Dihydrotanshinone I-Induced CYP1 Enzyme Inhibition and Alteration of Estradiol Metabolism

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Abbreviations

α-NF, α-naphthoflavone; CE, collision energy; DHTI, dihydrotanshinone I; DP, declustering Potential; E2, estradiol; EE2, ethynyl estradiol; NADPH, nicotinamide adenine dinucleotide phosphate; EROD, ethoxyresorufin O-deethylation; IS, internal standard; MRM, multiple-reaction monitoring; HPLC, high-performance liquid chromatography; P450, cytochrome P450 enzyme; 2-OHE2, 2-hydroxyestradiol; 4-OHE2, 4-hydroxyestradiol.
Abstract

Dihydrotanshinone I (DHTI) is a pharmacologically active component occurring in the roots of the herbal medicine *Salvia miltiorrhiza* Bunge. This study investigated DHTI-induced inhibition of CYPs1A1, 1A2 and 1B1, with the aim to determine the potential effects of DHTI on the bioactivation of estradiol (E2), possibly related to preventive/therapeutic strategy for E2-associated breast cancer. Ethoxyresorufin as a specific substrate for CYP1s was incubated with human recombinant CYP1A1, CYP1A2, or CYP1B1 in the presence of DHTI at various concentrations. Enzymatic inhibition and kinetic behaviors were examined by monitoring the formation of the corresponding product. Molecular docking was further conducted to define the interactions between DHTI and the three CYP1s. The same method and procedure were employed to examine the DHTI-induced alteration of E2 metabolism. DHTI showed significant inhibition of ethoxyresorufin *O*-deethylation activity catalyzed by CYPs1A1, 1A2 and 1B1, in a concentration-dependent manner (IC\textsubscript{50} = 0.56, 0.44 and 0.11 μM, respectively). Kinetic analysis showed that DHTI acted as a competitive type of inhibitor of CYP1A1 and CYP1B1, while it noncompetitive inhibited CYP1A2. The observed enzyme inhibition was independent of NADPH and time. Molecular docking analysis revealed hydrogen-bonding interactions between DHTI and Asp-326 of CYP1B1. Moreover, DHTI displayed preferential activity to inhibit 4-hydroxylation of E2 (a genotoxic pathway) mediated by CYP1B1. Exposure to DHTI could reduce the risk of genotoxicity induced by E2.
Keywords: Dihydrotanshinone I; Estradiol; 4-Hydroxyestradiol; Recombinant human cytochrome P450; Inhibition
Significance Statement

CYP1A1, CYP1A2, and CYP1B1 enzymes are involved in the conversion of E2 into 2-OHE2 and 4-OHE2 through oxidation. 2-OHE2 is negatively correlated with breast cancer risk, and 4-OHE2 may be a significant initiator and promoter of breast cancer. The present study revealed that DHTI competitively inhibits CYP1A1/CYP1B1 and non-competitively inhibits CYP1A2. DHTI exhibits a preference for inhibiting the genotoxicity associated with E2 4-hydroxylation pathway mediated by CYP1B1, potentially reducing the risk of 4-OHE2-induced genotoxicity.
1. Introduction

Salviae Miltiorrhizae Radix et Rhizoma (the dried rhizome of *Salvia miltiorrhiza* Bunge), is a natural herbal with many health-promoting properties. These properties include antitumor function, anti-inflammatory action, and lipid peroxidation inhibitory effect (Zhou et al., 2022). Additionally, Salviae Miltiorrhizae Radix et Rhizoma is widely used for treating cardiovascular diseases such as hyperlipidemia, angina pectoris, and coronary heart disease (Sun et al., 2021; Lv et al., 2022).

Tanshinones, a category of natural diterpenoid quinone compounds, have been identified as the main bioactive ingredients in Salviae Miltiorrhizae Radix et Rhizoma (Fu et al., 2022). The major tanshinones extracted from Salviae Miltiorrhizae Radix et Rhizoma include cryptotanshinone (CTS), dihydrotanshinone I (DHTI), tanshinone I (TI), and tanshinone IIA (TIIA) (Fu et al., 2022; Huang et al., 2022; Jiang et al., 2022; Ke et al., 2023). Among these, TIIA displays potent inhibition of CYP1A2 with IC$_{50}$ value of 0.09 μM and is a potent competitive inhibitor of CYP1A2 ($K_i = 1.0 \mu M$) (Ueng et al., 2003; Qiu et al., 2008). In addition, DHTI has shown inhibitory effects on cytochrome P450 enzymes, including CYPs2J, 3A, and 2C19 with IC$_{50}$ values of 6.39, 13.85, and 0.6 μM, along with multiple pharmacological activities (Hu et al., 2015; Wang et al., 2022).

Cytochrome P450 (P450) is a well-known heme-containing enzyme responsible primarily for the oxidation or reduction of various organic compounds (Lin et al., 2022; Zhang et al., 2022; Xing et al., 2023). The CYP1 family is composed of
CYP1A1, CYP1A2, and CYP1B1. Extrahepatic tissues are the major sites for the
distribution of CYPs1A1 and 1B1, whereas CYP1A2 mostly occurs in the liver (Lo et
al., 2013; Pan et al., 2014). These three members of the CYP1 family play an
important role in phase I metabolism of both exogenous and endogenous compounds,
particularly in the metabolic activation of procarcinogens (Juvonen et al., 2020).
Alterations in CYP1 activity have been linked to cancer risk.

