

Research Article

Effects of Fungicides on Rat's Neurosteroid Synthetic Enzymes

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Exposure to environmental endocrine disruptors may interfere with nervous system's activity. Fungicides such as tebuconazole, triadimefon, and vinclozolin have antifungal activities and are used to prevent fungal infections in agricultural plants. In the present study, we studied effects of tebuconazole, triadimefon, and vinclozolin on rat's neurosteroidogenic 5 α -reductase 1 (5 α -Red1), 3 α -hydroxysteroid dehydrogenase (3 α -HSD), and retinol dehydrogenase 2 (RDH2). Rat's 5 α -Red1, 3 α -HSD, and RDH2 were cloned and expressed in COS-1 cells, and effects of these fungicides on them were measured. Tebuconazole and triadimefon competitively inhibited 5 α -Red1, with IC₅₀ values of $8.670 \pm 0.771 \times 10^{-6}$ M and $17.390 \pm 0.079 \times 10^{-6}$ M, respectively, while vinclozolin did not inhibit the enzyme at 100×10^{-6} M. Triadimefon competitively inhibited 3 α -HSD, with IC₅₀ value of $26.493 \pm 0.076 \times 10^{-6}$ M. Tebuconazole and vinclozolin weakly inhibited 3 α -HSD, with IC₅₀ values about 100×10^{-6} M, while vinclozolin did not inhibit the enzyme even at 100×10^{-6} M. Tebuconazole and triadimefon weakly inhibited RDH2 with IC₅₀ values over 100×10^{-6} M and vinclozolin did not inhibit this enzyme at 100×10^{-6} M. Docking study showed that tebuconazole, triadimefon, and vinclozolin bound to the steroid-binding pocket of 3 α -HSD. In conclusion, triadimefon potently inhibited rat's neurosteroidogenic enzymes, 5 α -Red1 and 3 α -HSD.

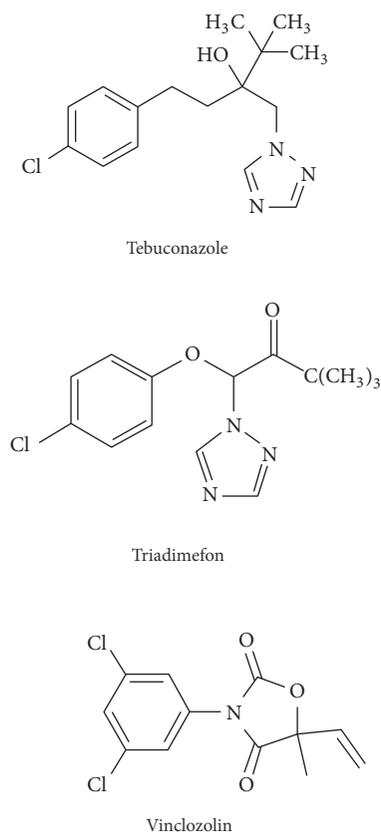
1. Introduction

Exposure to environmental endocrine disruptors may interfere with nervous system's activity. Fungicides such as tebuconazole (TEB) [1], triadimefon (TRI) [2], and vinclozolin (VCZ) [3] have a wide range of antifungal activities and are used to prevent fungal infections for agricultural plants. Therefore, exposure to these chemicals is very common. These fungicides contain at least one triazole or imidazole moiety in the chemical structure (Scheme 1). It is believed that these fungicides block the synthesis of fungus steroid, ergosterol. Ergosterol is a membrane component, and, therefore, these chemicals can disrupt cell membrane assembly of fungi to kill the fungi [4].

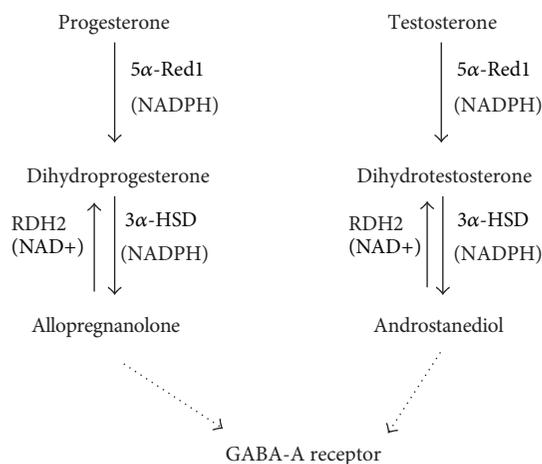
These fungicides may interfere with the steroid biosynthesis in mammals. For example, azole fungicides reduce the estrogen production via blocking aromatase [5, 6].

Neurosteroids are another set of steroids which have neurological activity [7]. These neurosteroids include allopregnanolone (ALLO) and 5 α -androstane-3 α , 17 β -diol (DIOL) [7]. Although the classic steroids such as progesterone, estrogen, and testosterone act via binding to their respective nuclear receptors (progesterone, estrogen, and androgen receptors), neurosteroids allosterically activate the membrane GABA-A receptors and potentiate the central inhibition, causing anxiolytic, anticonvulsant, analgesic, and sedative effects [7, 8]. GABA-A receptors are widely present in the nervous system to exert inhibitory action on nerve activity [7].

