homology modeling

Protein homology modeling is based on the concept of homologous proteins. Generally speaking, when the template protein is with over 60% homology to target protein, the 3D model we derived is accurate [15]. Once the model is generated, the accuracy of the model can be assessed through Ramachandran plots generated by PROCHECK, ERRAT, VERIFY-3D, etc. Since no crystal structure of zebrafish ER α has been reported in the PDB, the 3D structure of zebrafish ER α was obtained through homology modeling.

First, the protein sequence of zebrafish ER alpha (AAK16740.1) was retrieved from NCBI database (<https://www.ncbi.nlm.nih.gov/>). Second, it was submitted to BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find a proper template. The PDB database was used for identifying a segment of homologous protein with a high degree of homology to it. The total score, similarity and homology of the search results were all taken into consider. Finally, 2YJA was selected as the template protein with 62.06% homology and 44% sequence coverage with zebrafish ER α . Then, an automated mode of SWISS-MODEL (<https://www.swissmodel.expasy.org>) was performed for homology modeling the zebrafish ER α (Fig. S1). Lastly, the model was evaluated with SAVES. In addition, as estrogen receptors β has a similar structure to ER α [16], to further ensure the selectivity of the interaction between molecules and ER α , ER β was simultaneously homology modeled based on its template protein 1L2J for subsequent docking calculations.

2.2. Molecular docking

The Molecular Docking Method (MDM) is designed on the basis of the "lock-and-key" principle. It predicts the mechanism of receptor-ligand interaction through chemometric methods, which enables accurate prediction of protein receptor's binding sites and its binding modes [17].

According to the usage of pyrethroid pesticides, we selected permethrin, cypermethrin, methrin, cyfluthrin, fenpropathrin, ethofenprox, prallethrin and bifenthrin for study (Fig. S2). Estradiol was used as a positive control. Surfflex-Dock program of Sybyl-x 2.0 was used for docking to predict whether these pyrethroid pesticides were potential ligands of the zebrafish ER. Before docking, the modeled protein was pre-treated by removing water molecules and adding polar hydrogen atoms and charges. As there was no ligand in the modeled protein, automatic mode was chosen to generate the Protomol with empirical scoring functions. The volume of the Protomol and the degree of embedding in the receptor could be determined by two parameters, threshold and bloat, which were set to 0.45 and 0, while the other parameters were set to default values. Since the structure of the ligand is flexible, Surfflex-Dock can generate 100 docking conformations for each ligand and select the most likely bioactive conformation as the final conformation for further study. The ultimate Total Score is the criterion for assessing the reliability of docking. According to the SYBYL manual, when the docking score is greater than 4, there is an interaction between the receptor and the ligand. Prior to docking, each small molecule has been optimized in the MM94 force field. After the first round of docking, the highest and lowest scoring molecules were selected for further docking with ER β to exclude non-selective binding.

2.3. Molecular dynamics simulations

Molecular dynamics simulations (MDs) can provide high spatial resolution information and high temporal resolution structural dynamics information, which is crucial for grasping the microstructure of receptors and their kinetic information [18].

In this experiment, Amber 14 was used to investigate the stability of the complexes and the kinetic behaviour of the ligands through MDs. Based on the molecular docking results, the highest scoring bifenthrin and the lowest scoring permethrin were selected and their docking models with ER α were used as the initial structures for in-depth study.

The small molecule ligands were pre-processed using the ANTECHAMBER module. Energy minimization was performed with HF/6-31G* basis set in Gaussian 16 program [19]. The missing hydrogen atoms were filled in the Tleap program. The force field parameters for the protein were taken from the Amber ff03 force field [20] and that for the ligands were taken from the general Amber force field (GAFF) [21]. The initial model of the complex was placed in an truncated octahedral statistical system with a 10 Å layer of TIP3P water molecules added to mimic the solvent and Na $^+$ was added to neutralize the system. Then, the whole system was optimized using the SANDER program with a binding constant of 200 kcal·mol $^{-1}$ ·Å $^{-2}$ for 5000 steps of maximum descent [22]. Then 5000 steps of conjugate gradient optimization were carried out under unrestrained binding conditions to minimize the energy of the system. The system temperature was then gradually increased from 10 K to 310 K in 100 ps using Langevin kinetics. Fifty ns of molecular dynamics calculations were performed for the whole system at thermodynamic temperature (T = 310 K, 101 kPa) with an integration step of 2 fs. For each 500 fs, a molecular conformation was collected and saved to the results file. During the simulations, the SHAKE method was used to confine the hydrogen atoms and the Particle Mesh Ewald (PME) method was used to calculate the electrostatic energy [23].