Estradiol (E2) is a major hormone that controls women’s fertility, playing a
critical role in promoting the maturation of female accessory sex organs and the
emergence of secondary sexual characteristics. According to literature, the conversion
of E2 into 4-hydroxyestradiol (4-OHE2) and 2-hydroxyestradiol (2-OHE2) is
facilitated by CYPs1A1, 1A2, and 1B1, which are linked to tumorigenesis or
detoxification, respectively (Scheme 1) (Mao et al., 2019; Malekinejad et al., 2023).
The 4-hydroxylation metabolite of E2 reportedly increases the risk of estrogenic
cancers, such as breast cancer, which is the most prevalent form of cancer among
women and the primary cause of female mortality. (An et al., 2019; Katsura et al.,
2022). Studies have shown that CYP1B1 might be the key enzyme contributing to
E2-associated cancers (Wang et al., 2021; Yi et al., 2023). Since CYP1 enzymes play
a role in deactivating specific anticancer drugs and activating procarcinogens
metabolically, inhibiting CYP1 enzymes has been regarded as an effective strategy for
chemoprevention. Previous studies have shown that almost all CYP1 inhibitors and
associated prodrugs are planar molecules featuring a single aromatic ring, notable
examples of these inhibitors of CYP1B1 include isorhamnetin and 2,4,3',5'-tetramethoxystilbene (Cui and Li, 2014; Li et al., 2017). Due to the similarity in structure with the two inhibitors, we speculate that DHTI might induce CYP1 enzyme inhibition as well. In this study, we examined the interactions between three human recombinant CYP1 enzymes and DHTI, as well as investigated the changes in E2 metabolism in which DHTI participated, with the aims of searching for a chemotherapy and chemoprevention agent for E2-associated cancers.
2. Materials and Methods

2.1 Chemicals and reagents

Dihydrotanshinone I (purity: 98%) was obtained from Shanghai Jianglai Biologic Technology Co., Ltd (Shanghai, China). Shanghai Aladdin Industrial Corporation (Shanghai, China) provided resorufin ethyl ether, estradiol, α-naphthoflavone (α-NF), MgCl₂, K₂HPO₄, KH₂PO₄ and EDTA. Shanghai Baiquan Biotechnology Co., Ltd (Shanghai, China) provided us with human recombinant CYP1A1, CYP1A2, and CYP1B1. Shanghai Macklin Biological Technology Co., Ltd. (Shanghai, China) provided resorufin and propranolol. Ethynyl estradiol (EE2) was acquired from Adamas Technology Co., Ltd. (Shanghai, China). NADPH and L-ascorbic acid were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). All solvents and reagents were of either analytical or HPLC grades. We obtained purified water from Wahaha Co, Ltd located in Hangzhou, China. Thermo Fisher Scientific (Springfield, NJ) supplied all the organic solvents.

2.2 Determination of IC₅₀ values of CYP1s inhibition by DHTI

The inhibition of CYP1 enzyme was estimated using the ethoxyresorufin O-deethylation (EROD) assay (Scheme 1), based on a previously established method (Venkatapura Chandrashekar and Mehvar, 2020; Chen et al., 2022). In brief, CYP1A1, CYP1A2, or CYP1B1 (5.0 nM) underwent incubation with resorufin ethyl ether (ERF: 2.0 μM), and DHTI at concentrations ranging from 0-80 μM, fortified with MgCl₂ at the concentration of 3.2 mM in 0.1 M PBS (0.2 mL, pH 7.4). The incubations were
initiated by the introduction of NADPH at the concentration of 1.0 mM. Furthermore, α-NF (0-10 μM), a specific inhibitor of CYP1s was added to ERF following the previously described method to confirm the role of CYP1s in EROD catalytic metabolism. Following a 10-minute incubation reaction at 37 °C, the incubation reactions were terminated with 0.2 mL ice-cold acetonitrile involving 0.05 μg/mL of propranolol as the internal standard. The mixture was then centrifuged at room temperature for 10 min at a speed of 19,000 g. LC-MS/MS analysis was performed using the supernatants. The IC₅₀ values for α-NF and DHTI, which represent half maximal inhibitory concentration, were determined through curve fitting.

2.3 Determining the kinetic values for the inhibition of CYP1s caused by DHTI

DHTI-induced inhibition of CYP1A1, CYP1A2 or CYP1B1 was assessed using EROD assays. Specifically, CYP1A1 (5.0 nM) was combined with ERF (0.05-5 μM) while being exposed to different levels of DHTI (0, 0.2, 0.5, or 2.0 μM). In the presence of different amounts of DHTI (0, 0.1, 0.5, or 2.0 μM), CYP1A2 (5.0 nM) and ERF (0.05-5.0 μM) were combined. In the presence of different amounts of DHTI (0, 0.1, 0.2, or 1.0 μM), CYP1B1 (5.0 nM) and ERF (0.05-5.0 μM) were combined. The same method as described in section 2.2 was utilized for conducting sample preparation, reaction initiation, and reaction termination. Residual EROD activity was determined by monitoring the formation of resorufin analyzed by an LC-MS/MS system. The remaining enzyme activities were determined by utilizing a well-established standard curve for resorufin (Venkatapura Chandrashekar and
Mehvar, 2020). To generate the calibration standards, resorufin stock solutions were serially diluted with PBS to achieve final concentrations of 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 nM. The resultant samples were mixed with 0.2 mL ice-cold acetonitrile containing 0.05 μg/mL of propranolol, and calibration curves were constructed by plotting the peak area ratios of analytes to the internal standard (resorufin/IS) against the concentrations of resorufin. Linearity was evaluated using the $1/x$-weighted linear regression method.