ALLO and DIOL biosynthesis requires brain 5 α -reductase 1 (5 α -Red1) and 3 α -hydroxysteroid dehydrogenase (3 α -HSD). 5 α -Red1 is a smooth endoplasmic reticulum NADPH-dependent enzyme [9], catalyzing progesterone or testosterone into dihydroprogesterone and dihydrotestosterone,



SCHEME 1: The chemical structure of fungicides.



SCHEME 2: The biosynthesis and metabolism of neurosteroids, allopregnanolone and androstenediol, by three distinct enzymes: NADPH-dependent 5 α -reductase 1 (SRD5A1), NADPH-dependent cytosolic 3 α -hydroxysteroid dehydrogenase (AKRIC14), and NAD⁺-dependent microsomal retinol dehydrogenase 2 (RDH2).

respectively [10] (Scheme 2). 3 α -HSD, a cytosolic enzyme, catalyzes these two steroids into ALLO or DIOL, respectively [11]. In rat's brain, microsomal NAD⁺-dependent retinol dehydrogenase 2 (RDH2) catalyzes the reverse reaction of ALLO or DIOL back to dihydroprogesterone and dihydrotestosterone, thus controlling the levels of these neurosteroids [12] (Scheme 2). Therefore, in the present study, we

examined their direct effects on these neurosteroidogenic enzymes and their differential sensitivity.

2. Experimental Procedures

2.1. Chemicals. [³H]Testosterone, [³H] dihydrotestosterone, and [³H] DIOL were obtained from DuPont-New England Nuclear (Boston, MA). Testosterone, dihydrotestosterone, and DIOL were purchased from Steraloids (Newport, RI). TEB, TRI, and VCZ were purchased from Sigma-Aldrich (St. Louis, MO). TEB, TRI, and VCZ were dissolved in DMSO, which is used as a vehicle. Rat 3 α -HSD gene *Akr1c14* in the expression vector pRc/CMV was a gift from Penning T. M. (University of Pennsylvania, Philadelphia, Pennsylvania). Rat's 5 α -Red1 gene *Srd5a1* and RDH2 gene *Rdh2* in the expression vector pcDNA1.1 were constructed previously [13]. COS-1 cell line was purchased from ATCC (Manassas, VA).

2.2. Transient Transfection. COS-1 cells were maintained in DMEM medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum and 5% CO₂ at 37°C. For transfection, 1 × 10⁶ cells were seeded per well in a six-well plate and cultured for 24 h in media supplemented with charcoal-stripped fetal calf serum to obtain 50–80% confluence. Transfection was performed by the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. 1 μ g DNA per well showed maximal efficiency and, therefore, this quantity was used in the transfection assays.

2.3. Preparation of 5 α -Red1, 3 α -HSD, and RDH2 Proteins. Twenty-four hours after transfection, the COS-1 cells were scraped from dishes and were homogenized in 10 ml 0.01 mM phosphate-buffered saline containing (0.25 M) sucrose and nuclei and large cell debris were removed by centrifugation at 1500 ×g for 10 min. Microsomal and cytosolic fractions were harvested after subsequent centrifugation at 10,000 ×g for 1 h and at 105,000 ×g for 1 h twice. The protein concentrations in cell lysates and subcellular fractions were measured using a kit (number 500-0006, Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard. The concentrations of rat's 5 α -Red1, 3 α -HSD, and RDH2 proteins were 20 mg/ml. The proteins were used for the measurement of 5 α -Red1, 3 α -HSD, and RDH2 activities.

2.4. Measurement of 5 α -Red1, 3 α -HSD, and RDH2 Activities. 5 α -Red1 activity was measured by incubating 1000 nM testosterone spiked with 60,000 dpm of [³H] testosterone as the substrate, 10 μ g SRD5A1-containing microsomal protein, and 0.2 mM NADPH in 250 μ l PBS (pH = 7.2). 3 α -HSD activity was measured by incubating 1000 nM dihydrotestosterone spiked with 60,000 dpm of [³H]-dihydrotestosterone as the substrate, 10 μ g 3 α -HSD-containing cytosolic protein, and 0.2 mM NADPH in 250 μ l PBS (pH = 7.2). RDH2 activity was measured by incubating 1000 nM DIOL spiked with 630,000 dpm of [³H] DIOL as the substrate, 10 μ g RDH2-containing microsomal protein, and 0.2 mM NAD⁺ in 250 μ l PBS (pH = 7.2). 100 μ M fungicides were incubated in the

TABLE 1: The enzyme kinetic parameters and the half maximal inhibitory concentration (IC₅₀) of fungicides.