2.4. Free energy calculations

Molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) is a method for calculating free energy based on samples collected from molecular dynamics trajectories that combines molecular mechanics with a continuum media model [24]. In this study, we selected 5000 frames of each ligand, receptor and complex conformation in the last 5 ns of the equilibrium phase and analyzed them with MMPBSA.py module of Amber 14. The calculation equations are as follows.

$$(1) \Delta G = \Delta G_{GAS} + \Delta G_{solv} - T\Delta S$$

$$(2) \Delta G_{GAS} = \Delta E_{INT} + \Delta E_{VDW} + \Delta E_{ELE}$$

$$(3) \Delta E_{INT} = \Delta E_{bonds} + \Delta E_{angles} + \Delta E_{dihedrals}$$

Among them, ΔG_{GAS} is the mechanical free energy of the gas molecules, which contains internal energy ΔE_{INT} , van der Waals interactions ΔE_{VDW} and electrostatic interactions ΔE_{ELE} . The internal energy ΔE_{INT} includes bond energy ΔE_{bonds} , bond angle energy ΔE_{angles} and dihedral angle energy $\Delta E_{dihedrals}$. ΔG_{solv} is the solvation free energy, which can be subdivided into polarization energy ΔG_{polar} and non-polarization energy ΔG_{NP} . $T\Delta S$ refers to the entropic change. Since the entropy change during translation, rotation and vibration had little effect on the binding free energy, it was neglected in this study.

Additionally, to further investigate the interaction between key amino acid residues and ligands, the decomposed energy value contributed by each key amino acid residue were calculated through MM-PBSA method. The last 5 ns of the equilibrium MD trajectory was also selected for the analysis.

2.5. Fish and chemical exposure

For environmental estrogenic pollutants, the typical action mechanism is to act as a ligand to bind to the ER and regulate the transcriptional process of the responding genes through estrogen response elements [25]. Studies have shown that the expression of ERs genes can be significantly upregulated in the liver in response to estrogen [26], thus ERs genes can serve as typical biomarkers for the detection of estrogenic activity in organisms. Besides this, vitellogenin (VTG), a yolk precursor protein synthesised in the liver, has also been used as a biomarker for the analysis of estrogen activity in animals such as fish. Adult male zebrafish can synthesize VTG after the induction of exogenous estrogen [27] and the transcription of mRNA for the VTG gene will also be significantly up-regulated [28]. As ER α may act as the principal subtype in regulating VTG, which can act directly on the expression of the VTG gene and they are expressed in a time-dose-dependent manner, we can monitor the binding of pyrethroid pesticides with ER α by measuring the amount of VTG [29]. There are seven forms of VTG genes in the zebrafish genome, known as VTG 1 to VTG 7, of which VTG 1 is the most expressed and accounts for the absolute majority of its content. Therefore, VTG 1 is an important gene for the study of estrogenic effects [30].

2.5.1. Experimental materials

Permethrin (Purity > 95%, CAS: 52645-53-1, purchased from Hubei Yongkua Technology Ltd.); Bifenthrin (Purity > 95%, CAS: 82657-04-3, offered by Bioassay Office, College of Science, China Agricultural University); Zebrafish VTG ELISA kit (purchased from Shanghai Enzyme Link Biotechnology Ltd.); AB-type male zebrafish (purchased from Gaofeng Aquarium, Beijing). The experimental stock solution was prepared with acetone at a concentration of 10 mg/L and stored at 4 °C away from light for backup.

2.5.2. Pesticide exposure experiments

Here the model organism zebrafish were chosen for further investigation of the interaction of pyrethroid pesticides with the ER α .

The zebrafish were domesticated for 2 weeks before the experiment to eliminate the effects of endogenous steroids produced by the male zebrafish themselves. Male zebrafish (AB-type) were kept in 5 L beakers (with 15 L of continuously aerated dechlorinated tap water at 25 ± 1 °C, pH 7.4 ± 0.3 and dissolved oxygen 6.5 ± 0.2 mg/L) for a fortnight under a 14:10 h light-dark cycle and fed twice daily with a mixed diet. Their natural mortality should be less than 5%.

After the acclimation period, 4 L of test solution at concentrations of 0, 100, 300 and 900 ng/L were prepared per day using continuous aerated dechlorinated tap water. Male zebrafish were exposed to different concentrations of permethrin and bifenthrin test solutions and

three replicate experiments were set up for each concentration. Ensure that the concentration of acetone was less than 0.0025% (v/v). Each tank contained 10 male zebrafish. Culture for 15 days with the same photoperiod and temperature conditions as the acclimation period. Test solutions were changed daily to ensure accurate exposure concentrations each day. Feed 0.5 g of dry bait daily. At the end of the exposure period, we collected blood for further study.

