

Azole Fungicides: Potential Endocrine Disrupting Effects and Impact on Placental Steroidogenesis via Inhibiting Human and Rat 3β -Hydroxysteroid Dehydrogenase

Jingyun Yan

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Huan Chen

The Dingli Clinical College of Wenzhou Medical University

Zhongyao Ji

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Yunbing Tang

Wenzhou University

Shaowei Wang

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Jingyi Zheng

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Huitao Li

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Linxi Li

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Ren-shan Ge

renshan_ge@wmu.edu

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

Yaoyao Dong

Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University

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Abstract

Azole fungicides have been found to inhibit the activity of human 3β -hydroxysteroid dehydrogenase 1 (3β -HSD1), a key enzyme involved in steroid synthesis during normal pregnancy. However, the specific azole fungicides that exert this inhibitory effect and the structure-activity relationship (SAR) involved have not been fully elucidated. This study aimed to analyze the inhibitory potency, mode of action, SAR, and docking parameters of 9 azole fungicides on human 3β -HSD1 and its rat homolog 3β -HSD4. The results revealed that bromuconazole, climbazole, cyproconazole, and etaconazole A varying inhibitory potency against human 3β -HSD1, with IC_{50} values ranging from 6.21 μ M (climbazole) to 104.83 μ M (cyproconazole). These fungicides were identified as mixed/noncompetitive inhibitors of human 3β -HSD1. Among them, climbazole and bromuconazole were found to inhibit progesterone secretion by human JAr cells at 20 and 40 μ M. It was also observed that these fungicides significantly inhibited the activity of rat placental 3β -HSD4, although with reduced potency compared to their effect on human 3β -HSD1. Furthermore, docking analysis indicated that the azole fungicides bind to the NAD^+ /steroid binding site via hydrogen bonds, hydrophobic, and van der Waals interactions. The study demonstrated a negative correlation between LogP and IC_{50} value for 3β -HSD1, indicating that the lipophilicity of the chemicals plays a critical role in their inhibitory effect. Additionally, 3D-QSAR analysis highlighted the significance of hydrogen bond donor and acceptor in the binding process. In conclusion, it was shown that certain azole fungicides exhibit inhibitory effects on human 3β -HSD1, and this inhibition is dependent on their lipophilicity. These findings suggest that these fungicides could potentially act as potential endocrine disruptors in the context of steroid synthesis during pregnancy.

1. Introduction

Azole fungicides are a class of widely used agricultural chemicals that have found extensive application in various industries, including crop protection, personal care products, and pharmaceuticals (Fig. 1A) (Draskau and Svingen 2022; Gava et al. 2021). They include bromuconazole, climbazole, cyproconazole, etaconazole, carbendazole, propiconazole, systhane, and triadimefone (Fig. 1A). However, growing concerns have been raised about the potential endocrine-disrupting effects of these compounds, particularly their ability to interfere with key enzymes involved in steroid hormone biosynthesis (Draskau and Svingen 2022).

Azole fungicides have been shown to inhibit the activity of the enzyme cytochrome P450-19A1 (CYP19A1), also known as aromatase, which is responsible for conversion of androgens to estrogens (Zarn et al. 2003). This inhibition can lead to an imbalance in the levels of sex hormones, potentially affecting various physiological processes, including reproduction, development, and metabolism (Cao et al. 2017; Zarn et al. 2003).

In addition to their effects on CYP19A1, azole fungicides have also been found to target the enzyme 3β -hydroxysteroid dehydrogenase (3β -HSD), which plays a critical role in steroidogenesis. The human 3β -HSD enzyme exists in multiple isoforms with distinct tissue distributions, functions, and substrate affinity (Simard et al. 2005; Zhang et al. 2019; Zhu et al. 2019). In humans, the placenta contains a high-affinity isoform of 3β -HSD known as type 1 (h 3β -HSD1), which is primarily responsible for the production of progesterone (P4)

using pregnenolone (P5) as a substrate during pregnancy (Simard et al. 2005). This isoform plays a crucial role in maintaining pregnancy and supporting fetal development (Sang et al. 2023a). In the gonads (testes and ovaries) and adrenal glands, the predominant isoform of 3 β -HSD is the type II enzyme (h3 β -HSD2), which is a low-affinity enzyme (Simard et al. 2005; Thomas et al. 2002a; Thomas et al. 2002b). This isoform is involved in the synthesis of androgens and glucocorticoids in these tissues. Androgens are male sex hormones such as testosterone, while glucocorticoids are involved in regulating metabolism and the stress response.

The placental h3 β -HSD1 enzyme primarily catalyzes the conversion of P5 to P4 or dehydroepiandrosterone to androstenedione (Fig. 1B) (Zhu et al. 2019). This reaction involves the oxidation of the 3 β -hydroxyl group and the isomerization of the Δ^5 double bond to the Δ^4 position. The enzyme requires the cofactor NAD⁺ (nicotinamide adenine dinucleotide) as a coenzyme and utilizes catalytic residues, such as Ser, Tyr, and Lys, to facilitate the reaction (Simard et al. 2005). In the h3 β -HSD1, NAD⁺ is involved in the oxidation of P5 and during this process, NAD⁺ accepts electrons from P5, becoming reduced to NADH, and then NADH is used for isomerization of intermediate steroid to finally form P4 (Simard et al. 2005).

However, the inhibitory potency of different azole fungicides on placental h3 β -HSD1 has not been extensively studied. Since rat placenta contains human h3 β -HSD1 homolog 3 β -HSD type IV (r3 β -HSD4) and the enzyme shares high similarity with h3 β -HSD1 and catalyzes the similar reaction (Simard et al. 2005), we would evaluate the inhibitory effects of azole fungicides on both enzymes and performed structure-activity relationship (SAR) and three-dimensional quantitative structure-activity relationship (3D-QSAR) analyses. These studies will reveal that the specific structural features of the azole compounds, such as the nature and positioning of the substituents, can significantly influence their inhibitory activity on these enzymes.

2. Materials and methods

2.1. Materials and animals

We obtained pregnenolone, progesterone, and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA). Bromuconazole (CAS#116255-48-2, Cat#G21120100, purity of 98.8%) and etaconazole (CAS#60207-93-4, Cat#0492103 with a purity of 97.4%) were acquired from TM Standard (Shanghai, China). Epoxiconazole (CAS#133855-98-8, Cat#2030600, purity of 98.5%) was sourced from Yuanye (Shanghai, China). Climbazole (CAS#38083-17-9, Cat#001906, purity of 98%) and sythane (CAS#88671-89-0, Cat#M796746, purity of 99.9%) were obtained from Mackin (Shanghai, China). Cyproconazole (CAS#94361-06-5, Cat#C11300887, purity of 98%), propiconazole (CAS# 60207-90-1, Cat#P114492, purity of 99.9%), and triadimefene (CAS#43121-43-3, Cat#BWY395929, purity of 97%) were acquired from Aladdin (Shanghai, China). Carbendazole (CAS#10605-21-7, Cat#513039, purity of 98%) was obtained from J&K (Shanghai, China). Pregnant Sprague-Dawley rats weighing 250–300 g were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). The rat placentas were harvested from the dams on gestational day 20 following CO₂ euthanasia. The use of animal subjects was approved by the Experimental Animal Ethics Committee of Wenzhou Medical University, and all experimental procedures were performed in accordance with the guidelines outlined in the Manual for Nursing and Experimentation with Laboratory Animals. Human

full-term placentas were obtained from the Second Affiliated Hospital of Wenzhou Medical University, and their acquisition was conducted under the oversight and direction of the Clinical Research Committee of Wenzhou Medical University (protocol number: 2022-K-81-01).