### 2.4 Time- and NADPH-dependent inactivation of CYP1s by DHTI

The first incubation mixtures comprised of CYP1A1, CYP1A2 or CYP1B1 (0.1 μM), MgCl$_2$ (3.2 mM) and DHTI (0 and 50 μM) in 0.2 mL of 0.1 M PBS. The mixture samples were pre-reacted at 37 °C for 3 min, and reactions were triggered by adding NADPH (1.0 mM). At 0, 5, 10, and 20 min of incubation, 40 μL aliquots from the primary incubation mixtures were transferred to the secondary incubation mixtures consisting of ERF (2.0 μM), NADPH (0.5 mM), and MgCl$_2$ (3.2 mM) in 0.1 M PBS (0.12 mL, pH 7.4). The reactions were incubated for 10 min at 37 °C and terminated by adding an equal volume of ice-cold acetonitrile fortified with propranolol (0.05 μg/mL) as an internal standard. To determine the NADPH-dependence of the inhibitions of the three CYP1 enzymes, negative control experiments were performed by replacing NADPH with the same volume of 0.1M PBS (pH 7.4). The residual catalytic activities were determined following the method described earlier.
2.5 Computer modeling and docking were used to simulate the binding of DHTI with CYP1s

Computer modeling of DHTI was performed using a glide module that utilizes the scoring function from Schrödinger Maestro. The presumed active sites of CYP1 enzymes were targeted for docking DHTI by utilizing the crystal structures of CYP1A1 (PDB ID 4I8V), CYP1A2 (PDB ID 2HI4), and CYP1B1 (PDB ID 3PM0). The energy minimization during docking was performed by the Protein Preparation Wizard using the smart minimization method. Finally, Pymol 2.1 software was employed to display the docking models of DHTI with CYPs1A1, 1A2 and 1B1.

2.6 Determination of inhibition IC\textsubscript{50} values for 2-/4-hydroxylation catalyzed by CYP1s

The incubation samples were prepared by combining DHTI (0-80 μM), MgCl\textsubscript{2} (3.2 mM), NADPH (1.0 mM), and E2 (20 μM) fortified with CYP1A1, CYP1A2 or CYP1B1 (10 nM) in 0.1 mL of 0.1 M PBS with 0.1% ascorbic acid. The combinations were placed in a water bath at a temperature of 37 °C for a duration of 10 min. To halt the process, 500 μL of ethyl acetate, which included EE2 (25 nM) as the internal standard, was introduced. After vortexing for 3 min, the organic layers were collected and concentrated under nitrogen flow. Dissolve the residues in 0.1 mL of acetone containing dansyl chloride (1.0 mg/mL) and 0.1 mL of 0.1 M sodium bicarbonate buffer (pH 9.0). The reaction mixture samples were incubated at 60 °C for 20 min and then cooled on ice for 5 min. Following centrifugation at a speed of 19,000 g the force
of gravity for a duration of 10 minutes, the resultant liquid above the sediment was introduced into the LC-MS/MS system for examination.

2.7 Determination of kinetic parameters for DHTI-caused inhibition of E2 hydroxylation mediated by CYP1B1

Incubations, where E2 at concentrations ranging from 2.0 to 80 μM, CYP1B1 (10 nM), NADPH (1.0 mM), and MgCl₂ (3.2 mM) were included were carried out with various concentrations of DHTI (0, 0.02, 0.1, or 0.5 μM) at 37 °C for 10 min. The resulting metabolites (4-OHE2 and 2-OHE2) were extracted and treated using the identical derivatization method previously explained. Afterward, the resultant blends were subjected to centrifugation, followed LC-MS/MS analysis.

2.8 Instrumentation and chromatographic conditions

The inhibition of EROD and E2 hydroxylation activities of CYP1 enzymes catalyzed by DHTI was evaluated by monitoring the production of de-ethylation and hydroxylated metabolites, respectively. The resulting products were detected by AB Sciex (Applied Biosystems, Foster City, CA) 5500 triple quadrupole mass spectrometer connected online to an ExionLC system (Applied Biosystems, Foster City, CA). The separation through chromatography was achieved by employing a symmetry reverse-phase C₁₈ column (4.6×150 mm, 3 μm) from ACE® ExcelTM (Advanced Chromatography Technologies, Aberdeen, Scotland) at a temperature of 40 °C. Gradient elution was executed to separate resorufin using a mobile phase
consisting of phase A (0.1% formic acid water) and phase B (0.1% formic acid acetonitrile) with a flow rate of 0.6 mL/min. Gradient elution for resorufin was set as follows: 10% B from 0 to 2.0 min; 10-40% B from 2.0 to 6.0 min; 40-90% B from 6.0 to 8.0 min; 90% B from 8.0 to 10.0 min; 90-10% B from 10.0 to 10.5 min; and 10% B from 10.5 to 12.0 min. Gradient elution for derivatized 2-OHE2 and 4-OHE2 was conducted as follows, 12% B for 2.0 min, followed by 12-75% B from 2.0 to 6.0 min; 75% B from 6.0 to 8.0 min; 75-95% B from 8.0 to 10.0 min; 95% B from 10.0 to 11.0 min; 95-12% B from 11.0 to 12.0 min; and 12% B from 12.0 to 13.0 min, the flow rate was operated at 1.0 mL/min, with an injection volume of 5.0 μL, and the column temperature was maintained at 40 °C. Quantification was carried out utilizing an electrospray ionization source (ESI) operating in the mode of positive ions. MRM analysis was conducted using targeted analysis, which involved measuring mass-to-charge ratio (m/z), collision energies (CE), and declustering potential (DP): internal standard propranolol (260.3→116.3, 44, 77), resorufin (214.0→186.0, 35, 70), derivatized metabolites 2-/4-OHE2 (755.0→521.0, 44, 50), and derivatized EE2 (530.0→171.0, 35, 30), respectively. Both the MS and HPLC systems were under the control of Analyst 1.6.2 software.