2.5.3. Measurement of plasma VTG protein

After exposure experiments, blood was drawn from zebrafish tails into 1.5 mL centrifuge tubes. The blood sample was then centrifuged at 3000 rpm for 10 min and the upper plasma layer was stored at -20 °C for testing. Using the zebrafish VTG ELISA kit (Shanghai EnzymeLink Biotechnology Ltd), VTG levels in zebrafish were measured according to the instructions. Each sample contained blood samples from 10 zebrafish, and three parallel sets were set up for each concentration. The purified zebrafish VTG (30, 60, 120, 240, 480 µg/L) provided in the kit was utilized as a standard and the concentration of VTG in the plasma samples could be calculated from the linear portion of the zebrafish VTG standard curve. The results of the VTG concentration provided a direct indication of the magnitude of the effect of the pesticide molecule on the ER α .

3. Results and discussion

3.1. Homology modeling

We chose human ER α (2YJA) as the template protein for homology modeling, whose homology and similarity to zebrafish ER α were 62.06% and 44% respectively. After using the automated mode of SWISS-MODEL to derive the model protein, this resulting model was then evaluated by SAVES. PROCHECK was carried out based on the PDB high-resolution crystal structure parameters, which in turn gave a series of stereochemical parameters for the submitted model. The Ramachandran plots generated can be used to examine the distribution of Φ -dihedral angles in the zebrafish ER α backbone conformation so as to identify the structural plausibility of the protein. As shown in the Fig. S3A, 95.5% of the amino acids of the model protein are located in the core region, 4.1% in the allowed region, 0.5% in the maximum allowed region and no amino acids in the forbidden region. In general, when more than 90% of the amino acids are located in the core region, the modeled protein can be considered as a high quality model, i.e. the angles formed between the residues of the modeled protein are reasonable. ERRAT is an indicator to evaluate the reasonableness of the protein geometry by finding the non-bonding interactions between different pairs of atom types within 0.35 nm. Generally when the ERRAT score exceeds 50, the protein structure is reasonable. The zebrafish ER α model we built had an ERRAT-score of 98.2379, well above the threshold. VERIFY-3D compares the model tertiary structure with the amino acid primary structure and is used to assess the consistency of the two. When the number of residues with a 3D/1D value of 0.2 is greater than 80%, the protein model passes. The results showed that a total of 82.99% of amino acid residues scored more than 0.2 in VERIFY-3D (Fig. S4A). After the evaluation of Ramachandran plot, ERRAT and VERIFY-3D, it could be proved that the structure of zebrafish ER α obtained by homology modeling was reasonable and reliable and could be used for the subsequent analysis.

Moreover, zebrafish ER β has 62.06% sequence homology and 44% coverage to its template (1L2J). Its model's ERRAT-score was 98.96. The Ramachandran plot was shown in Fig. S3B. The results of VERIFY-3D were shown in Fig. S4B, where amino acid residues with a score greater than 0.2 accounted for 85.12%. From these results it can be seen that the structure of the modeled ER β was also credible.

3.2. Molecular docking

On the basis of the proteins obtained in the previous homology modeling step, we generated the active pockets through Automatic Mode of Sybyl-x 2.0. Several possible pocket sites were then derived through simulations with POCASA 1.1 and a ranking evaluation was given (Fig. S5). The results were visualised in the Pymol software as shown (Fig. S6). It could be seen that the highest ranked pocket in POCASA was cavity 126, consistent with the actual docked active pocket with the typical deep, narrow characteristics, whereas the other pockets were significantly shallower or less spacious. The Surfflex-Dock program was then run to flexibly dock a series of optimized pyrethroid pesticides to the modeled zebrafish ER α . The scoring results are shown in Table 1, and the docking pattern is plotted in Fig. S7. The scoring module uses four empirical scoring functions to evaluate the binding of the receptor to the ligand from different perspectives such as polar interaction, hydrophobic entropy and solvation. A total score is obtained by aggregating each function, which indicates the affinity of the ligand to the receptor. Typically, a Total score greater than 4 is regarded as a valid docking of the ligand to the receptor. Crash indicates the extent to which the ligand penetrates the protein preferably close to 0. Polar indicates the ability of an atom to form a hydrogen bond where a positive value means a strong hydrogen bonding ability and vice versa. The positive compound estradiol received the highest score, which was consistent with the objective fact, indicating that the docking result was relatively reliable. Based on these docking scores, it could be seen that the docking scores of fenpropathrin, bifenthrin, permethrin and prallethrin with the ER α were greater than 4, suggesting a valid docking, i.e. they were able to interact with the zebrafish ER α as ligands, theoretically demonstrating that these pyrethroid pesticides were potential environmental estrogens. At the same time, bifenthrin and permethrin both had a lower docking score with ER β (see Table S1.), indicating a selective interaction with ER α . As seen in the docking pattern diagram, for pesticide molecules with different structures, the conformation of their docking into the pocket also differed, thus affecting the degree of binding, which further exhibited the influence of compound structure on properties.