Parameters	5 α -Red1	3 α -HSD	RDH2
Apparent K_m (μ M)	1.397 \pm 0.35	3.148 \pm 0.197	2.850 \pm 0.037
Apparent V_{max} (pmol/mg·min)	3.494 \pm 0.287	66.69 \pm 1.589	529.5 \pm 2.612
IC ₅₀ (μ M)			
Tebuconazole	8.670 \pm 0.771	~100	>100
Triadimefon	17.39 \pm 0.079	26.493 \pm 0.076	>100
Vinclozolin	NI	~100	NI

Mean \pm SE, $n = 4$. NI: no inhibition at 100 μ M.

respective reaction mixture at 37°C for 60 min for the initial inhibition test. The inhibitory potency of fungicides was measured relative to the control (only DMSO). Each fungicide was dissolved in DMSO and an aliquot (1 μ l) of each fungicide was added to the reaction mixture at a final concentration of 0.4%, at which concentration DMSO did not inhibit 5 α -Red1, 3 α -HSD, or RDH2 activities. The reaction was stopped with 1 ml ice-cold ether. The steroids were extracted with ether after vigorous vortexing. The organic ether layer was transferred to the new glass tube and dried under nitrogen. The steroids were separated chromatographically on the thin layer plate in chloroform and methanol (90:3, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC) as previously described [14]. The percentage conversion of testosterone into dihydrotestosterone (for 5 α -Red1), dihydrotestosterone into DIOL (for 3 α -HSD), and DIOL into dihydrotestosterone (for RDH2) was calculated by dividing the radioactive counts identified as the respective steroids by the total counts of control DMSO.

2.5. Determination of Enzyme Kinetics. The enzyme kinetics was determined by adding 0.0315–10 μ M testosterone or dihydrotestosterone or DIOL for 5 α -Red1, 3 α -HSD, and RDH2. The Michaelis–Menten equation was used by GraphPad (Version 6, GraphPad Software Inc., San Diego, CA) to calculate the apparent Michaelis–Menten constant (K_m) and the apparent maximum velocity (V_{max}). The initial velocity (V_o) depends on the apparent K_m , V_{max} , and the substrate concentration ([S]) as $V_o = V_{max} [S] / (K_m + [S])$.

2.6. Determination of IC₅₀ Values and Inhibitory Modes. The half maximum inhibitory concentration (IC₅₀) of TEB or TRI to inhibit 5 α -Red1 was determined by adding 1000 nM of testosterone with 0.2 mM NADPH and 10⁻⁸–10⁻⁴ M TEB or TRI in 250 μ l phosphate-buffered saline (0.1 mM) containing 5 α -Red1 protein and incubating each reaction mixture for 60 min. The IC₅₀ value of TRI to inhibit 3 α -HSD was determined by adding 1000 nM of dihydrotestosterone with 0.2 mM NADPH and 10⁻⁸–10⁻⁴ M TRI in 250 μ l phosphate-buffered saline (0.1 mM) containing 3 α -HSD protein and incubating each reaction mixture for 60 min. For determining the mode of inhibition of 5 α -Red1, 10⁻⁹–10⁻⁵ M testosterone was added to the reaction mixture in the presence of TEB (10 and 20 μ M) or TRI (20 and 40 μ M) for 5 α -Red1. For determining the mode of inhibition of 3 α -HSD, 10⁻⁹–10⁻⁵ M

dihydrotestosterone was added to the reaction mixture in the presence of TRI (25 and 50 μ M) for 3 α -HSD.

2.7. Preparation of Protein and Ligand Structures and Docking. The crystal structure of rat's 3 α -HSD containing NADP⁺ and testosterone (PDB id lafs [15]) was used as a docking target for steroid substrate DIOL, TEB, TRI, and VCZ. These chemical structures were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) as ligands. Docking calculations were performed with SwissDock, a docking algorithm based on the docking software EADock DSS [16]. The docked file was visualized using the program Chimera 1.1.1 (San Francisco, CA) and the free energy was calculated.

2.8. Statistics. Each experiment was repeated four times. Data were subjected to a nonlinear regression analysis by GraphPad (Version 6, GraphPad Software Inc., San Diego, CA) for IC₅₀ values. Lineweaver–Burk plot was used for the mode of inhibition. Data were subjected to an analysis by ANOVA followed by ad hoc Tukey's comparison to identify significant differences between the control (CON) and TEB, TRI, or VCZ group. All data are expressed as means \pm SEM. The difference was regarded as significant at $P < 0.05$.