2.2. Microsomal preparation

To prepare microsomes, we sourced samples from both human and rat placentas, adhering to an established methodology (Xu et al. 2016). The placental tissue was sliced and homogenized through homogenization in a solution comprising 0.01 M PBS (pH 7.2) and 0.25 M sucrose. Subsequently, the homogenate underwent a series of centrifugation steps, including rotations at 700×g for 30 min, 14,500×g for 30 min, and 105,000×g for 1.5 hr. The resultant pellet containing proteins was reconstituted in PBS. Subsequently, the protein concentration was determined using the BCA protein kit (Cat # P0010, Beyotime Biotech Inc, Shanghai, China), following the manufacturer's guidelines.

2.3. Detection of h3β-HSD1 and r3β-HSD4 activity in microsomes

The presence and activity of h3β-HSD1 and r3β-HSD4 in microsomes were assessed according to the previously described protocol (Li et al. 2023). In brief, a basic 3β-HSD reaction contained 2 μg of microsomes, P5 and 0.2 mM NAD⁺ in 100 μL of PBS (0.01 M, pH 7.2) in a 1.5 mL Eppendorf microtube and the microtube was then placed in a shaking water bath set at 37°C and 75 rpm for reaction. First, Michaelis-Menten (MM) kinetics analysis was performed in the basic reaction comprising 0–1 μM P5 for 30 min. Second, for the initial screening, a solution of an azole fungicide dissolved in DMSO was added to the reaction mixture as a control, with a concentration of 100 μM in the reaction containing 0.2 μM P5 in the basic reaction for 30 min. Third, for dose response assay, a setup comprised 0.2 μM P5 and varying concentrations (0–100 μM) of a fungicide in the basic reaction for 30 min was performed. Lastly, for mode action (MOA) assay, 0–1 μM P5 and varying concentrations (0–100 μM) of a fungicide in the basic reaction for 30 min were performed. After incubation, the reaction was stopped by adding 10 μL of an internal standard called testosterone-d5 (T-d5) (Shanghai Zzbio Co., China) and 200 μL of acetonitrile (Merck Supelco, PA, USA) to the reaction mixture. The microtube was then vortexed and centrifuged at 12,000 ×g for 10 min. Following centrifugation, 100 μL of the supernatant was transferred to a sample bottle, and 10 μL of the sample was injected for analysis using HPLC/MS-MS assay.

2.4. P4 quantification via HPLC-MS/MS analysis

P4 levels were quantified utilizing state-of-the-art Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) techniques. The analysis was conducted on the Acquire UPLC system (Waters, USA), equipped with an Acquire BEH C18 column (2.1 mm × 50 mm, particle size: 1.7 μm), adhering to the established protocol (Li et al. 2023). Detection and quantification were executed employing the XEVO TQD triple quadrupole mass spectrometer (Waters, USA) with electrospray ionization (ESI). The mass spectrometer was configured to operate in the Multiple Reaction Monitoring (MRM) mode, utilizing unit mass resolution for precise detection. Masslynx 4.1 software facilitated data acquisition and instrument control throughout the analysis process. Quantitative assessment of P4 levels was achieved via the

standard curve method with the internal standard (IS) serving as the reference compound. This rigorous approach ensured accurate determination of P4 concentrations in the samples analyzed.

2.5. Calculation of enzymatic parameters

In the analysis of MM kinetics, we utilized the equation ($v = V_{\max} \frac{[P5]}{K_m + [P5]}$) for nonlinear regression analysis in GraphPad software 9.5 (GraphPad Inc., CA, USA) to determine the values of MM kinetics constant (K_m) and maximum velocity ($V_{\max}$). When conducting a screening assay for fungicides, the residual activity (R) was calculated relative to the control tube (DMSO), which was considered as 100%. To investigate the dose response of a fungicide, the IC_{50} value was determined using the equation: $R = \frac{100}{1 + 10^{-((\text{Log}[i] - \text{Log}IC_{50}))}}$, where $[i]$ is the inhibitor's concentration. For the Enzyme kinetics inhibition (mixed model) assay, the

equations were employed to calculate K_i values: $V_{\max App} = \frac{V_{\max}}{1 + \frac{[i]}{K_i}}$, $K_{m App} = K_m \frac{1 + \frac{[i]}{K_i}}{1 + \frac{[i]}{K_i}}$, and $v = V_{\max App} \frac{X}{K_{m App} + X}$, where $V_{\max App}$ is apparent $V_{\max}$, $K_{m App}$ is apparent K_m , K_i is inhibition constant, and α is the digital number for determination of MOA. A " $\alpha = 1$ " denotes noncompetitive inhibition; $\alpha > 1$ or $\alpha < 1$ signifies mixed inhibition; α approaching 0 indicates uncompetitive inhibition; $\alpha > e^{10}$ suggests the competitive inhibition. A Lineweaver-Burk plot was generated to validate the MOA.

2.6. Investigation of JAr cell culture and P4 production

Human choriocarcinoma JAr cells obtained from ATCC (USA) were cultivated following the established protocol (Li et al. 2023). The JAr cells were incubated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, USA). The cell cultures were maintained at a constant temperature of 37°C within a humidified atmosphere enriched with 5% CO₂. Upon achieving 80% confluence during exponential growth, the cells were subjected to treatment with varying concentrations of azole fungicides, ranging from 0 up to 80 µM, for 24 hrs. Subsequent to the treatment regimen, the cell culture supernatants were harvested for P4 level assessment as above.

2.7. Analysis of the correlation between the structural properties and IC_{50}

A comprehensive and searchable database called ZINC database was utilized for this analysis (Irwin and Shoichet 2005). The database contained various chemical structural features such as LogP (lipophilicity), molecular weight (MW, d), apolar desolvation energy (AD, kcal/mol), polar desolvation energy (PD, kcal/mol), HB donor number (HBD), HB acceptor number (HBA), rotatable bond count, heavy atom count, hetero atom count, topological polar surface area (PSA, Å²), and Fsp3. The obtained data from the above-mentioned analyses were then subjected to bivariate correlation analysis to explore the potential relationships between the structural properties of the fungicides and their IC_{50} values.

2.8. 3D-QSAR analysis

Compound data was gathered and separated into a training dataset [20 chemicals, comprising various compounds, including curcuminoids (Sang et al. 2023a), insecticides (Li et al. 2023; Zhai et al. 2023),

disinfectants (Li et al. 2023), hormones (Tang et al. 2022), and resveratrol derivatives (Su et al. 2023) in this investigation, Table S2] and a validation dataset (comprising azole fungicides in this study) to construct a 3D-QSAR pharmacophore model. Within the training dataset, three categories of molecular attributes - hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), and hydrogen bond acceptor lipid (HBA_lipid) - were identified for the formulation of a theoretical pharmacophore. The HypoGen algorithm in DS3.5 was employed to produce a series of theoretical pharmacophores based on the essential molecular features and potency values of the molecules in the training dataset. A common reference frame was established in alignment with the structural resemblance of ligands or the active site of the target protein, giving rise to the generation of the pharmacophore hypothesis. This hypothesis focused on identifying the shared characteristics among ligands that played a role in inhibiting h3 β -HSD1, encompassing HBD, HBA, and HBA_lipid. Through the evaluation of the positions and orientations of these features in relation to the aligned ligands, ten pharmacophore models were developed (Table S1). Validation of the pharmacophore model was conducted to confirm its effectiveness in predicting the activities of novel ligands through correlation and RMSD analysis. A "Fit value" was calculated using the formula: $\text{Fit} = \sum \text{of mapped features } f \text{ of weight}(f) [1 - \text{SSE}(f)]$, where $\text{SSE}(f) = \sum \text{over location constraints } c \text{ on } f \text{ of } (D(c) / T(c))^2$, with D representing the displacement of the feature from the centre of the location constraint, and T indicating the radius of the location constraint sphere for the feature (tolerance). A higher fit value denotes a more optimal fitting outcome. The experimental and predicted activities of 20 training set compounds calculated on the basis of the 3D-QSAR-derived Hypo1 model were listed in Table S2.