To further explore the effect of the binding pattern of the receptor and ligand on the docking scoring results, we selected the highest scoring bifenthrin and the lowest scoring permethrin for analysis. The docking pattern of bifenthrin to the zebrafish ER can be seen in the Fig. 1A, where the entire ligand molecule was deeply bound in the receptor protein in a U-shaped conformation. The carbonyl oxygen on the ester group of the ligand formed a hydrogen bond with the NH on the imidazole group of HIS-217 at a distance of 2.2 Å. In addition, the benzene ring portion of the ligand formed a T-shaped π - π stacking with the benzene ring of PHE-97. The rest of the structure of the ligand was largely surrounded by hydrophobic residues such as MET, ILE, LEU, PHE, ALA, TRP with a few polar residues like GLY, THR, GLU. The ligand was anchored in the protein pocket under hydrophobic interactions. From the Fig. 1B, the docking pattern of permethrin can be observed. The permethrin molecule also formed a T-shaped π - π stacking with the benzene ring of PHE-97. Its dichloromethyl portion might have a polar

interaction with surrounding THR-40 and GLY-214, which might have led to a shift in the molecular backbone, making it impossible for the carbonyl oxygen to form hydrogen bonds with the NH of HIS-217 anymore. The entire molecule did not form any hydrogen bonds, which is consistent with the Polar values resulting from molecular docking.

The superimposition of the conformations of the two molecules (Fig. 1C) suggested that bifenthrin and permethrin were bent in opposite directions in the docking pocket, which resulted in a difference in the type of protein residues interacting with each molecule that led to a significant difference in the mode of action. According to the docking results, hydrogen bonding interactions probably played a major role in the binding process and influenced the scoring results to a large extent. Specifically, the main reason for the difference in docking orientation might be related to the type of polar group and the form of benzene ring present in the molecule. According to the above analysis, the differences in the polar groups were responsible for the different electrostatic interactions with the surrounding residues, which indirectly influenced the interaction of the other groups (carbonyl groups) with the residues, ultimately leading to significant differences in the overall magnitude of the effect. This means that when designing novel drugs in the future, we need to take into account not only the interaction of key functional groups with residues, but also the impact of other structures, such as polar groups, on the positioning of the entire drug molecule in the active pocket.

3.3. Molecular dynamics simulations

To further verify the reliability of the molecular docking results and the stability of the complexes, we conducted MDs for 50 ns on the complexes of bifenthrin and permethrin with zebrafish ER and got the Root Mean Square Deviation (RMSD) of the main chain atoms and the Root Mean Square Fluctuation (RMSF) of the amino acid residues of the two complex systems. It can be seen from Fig. 2 that the RMSD values of the permethrin complex gradually converge to equilibrium after 20 ns, which means that the system has reached a balanced state. In contrast, the RMSD of the bifenthrin complex remained in a fluctuating situation throughout the simulation, which implies that the stability of the complex is not satisfactory. It can also be seen that the RMSD of the permethrin complex was lower than that of the bifenthrin complex for the majority of the simulation, suggesting a greater stability of the permethrin complex. Although the results of the MDs contradict with the scores of molecular docking, it actually illustrates the ligand-receptor interaction from another perspective. Molecular docking focuses on discussing parameters such as the type of forces and binding sites during static binding between ligand and receptor, whereas MDs focus more on the dynamics of the whole system, emphasizing energy minimization during the movement rather than at a specific moment in time. Though the conformational stability of the bifenthrin complex is higher than that of permethrin under the set parameters, the energy of the system may exhibit larger fluctuations as the conformation of the protein and small molecule changes during the movement, resulting in a lower stability of the overall dynamics compared to permethrin. The actual combination situation has to be proved by subsequent exposure experiments.

In order to understand the flexibility of the molecular docking complexes and the partial motion characteristics of their structures in more detail, we calculated the RMSF values of the amino acid residues of the receptor during MDs. The higher the value, the more flexible the complex is during the binding process. As shown in Fig. 3, the RMSF values of the different amino acid residues are roughly the same for both complex systems due to the same active pockets docked by the small molecules. Both systems showed a larger RMSD fluctuation around residues 25, 160 and 230, which might be associated with the stability of complex formation, while the valleys were basically around residues 50, 80, 140 and 180, implying that they might belong to conservative regions. Further comparison revealed that the RMSF of the bifenthrin

Table 1
Docking results of pyrethroid pesticides with zebrafish estrogen receptor α .