**2.9 Statistical Analysis**

The experiments were conducted in triplicate, and all acquired data represent the mean ± standard deviation (SD). Statistical significance at $p<0.05$ was determined using SPSS Statistics software. Michaelis-Menten kinetic properties were utilized to
conduct kinetic analyses, evaluating the inhibition of CYP1A1, CYP1A2, and
CYP1B1 induced by DHTI, as well as the changes in E2 metabolism. Lineweaver-Burk plots were used to represent all inhibition data. These graphs provide a quick and visual evaluation of the different types of enzyme inhibition. Competitive inhibitors show identical y-intercepts but demonstrate different slopes and x-intercepts in the two datasets. Plots resulting from noncompetitive inhibition exhibit identical x-intercepts but differ in slopes and y-intercepts. The v-S curves were fitted using the Michaelis-Menten equation by use of GraphPad Prism 9.0 software (GraphPad Co. Ltd., San Diego, CA) through non-weighted nonlinear least-squares regression. The calculation of the inhibitory categories and kinetic measurements ($K_I$ and $K_i$) was performed in the following manner.

Competitive inhibition: $v = \frac{V_{\text{max}} \cdot S}{S + K_m[1+(I/K_I)]}$

Noncompetitive inhibition: $v = \frac{V_{\text{max}} \cdot S}{[S+K_m][1+(I/K_I)]}$

Mixed type of inhibition: $v = \frac{V_{\text{max}} \cdot S}{S[1+(I/K_I)]+K_m[1+(I/K_I)]}$

The maximum velocity and DHTI concentration are represented by $V_{\text{max}}$ and $I$, respectively. The $K_m$ value represents the substrate concentration at half the $V_{\text{max}}$ of the reaction, while the inhibition constants consisted of $K_I$ and $K_i$, which represent the affinity of the inhibitor to both the enzyme-substrate complex and the enzyme, respectively.
3. Results

3.1 DHTI-caused inhibition of CYP1s

CYP1 inhibition caused by DHTI was evaluated using EROD assays. The results showed that DHTI inhibited the activities of CYPs1A1, 1A2 and 1B1 in a concentration-dependent type with IC$_{50}$ values of 0.56 ± 0.14, 0.44 ± 0.08, and 0.11 ± 0.01 μM, respectively (Table 1). Notably, the inhibition efficiency of DHTI against CYP1B1 was significantly higher than that against CYP1A1 and CYP1A2 (p<0.05) (Figure 1). The IC$_{50}$ values of positive control α-NF are listed in Table 1.

3.2 Analysis of the kinetics of CYP1s inhibition caused by DHTI

To further evaluate the inhibition caused by DHTI on the three CYP1 enzymes, kinetic experiments were conducted. GraphPad Prism 9.0 software was utilized to perform nonlinear regression analysis, assessing apparent inhibition patterns and $K_i$ values for competitive or noncompetitive and mixed inhibition. The correlation coefficient (r) ranging from 0.9088 to 0.9932 was used to assess the strength of the relationship between the two measurements. $K_m$ values for CYP1s catalyzed EROD activity were 0.42 ± 0.07 (CYP1A1), 0.95 ± 0.05 (CYP1A2), and 0.68 ± 0.27 μM (CYP1B1), respectively. The corresponding $V_{max}$ values were found to be 22.3 ± 1.98 (CYP1A1), 1.92 ± 0.01 (CYP1A2), and 1.21 ± 0.14 nmol/min/nmol P450 (CYP1B1), respectively. The constructed Lineweaver-Burk plots showed that DHTI induced the competitive inhibition of CYP1A1 ($K_i$ = 0.15 μM) and CYP1B1 ($K_i$ = 0.04 μM), while DHTI displayed a noncompetitive inhibitory effect on CYP1A2 with $K_i$ of 0.71 μM.
The inhibition of CYP1A1 and CYP1A2 by DHTI showed higher kinetic values ($K_i$) compared to CYP1B1, which is consistent with the above IC$_{50}$ results.

3.3 Inhibition of CYP1s by DHTI depends on incubation time and NADPH

As depicted in Figure 3, no progressive CYP1 inhibition mediated by DHTI was found with increasing incubation duration at 37 °C. Further, decreased enzyme activities were observed in the presence and absence of NADPH under the same incubation conditions. The finding suggests that DHTI inhibited CYP1A1, CYP1A2, and CYP1B1 without metabolic activation.