2.9. Molecular docking investigation of h3 β -HSD1 and r3 β -HSD4

The structural elucidation of h3 β -HSD1 and r3 β -HSD4 was conducted employing the Swiss-Model homology modelling platform, accessible online at <https://swissmodel.expasy.org>, since the crystal structures of both enzymes were not available. For h3 β -HSD1, modelling was predicated on the crystalline framework of MoeE5 (PDB id: 6kv9), bound with UDP-glucuronic acid and NAD⁺ (Sun et al. 2020). Conversely, the structure of r3 β -HSD4 was modeled using the Swiss-Model homology modelling engine, leveraging the crystal structure of dTDP-D-glucose 4, 6-dehydratase (PDB id: 1kew) (Allard et al. 2001). Comparison of primary sequence and 3D structural similarities between h3 β -HSD1 and r3 β -HSD4 was accomplished using Chimera software (version 1.1.0, UCSF, San Francisco, CA). To ascertain the structural integrity of the enzyme models, a Ramachandran plot analysis was executed utilizing Discovery Studio Client v19 (Accelrys Inc., San Diego, CA), as previously described (Ongtanasup et al. 2022). Docking simulations involving a paraben with h3 β -HSD1 and r3 β -HSD4 were performed employing Autodock 4.0 (Scripps Research Institute, CA, <http://autodock.scripps.edu>). Ligands were prepared utilizing AutoDock Tools. Binding affinities and energies of each ligand were computed and juxtaposed. The configuration of the docked fungicide with lowest binding energy (ΔG) was presented.

2.10. Statistical analysis

The study was repeated 3–4 times. An ANOVA was employed to analyze the enzyme outcomes, followed by a post-hoc Dunnett's multiple comparisons test to identify any notable variances. Pearson correlations were utilized in bivariate analyses to evaluate the connection strength between the characteristics of azole

fungicides and their corresponding IC_{50} values. Significant variances were indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. The results are presented as the mean $\pm$ standard error of the mean (SEM) for residual activity and MM kinetics or mean $\pm$ standard deviation (SD).

3. Results

3.1. Inhibition of h3 β -HSD1 activity by azole fungicides based on structure

To validate the assay, MM kinetics was performed. The results indicated that the apparent K_m for h3 β -HSD1 was 0.254 ± 0.051 μM (Fig. 2A), consistent with previously reported value (Li et al. 2023). The apparent V_{max} was determined to be 133.0 ± 10.19 pmol/mg/min (Fig. 2A). The effect of azole fungicides on h3 β -HSD1 activity was then assessed at an initial concentration of 100 μM . Among the tested azole fungicides, bromuconazole, climbazole, cyproconazole, and etaconazole showed significant inhibition of h3 β -HSD1 activity (Fig. 2B), while other azole fungicides did not exhibit any inhibitory effects. IC_{50} values were calculated for bromuconazole, climbazole, cyproconazole, and etaconazole, ranging from 6.21 μM (climbazole) to 104.83 μM (cyproconazole) (Table 1 and Fig. 2C-2F). Further analysis revealed K_i values of 18.43 μM for bromuconazole, 7.06 μM for climbazole, and 88.9 μM for etaconazole (Fig. 3), confirming their IC_{50} values. Lineweaver-Burk plot analysis demonstrated that bromuconazole, climbazole, and etaconazole acted as mixed/noncompetitive inhibitors (Fig. 3), indicating that they bind to the free enzyme and enzyme-complex in varying degrees. To evaluate the effects of fungicides on P4 secretion by human JAr cells, we focused on the two potent inhibitors, bromuconazole and climbazole. It was observed that climbazole significantly reduced P4 output even at low concentrations as low as 2.5 μM , while bromuconazole significantly suppressed P4 secretion at 40 and 80 μM (Fig. 4). This suggests that these inhibitors are capable of penetrating the cell membrane to inhibit h3 β -HSD1.

Table 1

The half-maximal inhibitory concentrations (IC_{50}), measured K_i constant (K_i), calculated K_i (Cal. K_i), lowest binding energy (ΔG), mode of action, and binding site

Compd.	IC_{50} μM	K_i μM	Cal. K_i μM	ΔG kcal/mol	Mode of action	Binding site
Human $h3\beta$-HSD1						
Bromuconazole	19.75 $\pm$ 0.37	18.43	19.20	-6.43	Mixed/noncompetitive	NAD ⁺ /Steroid
Climbazole	6.21 $\pm$ 0.19	7.06	6.34	-7.09	Mixed/noncompetitive	NAD ⁺ /Steroid
Cyproconazole	104.83 $\pm$ 3.76	ND	ND	ND	ND	ND
Etaconazole	78.24 $\pm$ 2.76	88.9	79.92	-5.59	Mixed/noncompetitive	NAD ⁺ /Steroid
Rat $r3\beta$-HSD4						
Bromuconazole	67.22 $\pm$ 0.89	63.60	79.59	-5.59	Mixed	NAD ⁺ /Steroid
Climbazole	52.33 $\pm$ 1.23	ND	58.72	-5.77	Mixed	NAD ⁺ /Steroid
Etaconazole	102.6 $\pm$ 2.54	109.6	99.95	-5.46	Mixed	NAD ⁺ /Steroid
ND = not determined. IC_{50} values: Mean $\pm$ SEM, n = 4.						

3.2. Inhibition of $r3\beta$ -HSD4 activity by azole fungicides based on structure

To validate the assay, MM kinetics for $r3\beta$ -HSD4 was performed. The results indicated that the apparent K_m for $r3\beta$ -HSD4 was $0.20 \pm 0.036 \mu M$ (Fig. 5A), consistent with previously reported value (Li et al. 2023). The apparent V_{max} was determined to be $626.2 \pm 40.30 \text{ pmol/mg/min}$ (Fig. 5A). The effect of azole fungicides on $r3\beta$ -HSD4 activity was then assessed at an initial concentration of $100 \mu M$. Among the tested azole fungicides, bromuconazole, climbazole, and etaconazole showed marked inhibition of $r3\beta$ -HSD4 activity (Fig. 5B), while other azole fungicides did not exhibit any inhibitory effects. IC_{50} values were calculated for $67.22 \mu M$ for bromuconazole, $52.33 \mu M$ for climbazole, and $102.60 \mu M$ for etaconazole (Table 1 and Fig. 5C-5E). Further analysis revealed K_i values of $63.60 \mu M$ for bromuconazole, $109.6 \mu M$ for etaconazole (Fig. 5F and 5H), confirming their IC_{50} values. Lineweaver-Burk plot analysis demonstrated that bromuconazole and etaconazole acted as mixed/noncompetitive inhibitors (Fig. 5G and 5I), indicating that they bind to the free enzyme and enzyme-complex in varying degrees.