NAME	molecular docking		
	Total score	Crash	Polar
Fenpropathrin	6.3044	-5.3086	0.0107
Cypermethrin	-0.0552	-11.2982	0.0104
Methrin	0.5483	-11.1207	0.0069
Bifenthrin	6.5208	-6.7326	0.6316
Cyfluthrin	3.1854	-8.3436	0.0000
Permethrin	4.5563	-6.2990	0.0046
Ethofenprox	3.1651	-7.1104	0.4030
Prallethrin	6.1873	-3.3417	0.0000
Estradiol	6.9175	-2.7538	2.4481

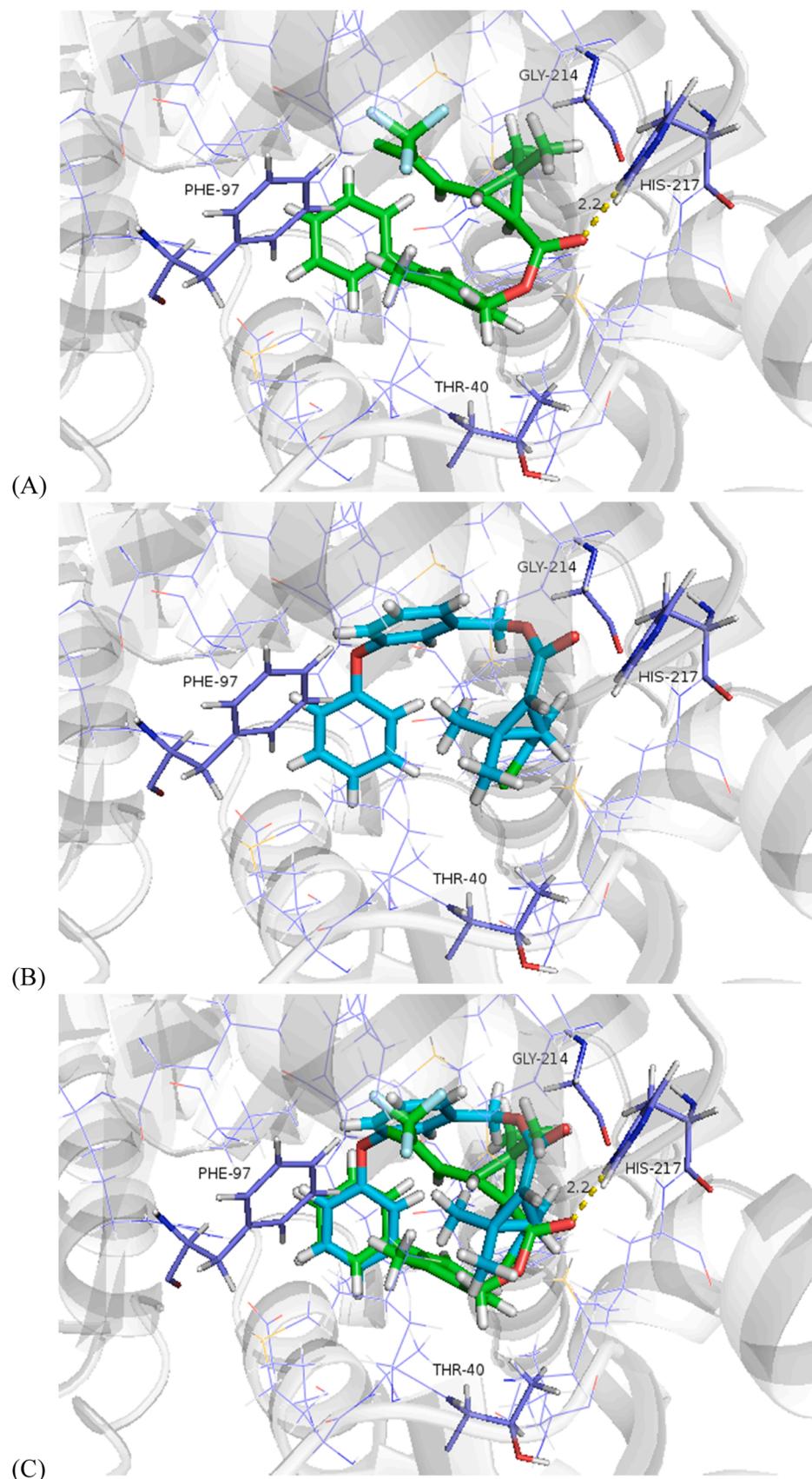


Fig. 1. (A-B) Docking pattern of bifenthrin and permethrin with the zebrafish estrogen receptor α . (C) Overlay of the docking pattern of the two. (Green stick - bifenthrin. Blue stick - permethrin. Residues within 5 Å of the ligand were showed as lines in lightblue.).

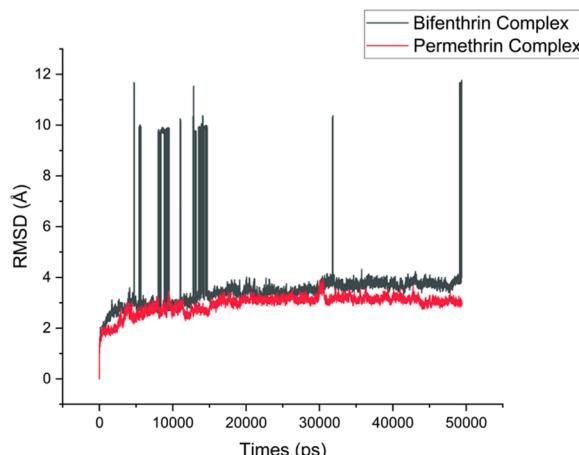


Fig. 2. Variation of the RMSD of the main chain atoms in bifenthrin complex and permethrin complex.