3.4 Molecular docking of DHTI with CYP1s

To provide additional insight into the observed CYP1 inhibition, we conducted computer modeling to examine the binding of DHTI to these enzymes. Our objective was to assess the affinity of DHTI for CYP1A1, CYP1A2, and CYP1B1 and identify the crucial amino acid residue(s) involved in the interaction with DHTI. We used Discovery Studio to analyze the interactions of CYP1 enzymes with DHTI (Figure 4). The molecular docking results demonstrated that DHTI had a good binding property and a high matching degree with the target proteins. The binding energy value order between DHTI and CYP1s was CYP1B1 (-9.233) < CYP1A2 (-9.005) < CYP1A1 (-7.421). The lowest binding energy of DHTI to CYP1B1 exhibited the lowest IC$_{50}$ value in activity inhibition (Table 1). We found that Asp-326 of CYP1B1 interacts...
with DHTI via a hydrogen bond. However, such hydrogen bond was not observed in CYP1A1 or CYP1A2 with DHTI. In addition, other mutual interactions, including π-alkyl, π-π, and others, between CYP1B1 and DHTI are described in Figure 4. There were no significant differences among the three CYP1 members during the process of the formation of hydrophobic interactions with DHTI. According to the docking findings, it appears that Asp-326 in CYP1B1 may play a significant role in the inhibition of the enzyme induced by DHTI.

3.5 DHTI-caused inhibition of 2-/4-hydroxylation of E2 mediated by CYP1s

Coincubation studies are often utilized to test the inhibitory effects of DHTI on CYP1-mediated E2 metabolism. As shown in Figure 5 and Table 3, DHTI displayed a significant inhibition of CYP1B1-catalyzed 2- and 4-hydroxylation of E2 with IC$_{50}$ values of 0.10 ± 0.02 μM and 0.08 ± 0.02 μM, respectively, which was significantly lower than those of CYP1A1 (IC$_{50}$ = 0.13 ± 0.01 and 0.24 ± 0.05 μM) and CYP1A2 (IC$_{50}$ = 0.14 ± 0.02 and 2.14 ± 0.51 μM). Furthermore, the IC$_{50}$ for inhibition of CYP1B1 4-hydroxylation activity was approximately 80% of that for 2-hydroxylation, suggesting that DHTI preferentially inhibited CYP1B1-mediated E2 4-hydroxylation activity.

3.6 Kinetic parameters of DHTI-caused CYP1B1-mediated inhibition of E2 hydroxylation
Based on the above results, it was apparent that DHTI induced a temporary blockage of CYP1B1-mediated 2-/4-hydroxylation of E2. To categorize the types of reversible inhibition, further experiments were performed to analyze the kinetics of DHTI at various concentrations in the CYP1B1 coincubation system. The plots of rate versus vs. substrate concentration in the CYP1B1-catalyzed 2-/4-hydroxylation of E2 exhibited hyperbolic curves (Figure 6A and 6C). The \( K_m \) and \( V_{max} \) values for 2-hydroxylation of E2 by CYPs1A1, 1A2, and 1B1 were 20.2 ± 4.46, 62.0 ± 8.89 and 70.7 ± 3.99 \( \mu \)M, and 3.46 ± 0.71, 8.19 ± 0.75 and 0.13 ± 0.01 nmol/min/nmol P450, respectively. The \( K_m \) and \( V_{max} \) values for 4-hydroxylation of E2 by CYPs1A1, 1A2 and 1B1 were 13.7 ± 3.65, 74.3 ± 6.93 and 64.2 ± 4.24 \( \mu \)M, and 0.20 ± 0.02, 0.78 ± 0.14 and 0.62 ± 0.04 nmol/min/nmol P450, respectively (Table 3). The catalytic efficiency (\( V_{max}/K_m \)) of CYP1-mediated 2- and 4-hydroxylation of E2 were 0.17 and 0.01 mL/min/nmol P450 (CYP1A1), 0.13 and 0.01 mL/min/nmol P450 (CYP1A2), and 0.002 and 0.01 mL/min/nmol P450 (CYP1B1), respectively (Table 3). The calculated activity ratios (2-/4-hydroxylation) for CYPs1A1, 1A2, and 1B1 were 17.0, 13.0 and 0.20, respectively. The findings indicate that CYPs1A1 and 1A2 mainly catalyzed the formation of 2-OHE2, while CYP1B1 was mainly responsible to produce 4-OHE2 (Scheme 2 and Table 3). The observed convergence of the Lineweaver-Burk plots at the same x-intercept point at different DHTI concentrations indicates that the inhibition of 2-/4-hydroxylation metabolism is a noncompetitive
type, with $K_i$ values of $0.27 \pm 0.01$ and $0.14 \pm 0.02$ $\mu$M, respectively (Figure 6 and Table 4).
4. Discussion

Varieties of danshen formulation are available in clinical practice, such as danshen capsules, danshen tablets, and danshen granules. Recommended doses of Danshen capsules, Danshen tablets and Danshen granules are 12 capsules (3.36 g), 12 tablets (3.12 g) and 18 g every day, respectively (Qiu et al., 2010; Zhou et al., 2018; Guo et al., 2020). The content of DHTI varies notably among these formulations. Specifically, the DHTI content ranges from 0.5337 to 1.173 mg/g in danshen granules, from 0.03427 to 0.08358 mg per tablet in danshen tablets, and from 0.0724 to 0.0986 mg/g in danshen capsules (Yang et al., 2022).