3.3. Bivariate correlation of structural parameters and IC_{50} values

The bivariate correlation analysis in this study focused on key structural parameters, including LogP and Fsp3, in relation to IC_{50} values. The correlation analysis was conducted using Pearson correlation

coefficients. Interestingly, the results revealed that only LogP and Fsp3 showed significant correlations with the IC₅₀ values. Specifically, LogP exhibited a negative correlation with h3β-HSD1 and r3β-HSD4, whereas Fsp3 demonstrated a positive correlation with h3β-HSD1 (Fig. S1). However, other parameters such as molecular weight, heavy atoms, hetero atoms, PSA, ring number did not show any correlation (data not shown).

3.4. 3D-QSAR

A pharmacophore modeling investigation was undertaken to target h3β-HSD1 with azole fungicides and other inhibitors, resulting in the development of the top 10 theoretical pharmacophores ranked, along with their respective statistical parameters (Table S1). Among these, Hypo1 emerged as the most superior hypothetical structure and was deemed the optimal model due to its outstanding parameters: a correlation coefficient of 0.96, RMSD of 1.94, and total cost of 109.87. Hypo1 comprised HBD and HBA components (Fig. 6A). When aligned with Hypo1, the most potent inhibitor, climbazole (IC₅₀: 6.21 μM, Fig. 6B), demonstrated a fit value of 4.89, with interactions observed with three HBAs and one HBD, whereas the least active compound, cyproconazole (IC₅₀: 104.83 μM), exhibited a lower fit value of 4.08 (Fig. 6C), with only 1 HBA interaction. The effectiveness of the Hypo1 model was confirmed through correlation analysis between the experimental and predicted IC₅₀ values of the training set molecules, indicating strong predictability ($r = 0.73$, $p < 0.0001$, Fig. 6D and Table S2). Overall, these discoveries strongly support a significant association between the actual and estimated activities of both the training and test set molecules in relation to the Hypo1 model.

3.5. Docking analysis of azole fungicides with h3β-HSD1 and r3β-HSD4

The structures of h3β-HSD1 and r3β-HSD4 were constructed utilizing the Swiss-Model engine. These two enzymes showed high similarity in their primary sequences (Fig. S2A) and 3D structures (Fig. S2B). Their structural integrity was verified through Ramachandran plots (Fig. S3), showing that over 94% of residues were situated within favorable regions, affirming the validity of their structures (Fig. S3). Initially, P5 was engaged in docking with h3β-HSD1, establishing HBs with specific catalytic residues such as Ser125 (Fig. S3B), thereby corroborating the robustness of the model. Similarly, P5 was docked with r3β-HSD4, also forming HBs with distinct catalytic residues like Thr125 (Fig. S3D), thus reinforcing the credibility of the model.

Upon docking azole fungicides with both h3β-HSD1 and r3β-HSD4, it was observed that bromuconazole, climbazole, and etaconazole exhibited binding to the steroid and NAD⁺ binding site, indicating their classification as mixed/noncompetitive inhibitors. Notably, climbazole emerged as the most potent inhibitor, establishing HBs with Ser124, Thr187, Tyr188, and Ile189, along with hydrophobic interactions involving six residues and van der Waals interactions with 8 residues of h3β-HSD1 (Fig. 7A and 7B). The calculated ΔG for climbazole was -7.09 kcal/mol, indicative of a robust interaction. Details regarding the interaction of other azole fungicides with h3β-HSD1 were presented in Supplementary Fig. S4 and they have similar types of interactions except that bromuconazole also has a halogen bond (Fig. S4B).

Moreover, climbazole exhibited HBs with Cys123, Thr125, Met188, and Ile190, in addition to hydrophobic interactions with four residues and van der Waals interactions with ten residues of r3 β -HSD4 (Fig. 7C and 7D). The calculated ΔG for climbazole with r3 β -HSD4 was -5.77 kcal/mol, further attesting to its strong interaction. Details regarding the interaction of other azole fungicides with r3 β -HSD4 were presented in Supplementary Fig. S5 and they have similar types of interactions except that bromuconazole also has a halogen bond (Fig. S5B).

4. Discussion

Azole fungicides are a class of chemicals commonly used to prevent fungal growth in crops. These fungicides work by inhibiting the lanosterol 14 α -demethylase enzyme, a key enzyme in the fungal ergosterol biosynthesis pathway (Draskau and Svingen 2022). While the use of azole fungicides has proven effective in protecting crops, there is growing concern about their effects on human health. Here, we screened nine azole fungicides, and found that climbazole was the most potent inhibitor of h3 β -HSD1, with an IC_{50} value of 6.21 μ M. In comparison, bromuconazole, etaconazole, and cyproconazole exhibited higher IC_{50} values, indicating lower inhibitory activity. Notably, the inhibition was identified as mixed/noncompetitive, suggesting a complex mechanism of action. Further investigation revealed that climbazole and bromuconazole were able to penetrate the JAr cell membrane to inhibit P4 output, highlighting their potential to disrupt steroid hormone synthesis in humans.

This study found that certain fungicides, namely bromuconazole, climbazole, etaconazole, and cyproconazole, inhibited a specific isoform of placental human 3 β -HSD, also called h3 β -HSD1. Interestingly, some of these azole fungicides also inhibit human gonadal isoform (h3 β -HSD2) with similar inhibitory trend (Sang et al. 2023b). Both type I (h3 β -HSD1) and type II (h3 β -HSD2) exhibit distinct substrate affinities, with h3 β -HSD1 as the high-affinity enzyme and h3 β -HSD2 as the low-affinity enzyme (Simard et al. 2005). This finding suggests potential implications for the interaction between these fungicides and both human 3 β -HSD isoforms.

Previous studies have also indicated that certain azole fungicides, such as triflumizole and difenoconazole, have the ability to inhibit human placental h3 β -HSD1 (Li et al. 2023; Zhai et al. 2023). The current study has identified an additional 4 azole fungicides that exhibit varying inhibitory potencies against h3 β -HSD1. It is crucial to recognize that the potential impact of this inhibition on h3 β -HSD1 could be associated with reproductive disorders. Further investigation and assessment are essential to comprehensively understand the scope and consequences of these inhibitory effects on placental h3 β -HSD1.

In contrast to their impact on h3 β -HSD1, climbazole and bromuconazole were additionally pinpointed as inhibitors of r3 β -HSD4. It is intriguing to note that the rat 3 β -HSD4 exhibits reduced sensitivity towards certain azole fungicides (Table 1). To illustrate, climbazole displayed an IC_{50} value of 6.21 μ M in inhibiting h3 β -HSD1, whereas it showed an IC_{50} value of 52.33 μ M in inhibiting r3 β -HSD4. Likewise, bromuconazole inhibited h3 β -HSD1 with an IC_{50} value of 19.75 μ M, while it inhibited r3 β -HSD4 with an IC_{50} value of 67.22 μ M. Despite the high similarity in primary sequences and 3D structures between h3 β -HSD1 and r3 β -HSD4 (Fig.S2), subtle distinctions such as variations in catalytic residues (Ser124 in h3 β -HSD1 vs. Thr125 in r3 β -

HSD4) may contribute to this discrepancy (Fig. S2A). Notably, insensitivity to inhibitors for $\text{r}3\beta\text{-HSD4}$ has been observed with curcuminoids (Sang et al. 2023a). This differential sensitivity may have important implications for interspecies variations in response to azole fungicide exposure. Understanding the differences in inhibitory effects between human and rat enzymes is crucial for assessing the potential risks associated with azole fungicide exposure.