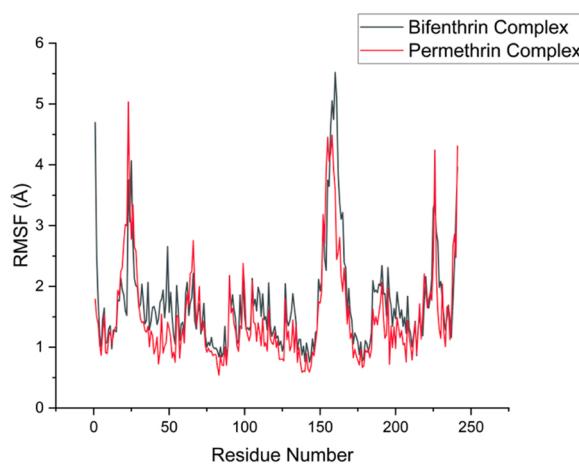


Fig. 3. The RMSF of amino acid residues in bifenthrin complex and permethrin complex.

complex system was generally higher than that of the permethrin complex overall, and it displayed a significantly higher RMSF near residue 50, indicating that most of the amino acid residues of the bifenthrin complex system were at a higher degree of freedom and were less stable throughout the kinetic process.

In addition, the stabilized conformations of the complexes of bifenthrin and permethrin were extracted separately after 50 ns MDs and were analyzed in superposition with the respective initial complex conformations. As shown in Fig. 4A, bifenthrin experienced a nearly 180° conformational inversion in the active pocket, resulting in the disruption of its hydrogen bonding interaction with HIS-217 and its $\pi-\pi$ stacking effect on PHE-97. Furthermore, the entire protein backbone also underwent a large shift. All of these implied that the stability of the bifenthrin complex was damaged to a rather high extent. In contrast, as shown in Fig. 4B, permethrin only experienced a flip of approximately 90°, and after 50 ns of simulation, the surrounding key amino acids were still basically within the range of 5 Å, allowing the whole system to remain generally stable. The above analyzes also reinforced the results of the MD calculations.

3.4. Binding free energy calculations

For better understanding of the mechanism of action between the ligand and the receptor, we calculated the binding free energy and the energy components of bifenthrin and permethrin with the zebrafish ER

based on the Amber 14 and MM-PBSA methods and selected the last 5 ns trajectories of the equilibrium phase for analysis as shown in Table 2. Generally speaking, the smaller the value of the binding free energy, the higher the affinity of the ligand and the more stable the binding to the receptor. It could be seen that the binding free energy of bifenthrin to the receptor was -35.66 kcal/mol compared to -38.61 kcal/mol for permethrin, indicating that the binding of permethrin was much more stable, which was consistent with the RMSD results. This result was probably related to the greater number of polar amino acid residues around the permethrin which generated a stronger van der Waals interaction. Though the trifluoromethyl group of bifenthrin, as a polar group, might also generate electrostatic forces with the surrounding polar amino acids, this interaction obviously did not contribute as much as the van der Waals interaction to the overall binding free energy, which was also evident from the energy components. In both systems, van der Waals forces served as the main driving force for ligand binding, electrostatic interaction and non-polar solvation also made some contribution to the stability of binding, while polar solvent solvation was the main inhibitory force for binding.

3.5. Decomposition free energy calculations

In this study, we used the MM-PBSA method to calculate the energy contribution of individual amino acid residues in the 5 Å range of the ligand using Amber 14, in order to investigate the energy contribution of each key amino acid residue in the binding process in depth. The results are presented in Table S2-S3 and Fig. 5. For the permethrin complex, the amino acids with high binding free energy contributions were LEU-39, LEU-77, MET-81, LEU-218, PHE-97, LEU-80, ILE-117, TRP-76, GLY-214 and ALA-43, all of which appeared to correspond to the valleys of the RMSF image, i.e. they were in the conserved regions of the protein, consistent with the previous analysis. Consistent with the previous analysis. As shown from the energy components, the forces between the amino acid residues and the ligand were essentially in the form of van der Waals forces and followed by electrostatic interactions.