3. Results

3.1. Effects of Fungicides on 5 α -Red1. The conversion of testosterone into DHT is catalyzed by 5 α -Red1, which requires NADPH as a cofactor; the apparent K_m and apparent V_{max} of 5 α -Red1 were 1.397 \pm 0.35 μ M (mean \pm SE, $n = 4$) and 3.494 \pm 0.287 pmol dihydrotestosterone/mg protein/min (mean \pm SE, $n = 4$), respectively (Table 1 and Figure 1(a)). As presented in Figure 1(b), when the highest concentration (100 μ M) was tested, TEB and TRI inhibited rat's 5 α -Red1 to 26.94 \pm 5.30% and 19.31 \pm 3.6% of the control value, respectively, but VCZ only to 74.17 \pm 5.57% of the control value. We further calculated the IC₅₀ values of TEB (Figure 1(c)) and TRI (Figure 1(d)), which were 8.670 \pm 0.771 and 17.390 \pm 0.079 μ M, respectively (Table 1). The modes of inhibition of TEB and TRI on 5 α -Red1 were found to be competitive against testosterone (Figures 2(a) and 2(b)).

3.2. Effects of Fungicides on 3 α -HSD Activity. The conversion of dihydrotestosterone into DIOL is catalyzed by 3 α -HSD, which requires NADPH as a cofactor; the apparent K_m and apparent V_{max} of 3 α -HSD were 3.148 \pm 0.197 μ M (mean \pm

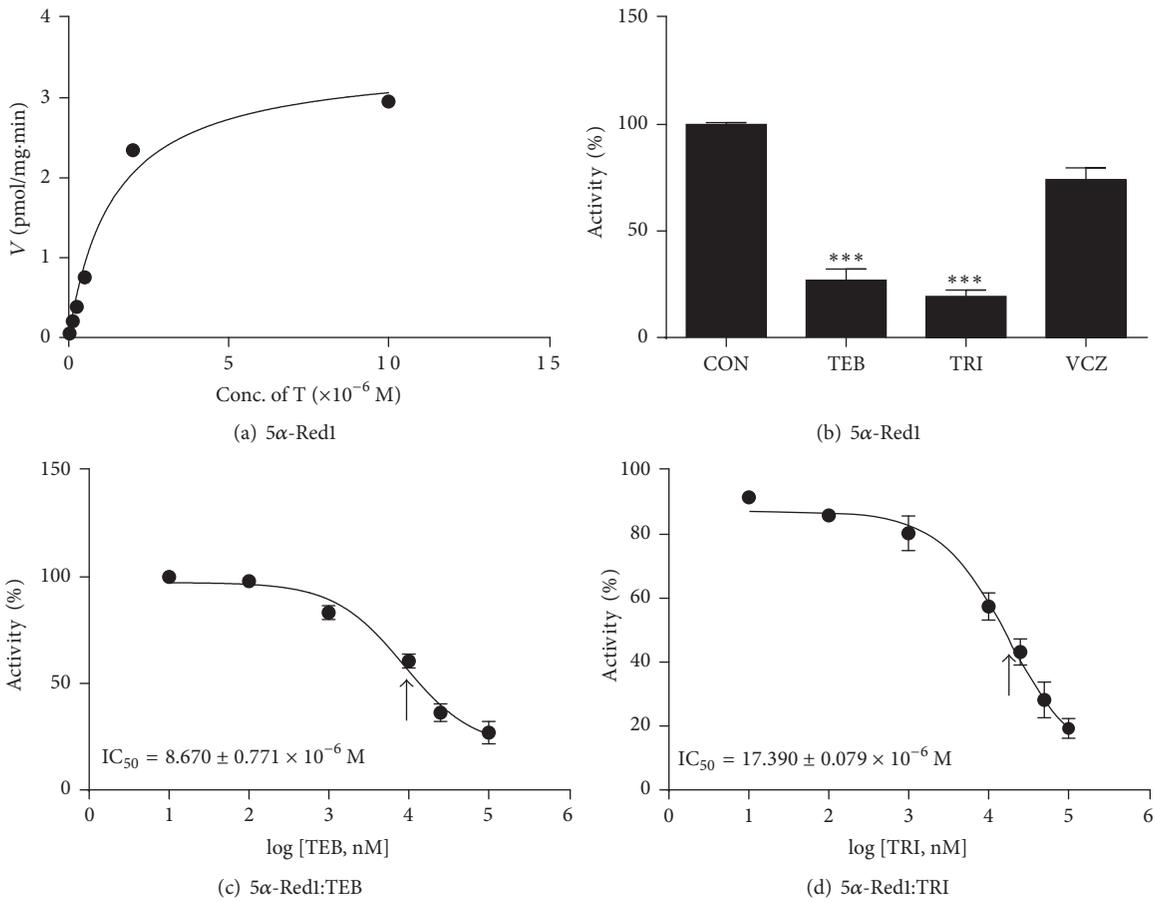


FIGURE 1: Kinetics of SRD5A1 and the inhibition of fungicides. Panel (a): kinetics of SRD5A1 with testosterone (T) as the substrate. Panel (b): % inhibition by tebuconazole (TEB), triadimefon (TRI), and vinclozolin (VCZ) at 100 μ M. Panels (c) and (d): IC_{50} values of TEB and TRI. Mean \pm SEM; *** indicates a significant difference compared to the control (CON) at $P < 0.001$.