The majority of CYP1 inhibitors are characterized by the presence of two or more combined aromatic systems, which exhibit molecular planarity, and DHTI is such a polyaromatic hydrocarbon (PAH) compound. Although DHTI has been proven to show remarkable inhibition of multiple P450 enzymes, its underlying effect on CYP1 activity remains unknown. Estrogen exposure has long been associated with an increasing risk of postmenopausal breast cancer, particularly since the publication of the WHI report in 2002. Two carcinogenic mechanisms are proposed to largely result in the estrogenic cancer. One is the hormonal mechanism of classic estrogen receptor (ER) binding and estrogen signaling, the other is the chemical mechanism of which estrogen as a procarcinogen is converted to the corresponding reactive quinones. E2, a prominent estrogen in women, has long been established as the standard treatment for postmenopausal symptoms for several decades. However, long-term exposure to
estrogen, especially in post-menopausal women, is known to play an important role in breast cancer etiology. Breast cancer is now the most common cancer among women in China, accounting for 9.6% of global breast cancer deaths.

CYP1 enzymes play a crucial role in phase I drug metabolism, overseeing the biotransformation of various endogenous and exogenous compounds. These enzymes can be found not just in tissues outside the liver but also have a wide distribution within liver tissues. CYPs1A1, 1A2, and 1B1 enzymes are primarily responsible for catalyzing the oxidation of E2 to produce 2-/4-OHE2. 2-OHE2 is negatively correlated with breast cancer risk, and 4-OHE2 might play a significant role as an initiator and promoter of breast cancer. The compound 3,4-E2-quinone (3,4-E2-Q) from the biotransformation of 4-OHE2, a known electrophile that leads to DNA adduction and tumorigenesis (Dunlap et al., 2017; An et al., 2019). This metabolic pathway can also generate reactive oxygen species (ROS), leading to oxidative DNA harm. 2-Methoxyestradiol (2-MeOE2), produced by the enzymatic activity of COMT on catechol, functions to prevent further oxidation of 2-OHE2. At the same time, 2-MeOE2 hinders CYP1B1, the enzyme accountable for the 4-hydroxylation process, and is now recognized as a potential candidate for combating metastatic breast cancer. Therefore, 4-OHE2 and 2-OHE2 can be considered genotoxic and detoxification biomarkers, respectively (Scheme 2). E2 undergoes metabolism through CYP1-mediated hydroxylation, which represents the initial and primary step in the metabolic process within human breast tumors. Given the importance of this pathway
in the context of breast cancer prevention and treatment, there has been substantial
interest in investigating the inhibitory impact of exogenous compounds, including
flavonoids, on CYP1B1 in recent years (An et al., 2019).

In the present study, we investigated the inhibitory effects of DHTI on CYP1A1,
CYP1A2, and CYP1B1. Our results demonstrated that DHTI effectively inhibited
EROD activities catalyzed by CYP1A1, CYP1A2, and CYP1B1 in a
concentration-dependent manner, with IC\(_{50}\) values of 0.56, 0.44 and 0.11 μM,
respectively (Figure 1 and Table 1). However, the current conditions did not reveal
any time-dependent and NADPH-dependent inhibition of CYP1s by DHTI (Figure 3),
which suggests that DHTI functions as a reversible inhibitor for CYPs1A1, 1A2, and
1B1 (Tanna et al., 2021). Further kinetic study revealed that DHTI competitively
inhibited CYPs1A1 (\(K_i = 0.15 \mu M\)) and 1B1 (\(K_i = 0.04 \mu M\)), suggesting that DHTI
might occupy the same binding site as the ERF probe substrate. However, DHTI
showed noncompetitive-type inhibition of CYP1A2 with \(K_i\) value of 0.71 μM. Our
observation results indicate that the inhibitory effect of DHTI on CYP1B1 is more
significant than on CYPs1A1 and 1A2 (\(p<0.05\)). In addition, we performed molecular
modeling for DHTI and CYP1s to help elucidate the differences in enzyme inhibition
induced by DHTI. According to previous reports, π-π interactions and hydrogen
bonding identified as significant contributors participate in the interaction of CYP1
enzymes with alkoxy derivatives of heterocyclic compounds (Lo et al., 2013). Our
findings indicate that DHTI formed hydrogen bonds with amino acid residue Asp-326
of CYP1B1, but no such hydrogen bonds were observed for the interaction of DHTI with this amino acid residue in CYPs 1A1 and 1A2. The score results also demonstrated that the affinity of DHTI to CYP1B1 was higher than that of CYPs 1A1 and 1A2. The effectiveness of the observed enzyme inhibition may be attributed to the formed hydrogen bonding between CYP1B1 and DHTI, as indicated by docking scores and molecular interactions with amino acid residues within the active sites of both enzymes.

2-OHE2 and 4-OHE2 are unstable, due to spontaneous oxidation by air. Analytical methods with high sensitivity and specificity are required for the assessment of the two metabolites in complex biological matrices. A derivatization approach with reagent dansyl chloride was established to stabilize the analytes and assist their ionization in mass spectrometry. By utilizing the LC-MS/MS technique, we were able to evaluate the levels of both 2-/4-OHE2 metabolites simultaneously (Scheme 3) (Ziegler et al., 2015).