Understanding the effects of these fungicides on placental $\text{h}3\beta\text{-HSD1}$ is crucial, as the enzyme plays important roles in steroid hormone biosynthesis, particularly in the context of pregnancy and fetal development. Further investigation into the specific mechanisms and consequences of inhibition by these fungicides on placental $\text{h}3\beta\text{-HSD1}$ is warranted to assess any potential implications for reproductive health and pregnancy outcomes.

The research involved potential interactions between environmental chemicals such as azole fungicides and human biology, future studies are necessary to fully comprehend the risks and implications associated with exposure to these fungicides, particularly in vulnerable populations such as pregnant individuals.

Docking studies have provided valuable insights into the molecular interactions between azole fungicides and the target enzymes. Key interactions include van der Waals forces, hydrogen bonds, and hydrophobic interactions, which play a crucial role in determining the binding affinity and potency of the inhibitors. Furthermore, 3D-QSAR analysis has revealed important features such as HBD and HBA, which contribute to the specificity and selectivity of azole fungicides towards their target enzymes.

In conclusion, our study highlights the inhibitory effects of azole fungicides on placental $\text{h}3\beta\text{-HSD1}$ and $\text{r}3\beta\text{-HSD4}$ enzymes, shedding light on their potential impact on steroid hormone production. The differential sensitivity of human and rat enzyme to azole fungicides underscores the importance of considering interspecies variations in risk assessment. Future research should focus on elucidating the long-term consequences of azole fungicide exposure on human health and developing strategies to mitigate any potential adverse effects.

Abbreviations

$3\beta\text{-HSD1}$, 3β -hydroxysteroid dehydrogenase 1; $3\beta\text{-HSD4}$, 3β -hydroxysteroid dehydrogenase 4; BCA, bicinchoninic acid; ESI, electrospray ionization; DMSO, dimethyl sulfoxide; HB, hydrogen bonds; IC_{50} , half maximal inhibitory concentration; IS, internal standard; K_i , inhibition constant; K_m , Michaelis-Menten constant; DG: lowest binding energy; MRM, multiple reaction monitoring; NAD^+ , nicotinamide adenine dinucleotide; P4, progesterone; P5, pregnenolone; PBS, phosphate buffer saline; SAR, structure-activity relationship; 3D-QSAR, 3D-quantitative structure-activity relationship; SD, standard deviation; SEM, standard error; UPLC-MS/MS; ultra-performance liquid chromatography/tandem mass spectrometry

Declarations

Author contribution

Jingyun Yan, Huan Chen, Zhongyao Ji: Investigation, Writing – original draft preparation. Yunbing Tang, Jingyi Zheng, Huitao Li: Investigation, Analysis. Shaowei Wang: HPLC-MS/MS supervision. Linxi Li, Yaoyao Dong, and Ren-shan Ge: Conceptualization, review & editing and Funding.

Declaration of Competing Interest

The authors declare no competing interests.

ASSOCIATED CONTENT

Supporting Information:

The Supporting Information (Table S1-S2 and Fig.S1-Fig.S5) is available.

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Author contributions

All authors agree to be accountable for all aspects of work, ensuring integrity and accuracy.

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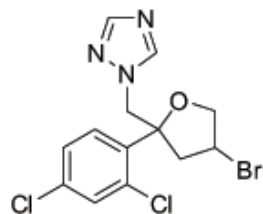
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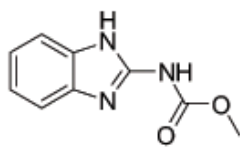
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Figures

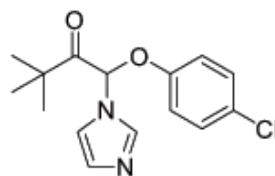
A. Structures of azole fungicides



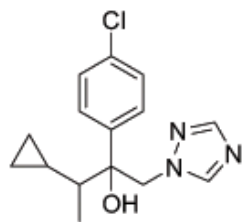
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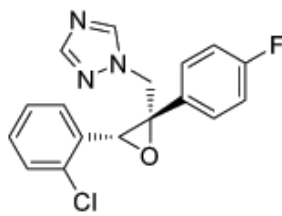
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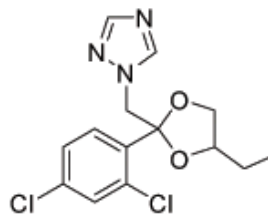
Climbazole
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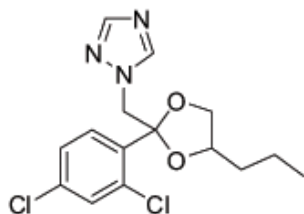
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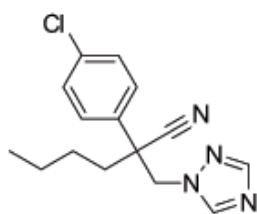
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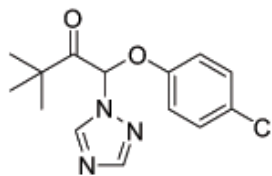
Etaconazole
(CAS: 60207-93-4)



Propiconazole
(CAS: 60207-90-1)



Systhane
(CAS:88671-89-0)



Triadimefone
(CAS:43121-43-3)

B. 3 β -HSD reaction

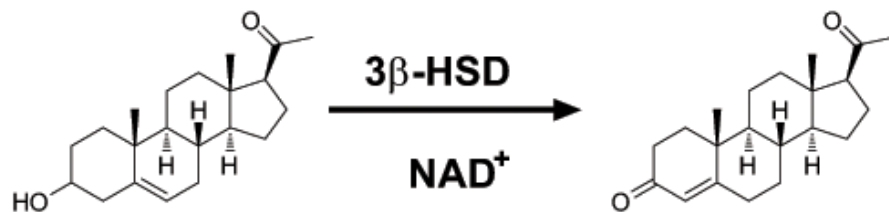


Figure 1

Chemical structures of azole fungicides and reaction scheme of 3 β -hydroxysteroid dehydrogenase (3 β -HSD). A, structures of azole fungicides; B, Human placental 3 β -HSD1 and rat placental 3 β -HSD4 catalyze pregnenolone (P5) to progesterone (P4).