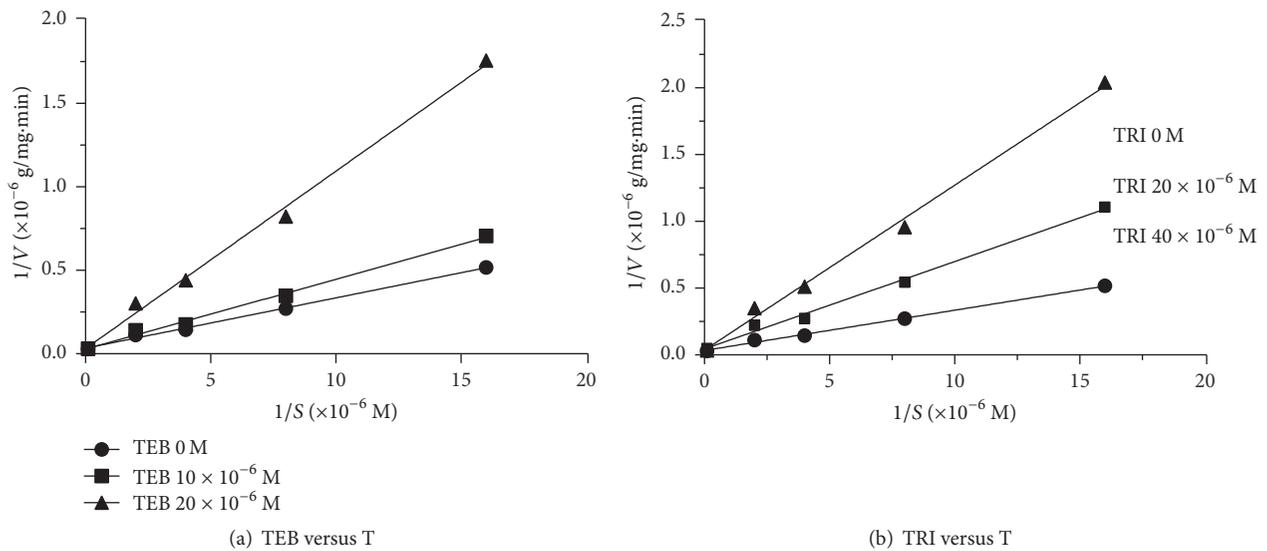


FIGURE 2: The inhibitory mode of tebuconazole (TEB) and triadimefon (TRI) on rat's SRD5A1. Lineweaver-Burk plots in presence of testosterone and TEB (Panel (a)) as well as testosterone and TRI (Panel (b)). Values were obtained from four samples.