IC50 value represents the concentration of an inhibitor required to inhibit the corresponding target enzyme by 50%. In the present study, we found that DHTI can slow down the formation of E2 2-OHE2 (IC50 = 0.10 μM) and of 4-OHE2 (IC50 = 0.08 μM). Previous studies have shown that the dose applied to rats (14.5 g/kg) was equivalent to that for the dose in patients (2.30 g/kg), and pharmacokinetic studies showed that the Cmax of blood DHTI can reach the levels in the range of 9.1 μg/L (approximately 0.03 μM) - 21.99 μg/L (approximately 0.08 μM) in rats given danshen.
formulation (Ma et al., 2016; Li et al., 2023). Clearly, the reported $C_{\text{max}}$ values reached or almost reached the IC$_{50}$ required for the inhibition of CYP1B1 responsible for the formation of E2 2-OHE2 and 4-OHE2, we speculate that this may also apply to humans. Particularly, DHTI preferentially inhibits CYP1B1-mediated E2 4-hydroxylation, thereby attenuating the genotoxicity associated with 4-OHE2.

Further enzyme incubation studies demonstrated that CYP1A1 and CYP1A2 were the primary enzymes responsible for the formation of 2-OHE2, whereas CYP1B1 mainly catalyzes the production of 4-OHE2 (Scheme 2), this is in accordance with related research (Itoh et al., 2010). The catalytic efficiency of E2 2-/4-hydroxylation by CYP1A1 was found to be approximately 17-fold greater than those of CYP1B1 (0.20-fold) and CYP1A2 (13-fold) (Table 3). However, DHTI-induced inhibition of CYP1B1 E2 4-hydroxylation displayed an IC$_{50}$ value of approximately 80% of that of 2-hydroxylation, suggesting that DHTI more effectively inhibited 4-OHE2 production than 2-OHE2 formation. Through kinetic analysis, it was discovered that DHTI acted as a noncompetitive inhibitor for E2-2-/4-hydroxylation, exhibiting $K_i$ values of 0.27 μM and 0.14 μM, respectively. Scheme 2 demonstrates the impact of DHTI on the prevention/treatment of E2 chemicals. The results of our research showed that the inhibitory impact of DHTI on the metabolism of E2 oxidation was mainly caused by the 4-hydroxylation pathway mediated by CYP1B1. The accumulation of 4-OHE2 may be reduced due to the inhibition of CYP1B1 caused by DHTI. Given together, our findings suggest that
DHTI inhibition of CYP1B1 may be a possible preventive/therapeutic strategy in human breast cancer.
5. Conclusion

In conclusion, DHTI exhibit competitive inhibitory effects on CYP1A1 and CYP1B1 and act as noncompetitive inhibitors of CYP1A2. The selective inhibition of CYP1B1-mediated E2 4-hydroxylation by DHTI implies that 4-OHE2-related genotoxicity may be attenuated after exposure to DHTI.
**Authorship Contributions**

Liu, Zheng, Li, Mao, and Peng participated in the study design; Liu, Chen, Zhang, Ran, Cheng, Wang, and Liao performed the experiments; Liu and Mao performed the data analysis; Liu and Zheng wrote or participated in the writing of the manuscript.

**Footnotes**

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**Data Availability Statement**

The authors declare that all the data supporting the findings of this study are contained within the paper.

**Conflict of Interest**

The authors declare no conflicts of interest.
Reference


Xing Y, Yu Q, Zhou L, Cai W, Zhang Y, Bi Y, Zhang Y, Fu Z, and Han L (2023) Cytochrome P450-mediated herb-drug interaction (HDI) of Polygonum...


Scheme Legends

Scheme 1. DHTI, E2 and ERF chemical structures.

Scheme 2. Hypothesized impact of DHTI on E2 chemical carcinogenesis. CYPs1A1 and 1A2 are mainly involved in the generation of detoxification biomarker (2-OHE2). CYP1B1 predominantly catalyzes the production of a genotoxic biomarker (4-OHE2), which can undergo further oxidation to form the genotoxic 3,4-E2-Q. DHTI showed potent inhibition of the 4-hydroxylation pathway and weak inhibition of the 2-hydroxylation pathway of E2, as shown with red and green arrows.

Scheme 3. Derivatization reactions of 2-OHE2, 4-OHE2 and EE2 (IS) with dansyl chloride.

Figure Legends

Figure 1. DHTI (A) and α-NF (B) induced inhibition of EROD activity catalyzed by CYP1s. The data are presented as the mean ± SD (n = 3).

Figure 2. Kinetic analysis of DHTI-induced inhibition of EROD activity within a human recombinant CYP1A1, CYP1A2, or CYP1B1 system is illustrated. (A), (C) and (E) present velocity (v) vs. ERF concentration (S) plots for activity in the presence of DHTI at various concentrations as indicated. (B), (D) and (F) display the Lineweaver-Burk plots depicting DHTI-induced inhibition of EROD activity catalyzed by CYP1A1, CYP1A2, and CYP1B1. The data are presented as the mean ± SD (n = 3).
Figure 3. Time- and NADPH-dependent inhibition profiles of CYP1A1 (A), CYP1A2 (B), and CYP1B1 (C) by DHTI are presented. CYP1A1, CYP1A2, or CYP1B1 (0.1 μM) were incubated with either vehicle (●) or DHTI (50 μM) in the presence (●) or absence (○) of NADPH at 37 ºC for 0, 5, 10, and 20 min. The initial enzyme activity at 0 min was normalized to 100%. The data are shown as the mean ± SD (n = 3).