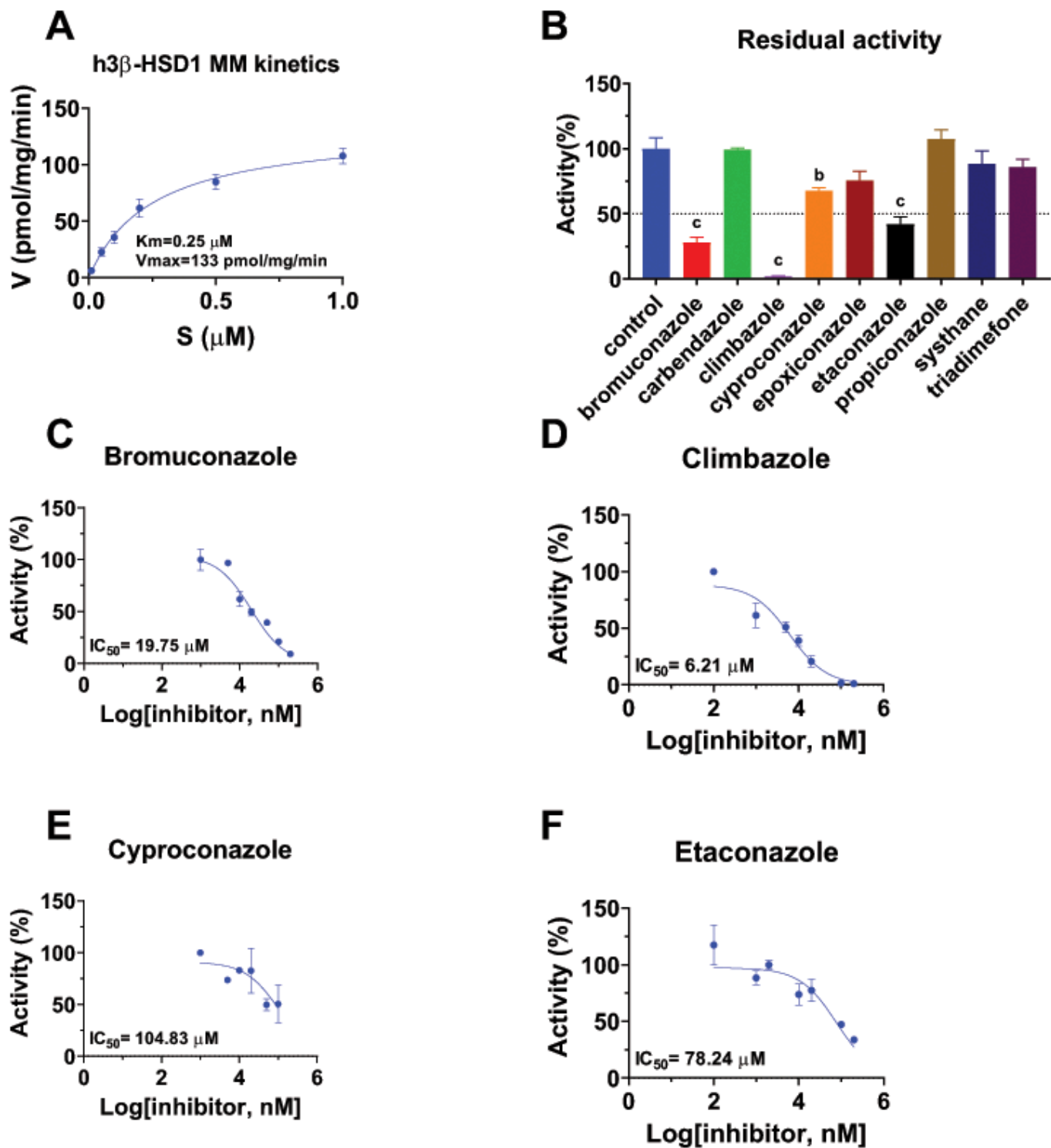


Figure 2

Effects of azole fungicides on human placental microsomal 3b-HSD1 activity at 100 mM, and dose response. A, Michaelis-Menton constant (Km) of 3b-HSD1; B, residual activity relative to the control (DMSO); Compared with the control, *P < 0.05, **P < 0.01, ***P < 0.001 (n = 4, mean ± SEM); C-F: IC₅₀ values of bromuconazole, climbazole, cyproconazole and etaconazole (n = 4, mean ± SEM), respectively.

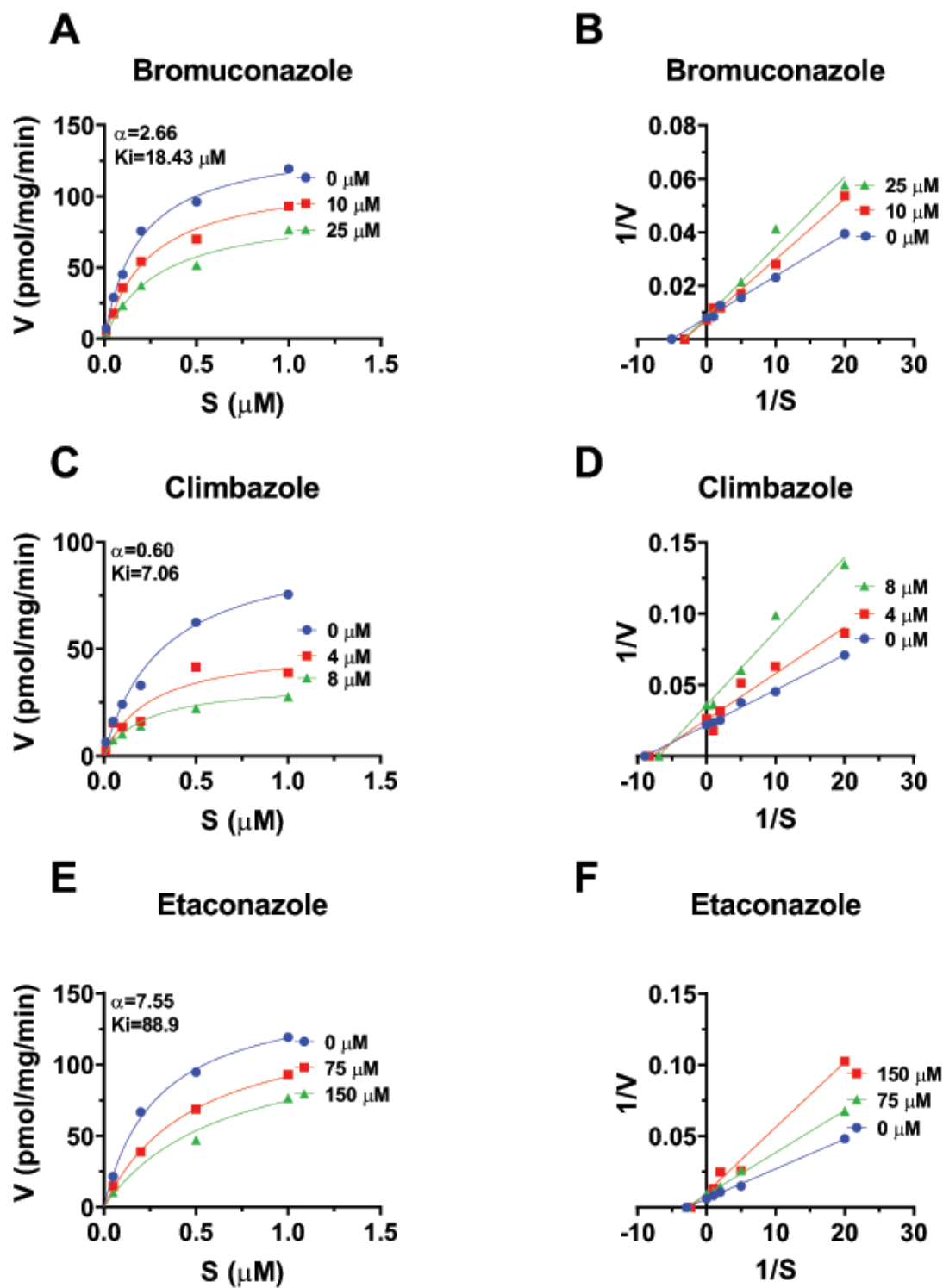


Figure 3

Enzyme kinetics-inhibition and Lineweaver-Burk plot analysis of azole fungicides on human 3b-HSD1. Enzyme kinetics-inhibition analysis (mixed model): (A) bromuconazole; (C) climbazole; (E) etaconazole; Lineweaver-Burk plot: (B) bromuconazole; (D) climbazole; (F) etaconazole; Mean $\pm$ SEM, n=4.