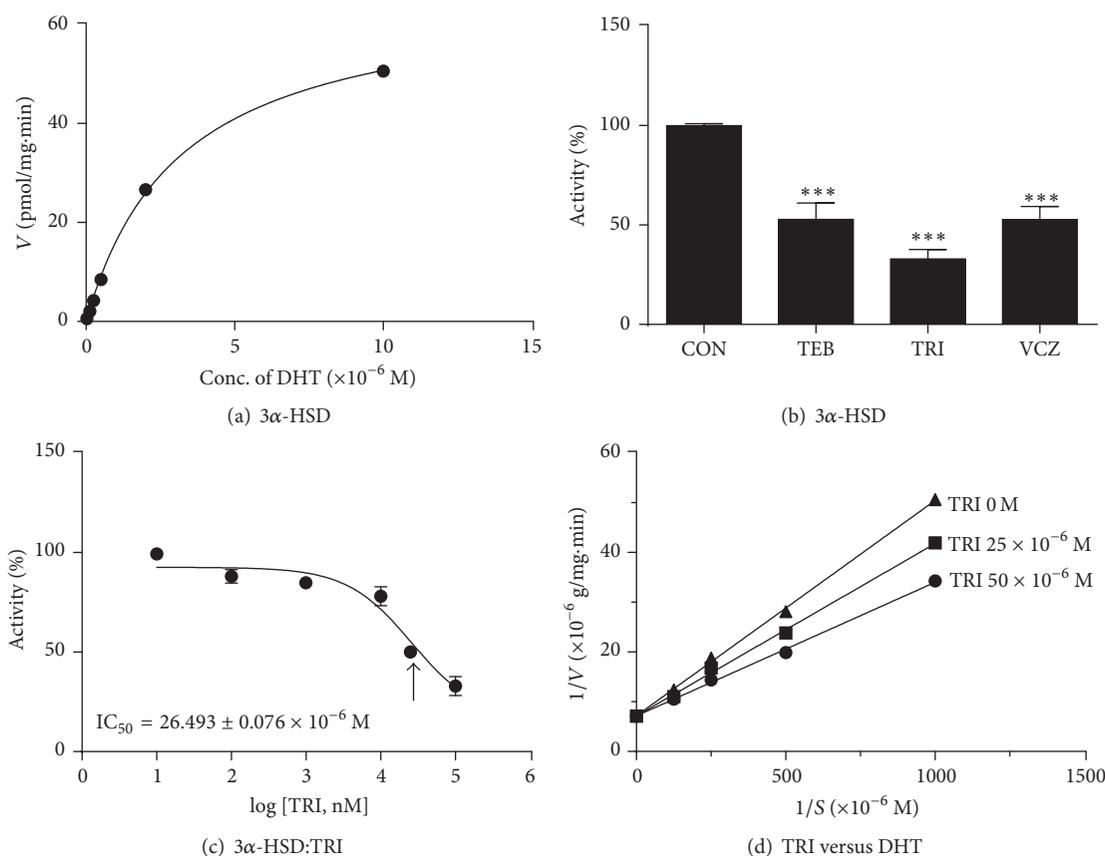


FIGURE 3: The kinetics of AKRIC14 and the inhibition of fungicides. Panel (a): kinetics of AKRIC14 with dihydrotestosterone (DHT) as the substrate. Panel (b): % inhibition by tebuconazole (TEB), triadimefon (TRI), and vinclozolin (VCZ) at 100 μM . Panel (c): IC_{50} value of TRI. Panel (d): the mode of inhibition of TRI versus DHT. Mean \pm SEM; *** indicates a significant difference compared to the control (CON) at $P < 0.001$.

SE, $n = 4$) and 66.69 ± 1.587 pmol DIOL/mg protein/min (mean \pm SE, $n = 4$), respectively (Table 1 and Figure 3(a)). TRI inhibited rat's 3α -HSD to $32.95 \pm 4.80\%$ of the control value, while TEB and VCZ caused about $52.78 \pm 8.278\%$ and $52.65 \pm 6.70\%$ of the control value, respectively (Figure 3(b)). We further calculated the IC_{50} value of TRI, which was $26.493 \pm 0.076 \mu\text{M}$ (Table 1 and Figure 3(c)). The mode of inhibition of TRI on 3α -HSD was found to be competitive against dihydrotestosterone (Figure 3(d)).

3.3. Effects of Fungicides on RDH2 Activity. The conversion of DIOL into dihydrotestosterone is catalyzed by RDH2, which requires NAD^+ as a cofactor; the apparent K_m and apparent V_{max} of RDH2 were $2.850 \pm 0.037 \mu\text{M}$ (mean \pm SE, $n = 4$) and 529.5 ± 2.612 pmol dihydrotestosterone/mg protein/min (mean \pm SE, $n = 4$), respectively (Table 1 and Figure 4(a)). TRI and TEB only inhibited RDH2 to $65.79 \pm 1.69\%$ and $53.35 \pm 5.03\%$ of the control value, while VCZ did not inhibit the enzyme activity ($85.51 \pm 2.20\%$ of the control value, Figure 4(b)).

3.4. Docking of Fungicides to 3α -HSD. Because among three enzymes only the crystal structure of rat's 3α -HSD is available, we docked DIOL to 3α -HSD first. DIOL was found

to bind to the dihydrotestosterone-binding pocket, with free energy of -7.73 Kcal. Further docking analysis for TEB (Figure 5(a)), TRI (Figure 5(b)), and VCZ (Figure 5(c)) showed that all these three chemicals bound to the steroid-binding pocket, with free energies of 7.28, -7.63 , and -7.34 . These data indicate that TRI has the highest binding affinity with 3α -HSD. TRI interacts with Try310, Trp227, His117, Tyr55, Leu54, Thr24, and Asn306 residues of 3α -HSD (Figure 6). The Tyr310 and Trp227 residues were believed to hold the steroid structure, and His117 and Tyr55 residues were believed to catalyze the 3α -position of the steroid [15].

4. Discussion

In the brain, the neurosteroidogenic enzymes 5α -Red1 [17], 3α -HSD [11, 17], and RDH2 [12] are involved in the biosynthesis and metabolism of neurosteroids. 5α -Red1 and 3α -HSD are responsible for the neurosteroid biosynthesis to form 3α -reduced neurosteroids, while RDH2 is responsible for the neurosteroid metabolism to remove the 3α -reduced neurosteroids. These neurosteroidogenic enzymes showed different sensitivity to some fungicides. Here, we demonstrated that TEB and TRI potently inhibited 5α -Red1, the irreversible step of neurosteroid biosynthesis. Furthermore, TRI also