Figure 4. Molecular docking simulations of DHTI with CYP1A1 (A), CYP1A2 (B), and CYP1B1 (C) are depicted. Oxygen atoms are represented in red, and the heme prosthetic group of the CYP enzyme is displayed in light blue, with iron in dark red.

Figure 5. DHTI-Induced inhibition of 2-hydroxylation (A) and 4-hydroxylation (B) of E2 mediated by CYP1 enzymes is illustrated. CYP1A1, CYP1A2, or CYP1B1 (10 nM) were incubated with various concentrations of DHTI (0-80 μM) and E2 (20 μM), followed by the measurement of residual enzyme activity. The data are presented as the mean ± SD (n = 3).

Figure 6. Kinetic analysis of DHTI-induced inhibition of E2 hydroxylation activity in a human recombinant CYP1B1 system is presented. (A) and (C) depict velocity (v) vs. E2 concentration (S) plots of activity with various DHTI concentrations as indicated. (B) and (D) demonstrate Lineweaver-Burk plots illustrating DHTI-induced inhibition of E2 hydroxylation activity mediated by CYP1B1. The data are presented as the mean ± SD (n = 3).
### Table 1. DHTI- and α-NF-induced inhibition of EROD activity ratio of CYP enzyme.

<table>
<thead>
<tr>
<th>CYP1 enzymes</th>
<th>1A1 (μM)</th>
<th>1A2 (μM)</th>
<th>1B1 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHTI</td>
<td>0.56 ± 0.14**</td>
<td>0.44 ± 0.08**</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>α-NF</td>
<td>0.14 ± 0.02</td>
<td>0.02 ± 0.005</td>
<td>0.02 ± 0.001</td>
</tr>
</tbody>
</table>

The data is presented as the mean ± SD (n = 3); *p<0.05, **p<0.01 compared with CYP1B1.
Table 2. Kinetic parameters of DHTI-induced inhibition of EROD activity ratio of CYP enzyme.

<table>
<thead>
<tr>
<th>CYP1 enzymes</th>
<th>Type of inhibition</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>Competitive</td>
<td>0.15 ± 0.03*</td>
</tr>
<tr>
<td>1A2</td>
<td>Non-competitive</td>
<td>0.71 ± 0.07**</td>
</tr>
<tr>
<td>1B1</td>
<td>Competitive</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

The data is presented as the mean ± SD ($n = 3$); *$p<0.05$, **$p<0.01$ compared with CYP1B1.
<table>
<thead>
<tr>
<th>CYP1 enzymes</th>
<th>E2 hydroxylation site</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/min/nmol P450)</th>
<th>$V_{max}/K_m$ (ml/min/nmolP450)</th>
<th>Activity ratio (2-4-)</th>
<th>IC$_{SO}$ (μM)</th>
<th>IC$_{50}$ ratio (2-4-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>2-OHE2</td>
<td>20.2 ± 4.46</td>
<td>3.46 ± 0.71</td>
<td>0.17</td>
<td>17.0</td>
<td>0.13 ± 0.01*</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>4-OHE2</td>
<td>13.7 ± 3.65</td>
<td>0.20 ± 0.02</td>
<td>0.01</td>
<td></td>
<td>0.24 ± 0.05**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-OHE2</td>
<td>62.0 ± 8.89</td>
<td>8.19 ± 0.75</td>
<td>0.13</td>
<td></td>
<td>13.0</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>4-OHE2</td>
<td>74.3 ± 6.93</td>
<td>0.78 ± 0.14</td>
<td>0.01</td>
<td></td>
<td></td>
<td>2.14 ± 0.51**</td>
</tr>
<tr>
<td>1B1</td>
<td>2-OHE2</td>
<td>70.7 ± 3.99</td>
<td>0.13 ± 0.007</td>
<td>0.002</td>
<td></td>
<td>0.20</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>4-OHE2</td>
<td>64.2 ± 4.24</td>
<td>0.62 ± 0.04</td>
<td>0.01</td>
<td></td>
<td></td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

The data is presented as the mean ± SD ($n = 3$); *$p$<0.05, **$p$<0.01 compared with 4-OHE2 of CYP1B1.
Table 4. Kinetic parameters of DHTI-induced inhibition of E2 hydroxylation activity ratio of CYP1B1.

<table>
<thead>
<tr>
<th>E2 hydroxylation site</th>
<th>Type of inhibition</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B1</td>
<td>2-OHE2</td>
<td>Non-competitive</td>
</tr>
<tr>
<td></td>
<td>4-OHE2</td>
<td>Non-competitive</td>
</tr>
</tbody>
</table>

The data is presented as the mean ±SD ($n = 3$); *$p<0.05$, **$p<0.01$ compared with 2-OHE2.
Scheme 1
Scheme 2
Scheme 3

Dansyl chloride + derivatized 2-OHE2/4-OHE2/EE2

2-OHE2: \( R_1=\text{OH}, R_2=\text{H}, R_3=\text{H} \)

4-OHE2: \( R_1=\text{H}, R_2=\text{OH}, R_3=\text{H} \)

EE2: \( R_1=\text{H}, R_2=\text{H}, R_3=\text{C}\equiv\text{CH} \)
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A. 2-OHE2

B. 4-OHE2

DHTI (µM) vs. % control for 2-OHE2 and 4-OHE2 hydroxylation activities by CYP1A1, CYP1A2, and CYP1B1.
Figure 6