3.6. Exposure experiments

To determine the binding of pesticides to ER α more visually and to further assess the estrogenic effects of different pyrethroid pesticides, we exposed male zebrafish to pesticides for 15 days at test solution concentrations of 0, 100, 300 and 900 ng/L of bifenthrin or permethrin respectively. The final results of their blood VTG concentrations measured are shown in Fig. 6. It could be found that both pesticides showed a certain amount of estrogenic activity, causing a significant increase in the blood VTG concentration, and the estrogenic activity increased continuously with the increasing concentration of the exposed pesticide within the experimental range. The higher estrogeno-mimetic activity shown by permethrin under the same concentration conditions might also indicate a better binding of permethrin to the ER, which was in accordance with the results of MDs.

Apart from this, we also found that the higher the exposed pesticide concentration, the greater the motility of the male zebrafish, whose liveliness was significantly stronger than that of the blank control. However, the exact mechanism of this is unclear.

4. Conclusion

In this research, we investigated the interactions of representative pyrethroid pesticides with the zebrafish ER by means of homology modeling, molecular docking, MDs, binding and decomposition free energy calculations, and exposure experiments. Through computational simulations, we found that there is indeed a non-negligible interaction between pyrethroid pesticides and the zebrafish estrogen receptor at an atomic level. In order to investigate the interaction in more detail, we selected the molecules with the highest and lowest molecular docking

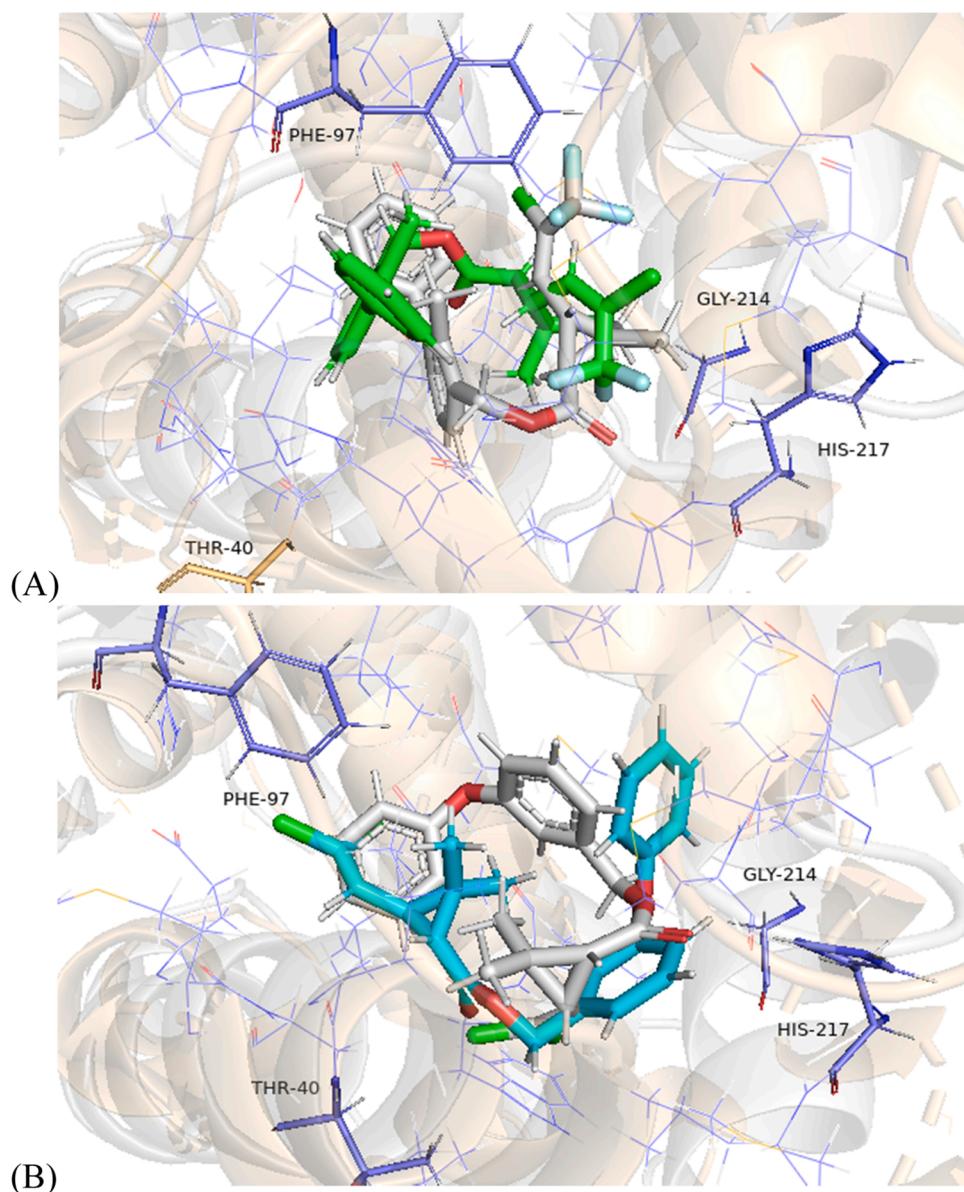


Fig. 4. Overlay of the docking pattern of the primary complex conformation and the final stabilized complex conformation of bifenthrin (A) and permethrin (B). (Green stick - bifenthrin in final conformation. Blue stick - permethrin in final conformation. White stick - corresponding molecule in primary conformation. Lightorange cartoon - ER α in final conformation. White cartoon - ER α in primary conformation. Residues within 5 Å of the ligand were showed as lines in lightblue.).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