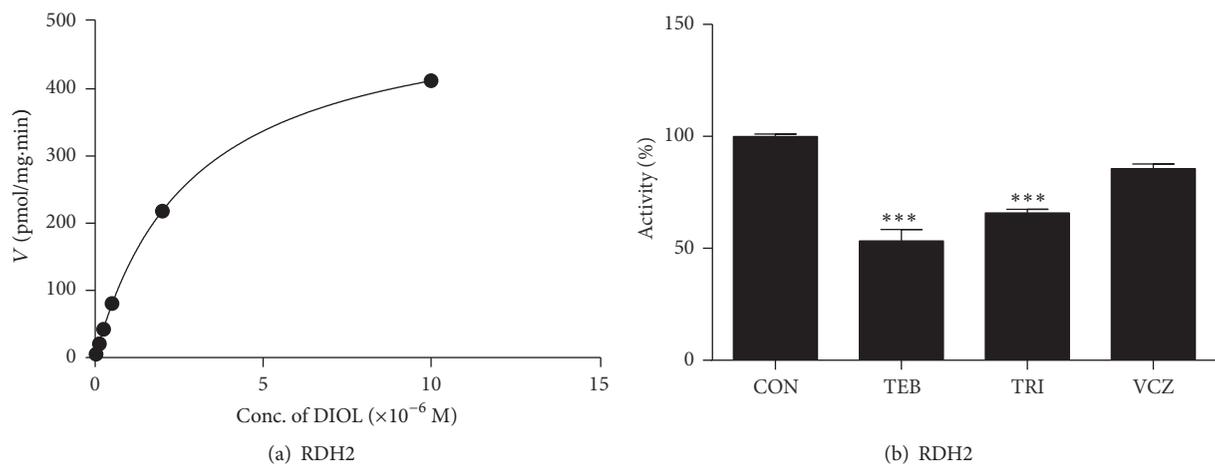


FIGURE 4: The kinetics of RDH2 and the inhibition of fungicides. Panel (a): kinetics of RDH2 with androstenediol (DIOL) as the substrate. Panel (b): % inhibition by tebuconazole (TEB), triadimefon (TRI), and vinclozolin (VCZ) at $100 \mu\text{M}$. Mean \pm SEM; *** indicates a significant difference compared to the control at $P < 0.001$.

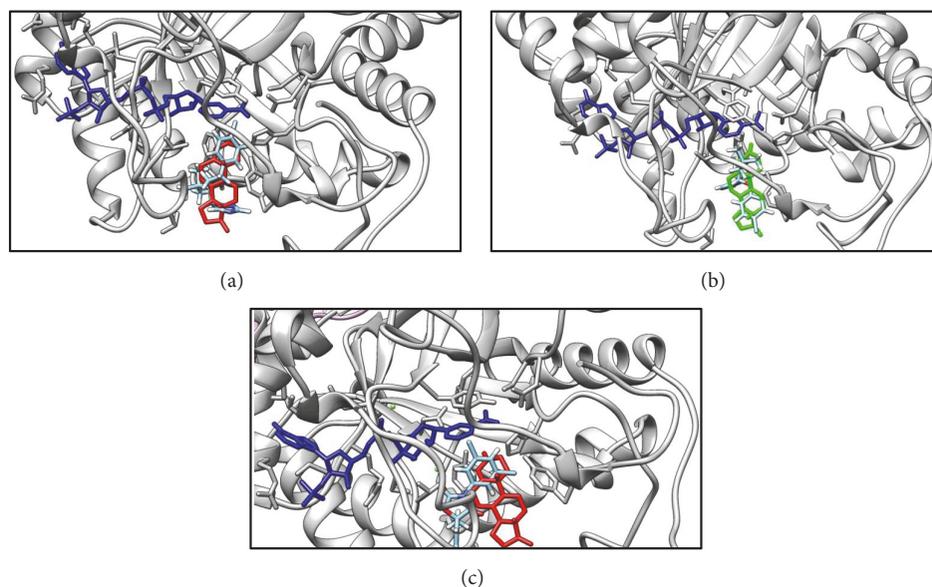


FIGURE 5: Docking analysis for the binding to rat's AKR1C14 (1AFS). Panel (a): tebuconazole; blue structure, NADPH; red structure, testosterone; sky-blue structure, tebuconazole. Panel (b): triadimefon; blue structure, NADPH; green structure, dihydrotestosterone; sky-blue structure, triadimefon. Panel (c): vinclozolin; blue structure, NADPH; red structure, testosterone; sky-blue structure, vinclozolin.

potently inhibited 3α -HSD, thus leading to the reduced level of neurosteroids. VCZ was the weakest fungicide to inhibit 5α -Red1 and 3α -HSD.

Interestingly, the enzyme 5α -Red1 is the most sensitive to the inhibition by TEB compared to 3α -HSD and RDH2. The IC_{50} values of TEB for 5α -Red1, 3α -HSD, and RDH2 were 8.67, ~ 100 , and $\sim 100 \mu\text{M}$. 5α -Red1 and 3α -HSD share equal sensitivity to the inhibition by TRI compared to RDH2. The IC_{50} values of TRI for 5α -Red1, 3α -HSD, and RDH2 were 17.39, 26.49, and $\sim 100 \mu\text{M}$. The reason for this difference is still unclear. This is possibly due to the difference of these enzyme structures. 5α -Red1 is the rate-limiting irreversible step for the formation of many neurosteroids. Animal study

suggests subsequent 3α -reduction of dihydroprogesterone and dihydrotestosterone by 3α -HSD into steroid metabolites which have neuroactive function via enhancing GABA suppression. These neuroactive steroids promote GABA effects by allosteric modulation at GABA-A receptors, thus exerting anticonvulsant, antidepressant, and anxiolytic effects [18]. In socially isolated mice, 5α -Red1 is downregulated in glutamatergic pyramidal neurons that converge on the amygdala from cortical and hippocampal regions possibly causing anxiety, aggression, and cognitive dysfunction [19, 20].