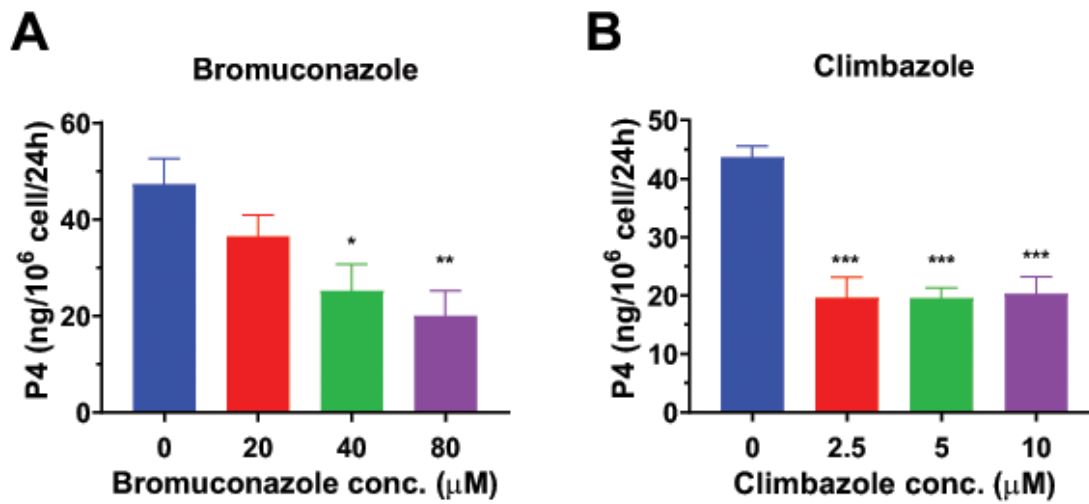


Figure 4

Treatment protocol and effects of azole fungicides on progesterone production by human JAr cells . A, treatment protocol; progesterone output: (A) bromuconazole; (B) climbazole; Compared with the control, ** $P < 0.01$, *** $P < 0.001$ ($n = 4$, mean $\pm$ SEM) for each concentration.

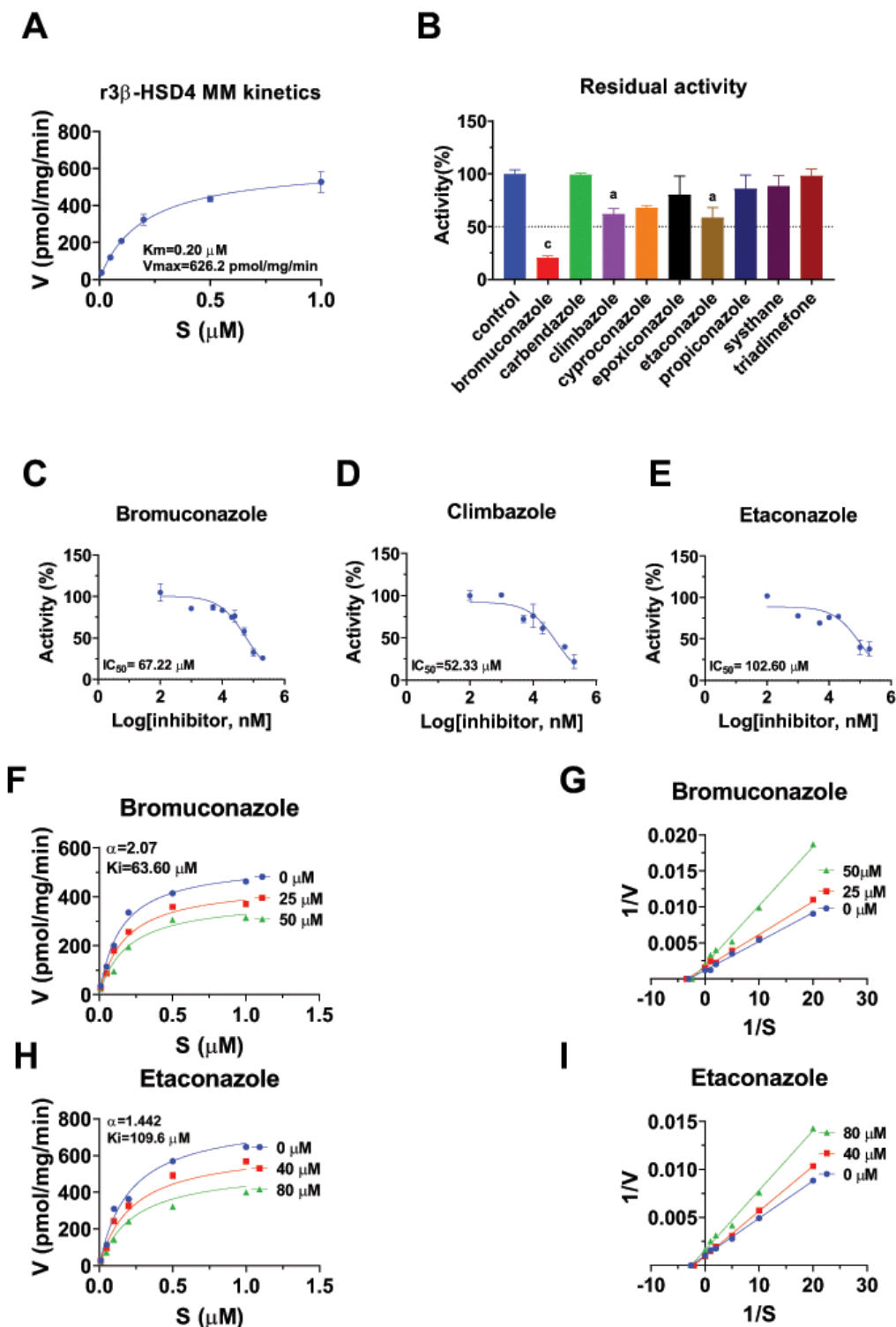
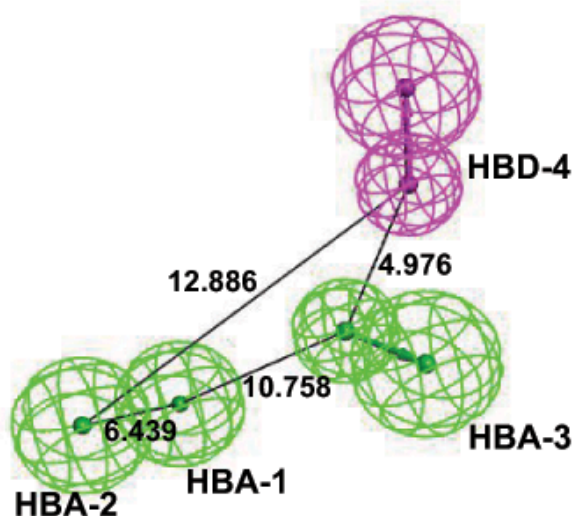