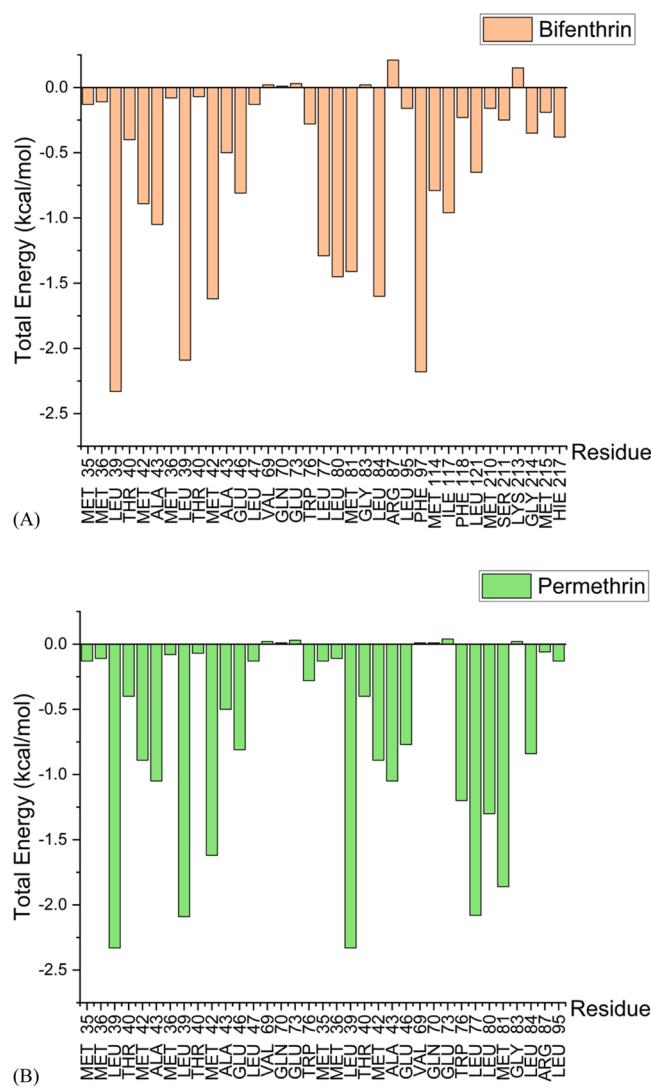
Table 2
Binding free energy and energy components (kcal/mol).

Energy	Bifenthrin	Permethrin
ELE	-2.26	-1.36
VDW	-48.50	-50.06
INT	-0.00	0.00
GAS	-50.76	-51.42
SUR	-6.62	-6.62
CAL	21.72	19.42
SOL	15.10	12.81
TOT	-35.66	-38.61

Note: ELE is electrostatic energy; VDW is van der Waals energy; INT includes bond, bond angle and dihedral angle energied; GAS is the sum of these three energies; SUR is non-polar solvation energy; CAL is polar solvation energy; SOL is the sum of SUR and CAL, including both of the solvation energies; TOT is total binding free energy. All of the results were carried out through MM-PBSA methods.

scores, i. e. bifenthrin and permethrin, for further study. By analyzing the conformation of the docking complexes, we concluded that the magnitude of the interaction between molecules and proteins is mainly

related to the type of functional groups and framework structure of the molecules. Subsequently, MDs based on the Amber14 program were carried out and it's showed that the binding of permethrin to the ER was more stable. This result, although different from the docking result, may indicate that the dynamic stability of the bifenthrin complex is inferior to that of permethrin, meaning that although the binding energy of bifenthrin is lower in a given case, the fluctuation of its system energy may be greater throughout its movement as the protein, small molecule conformation changes, leading to a decrease in its overall dynamic stability. Furthermore, calculations of the free energy using the MM-PBSA module also suggested that the binding stability of the permethrin was better. Moreover, it was evident from the energy breakdown that van der Waals forces were the main driving force for ligand binding in both systems, while polar solvent solvation was the main inhibiting force for binding. To further examine and determine the binding of the two complex systems, zebrafish exposure experiments were conducted on the basis of the above. The results showed that both pesticides had some estrogenic activity and that this activity was positively correlated with the pesticide concentration. The activity of permethrin was higher than that of bifenthrin, that is, the reproductive toxicity of permethrin is higher than that of bifenthrin, and if it



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