VCZ was the weakest inhibitor for 5α -Red1 and 3α -HSD, with IC_{50} about $100 \mu\text{M}$. However, VCZ almost did not inhibit RDH2 when $100 \mu\text{M}$ was used. The reason why the potency

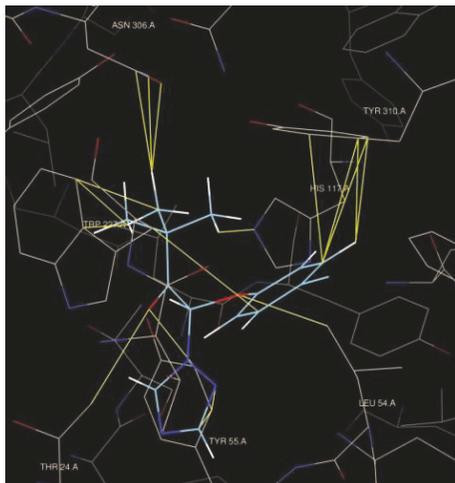


FIGURE 6: Docking analysis for the binding of triadimefon to rat's AKRIC14 (1AFS). The residues of AKRIC14 interacting with triadimefon were listed.

of VCZ is different from those of TEB and TRI is unclear. This is possibly due to the different chemical structures, in which TEB and TRI contain one triazole and VCZ contains one imidazole in the chemical structure.

TEB and TRI competitively inhibited 5α -Red1 when testosterone was provided. TEB and TRI also competitively inhibited 3α -HSD. Docking study further confirmed that these three chemicals bound to the steroid-binding pocket of 3α -HSD. TRI interacts with Try310, Trp227, His117, Tyr55, Leu54, Thr24, and Asn306 residues in the steroid-binding pocket of 3α -HSD. The Tyr310 and Trp227 residues were believed to maintain stability of the steroid, and His117 and Tyr55 residues of 3α -HSD were believed to catalyze the 3α -position of the steroid [15]. The free energy calculation further showed the lowest binding energy for TRI, which was comparable to DIOL, indicating that TRI has high affinity for 3α -HSD.

The homeostasis of neurosteroids including ALLO and DIOL depends on the catalysis of their biosynthetic enzymes, 5α -Red1 and 3α -HSD, as well as the metabolizing enzyme RDH2. Since 5α -Red1 is the rate-limiting step for neurosteroid formation, this inhibition by TEB and TRI is critical for the production of neurosteroids. Indeed, evidence shows that these fungicides can affect brain function. Rats after exposure to triadimefon developed a deficit in spatial learning and reference memory [21]. Rats after perinatal exposure to tebuconazole produced neurobehavioral deficits and neuropathology [22]. Triadimefon also disrupted the transporter of extracellular dopamine, dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in adult rat's striatum [23]. Goldfish after acute and chronic exposure to VCZ developed dysfunction of neuroendocrine regulation of reproduction [24]. Therefore, the disruption of neurosteroid biosynthesis by these fungicides could lead to neurological dysfunction.

In conclusion, TEB and TRI are inhibitors of 5α -Red1 and 3α -HSD. TEB inhibited 5α -Red1 activity more potently than

the activities of 3α -HSD and RDH2. Their negative effects on the neurosteroid accumulation were worthy of further research.

Abbreviations

3α -HSD:	3α -Hydroxysteroid dehydrogenase
ALLO:	Allopregnanolone
DHT:	Dihydrotestosterone
DIOL:	Androstanediol
IC ₅₀ :	Half maximum inhibitory concentrations
RDH2:	Retinol dehydrogenase 2
5α -Red1:	5α -Reductase 1
TEB:	Tebuconazole
T:	Testosterone
TRI:	Triadimefon
VCZ:	Vinclozolin.

Disclosure

Ping Huang is a co-corresponding author.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

Xiuwei Shen and Fan Chen equally contributed to this work.

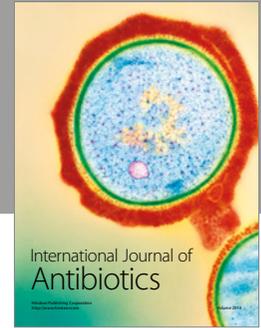
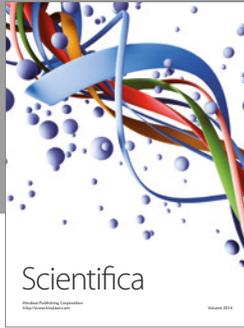
Acknowledgments

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