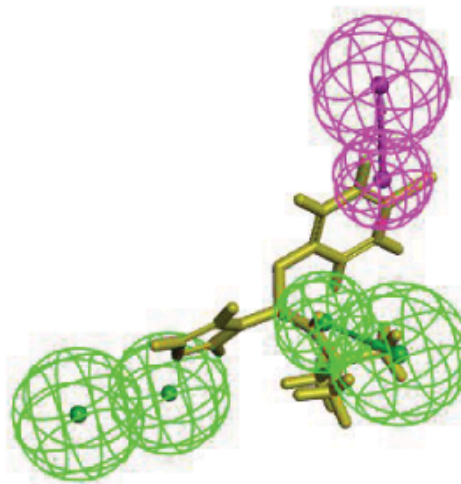
Figure 5

Rat placental 3β -hydroxysteroid dehydrogenase 4 (3β -HSD4) kinetics and effects of azole fungicides on 3β -HSD4 activity at 100 mM, and dose response. A, Michaelis-Menton constant (K_m) of 3β -HSD4; B, the residual activity relative to control (DMSO); Compared with the control, $***P < 0.001$ ($n = 4$, mean $\pm$ SEM); C-E: IC_{50} values of Bromuconazole, Climbazole and Etaconazole ($n = 4$, mean $\pm$ SEM); F, H, enzyme kinetics-inhibition analysis (mixed model); G, I, Lineweaver-Burk plot analysis of bromuconazole and etaconazole, respectively.

A Hypo1



B Climbazole



$IC_{50} = 6.21 \text{ } \mu\text{M}$
Fit value = 4.89

C Cyproconazole



$IC_{50} = 104.83 \text{ } \mu\text{M}$
Fit value = 4.08

D

LogActiv vs. LogEstimate

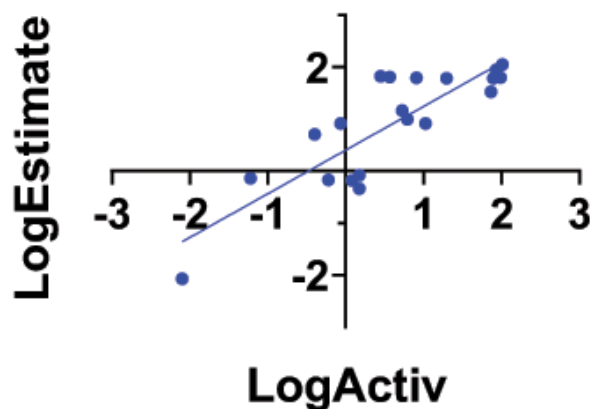
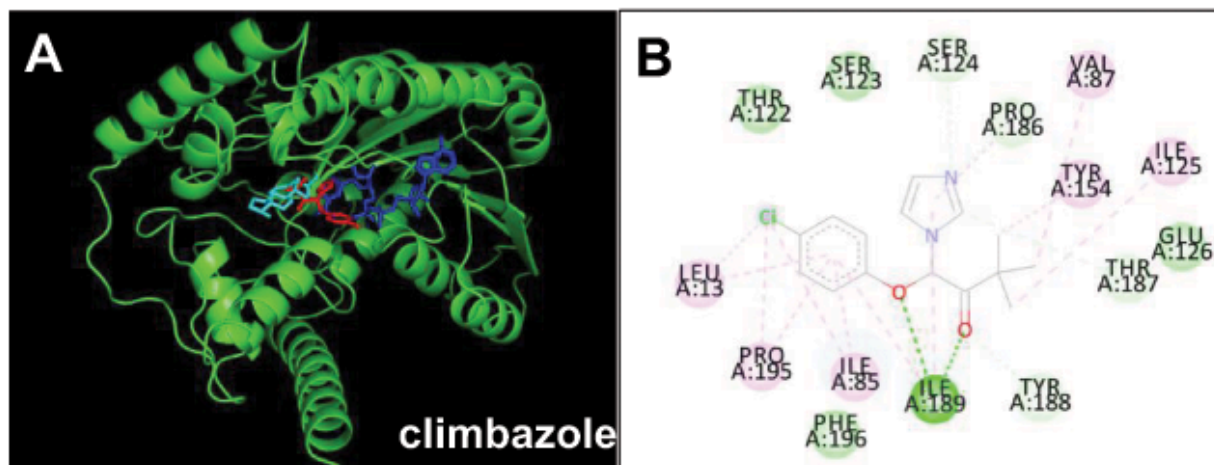


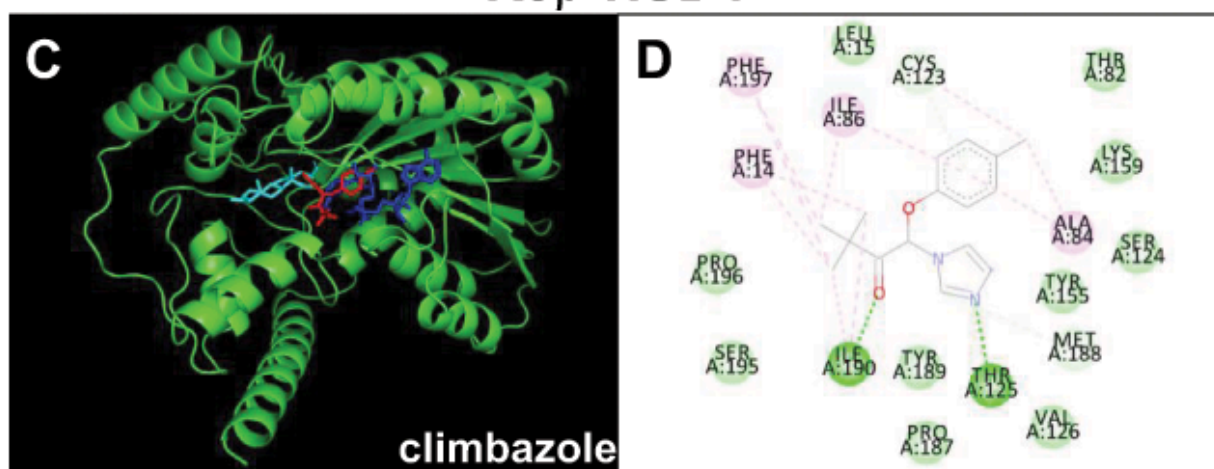
Figure 6

3D-Qsa results: A) Hypo1 comprised HBD and HBA components. B) Aligned with Hypo1, the most potent inhibitor, climbazole (IC_{50} : 6.21 mM); C, the least active compound, cyproconazole (IC_{50} : 104.83 mM). D, The effectiveness of the Hypo1 model was confirmed through correlation analysis between the experimental and predicted IC_{50} values of the training set molecules, indicating strong predictability ($r = 0.73$, $p < 0.0001$)

H3 β -HSD1



R3 β -HSD4



hydrogen bond
 carbon hydrogen bond

van der Waals
 hydrophobic interaction

Figure 7

Molecular docking of climbazole with human 3b-HSD1(A) and rat 3b-HSD4 (C). Each climbazole (red) occupies pregnenolone (cyan)-binding site; (B) and D: 2D superimposed structures: each climbazole analog (purple) overlaps pregnenolone (P5, cyan) contact residues (circled in red). Green dash-line indicates the hydrogen bond with distance

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FIGURES1Regression.jpg](#)
- [FIGURES4DockingH.jpg](#)
- [FIGURES5DOCKINGR.jpg](#)

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