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A hydroxylated flavonol, fisetin inhibits the formation of a carcinogenic estrogen metabolite

Xin Meng *, Hui Sun, Lianrong Yang, Rui Yin, Lehui Qi

School of Pharmacy, Heilongjiang University of Chinese Medicine, No. 24 Heping Road, Harbin 150040, PR China

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ABSTRACT

Fisetin can be found in a wide variety of plants and possesses strong efficacy against many cancers. 17β-Estradiol (E2) is hydrolyzed to 4-hydroxy-E2 (4-OHE2) via cytochrome P450 (CYP) 1B1 in vivo. In estrogen target tissues including the mammary gland, ovaries, and uterus, CYP1B1 is highly expressed, and 4-OHE2 is predominantly formed in cancerous tissues. Herein, we investigated the inhibitory activity of fisetin and flavone against CYP1B1 using estrogen E2 as substrate in vitro to reveal structure-activity relationship between structure of flavonoids and inhibition. The results showed that fisetin possessed inhibitory effect on CYP1B1 activity. Compared with flavone, the inhibition of fisetin was stronger. The V_{max} and K_i values were 1.950 ± 0.157 pmol/μg protein/min and 4.925 ± 0.689 nM for fisetin and 2.277 ± 0.231 pmol/μg protein/min and 9.148 ± 2.150 nM for flavone, respectively. By kinetic analyses, both fisetin and flavone displayed mixed inhibition. Taken together the data suggested that fisetin is able to inhibit the formation of carcinogenic 4-OHE2 from E2, which reveals one of its anti-cancer mechanisms and helps to reveal the relationship between the structure of flavonoids and the inhibition CYP1B1 for discovering new drugs in cancer therapy and prevention.

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1. Introduction

Fisetin, 3,3′,4′,7-tetrahydroxyflavone (Fig. S1), a small phytochemical molecule, can be found in a wide variety of plants [1]. Studies have shown that fisetin possessed strong efficacy against many cancers, suggesting that fisetin is a promising and potential anticancer agent [2–4]. In recent studies, the prolonged exposure of estrogens is well known to play a role in breast, ovary, and endometrial cancers etiology [5]. The mechanism of estrogen carcinogenicity is related to the initiation and promotion process affected by estrogen metabolites [6,7]. 17β-Estradiol (E2), one of the major estrogens, is hydrolyzed for catechol estrogens by cytochrome P450 (CYP) 1B1, and mainly converted to 4-hydroxy-E2 (4-OHE2) [8]. Several types of DNA damage are caused by 4-OHE2, such as oxidized DNA bases, DNA strand breakage, and adduct formation by reactive aldehydes derived from lipid hydroperoxides [9,10]. It was found that 4-OHE2 was capable of causing a loss of heterozygosity at doses as low as 0.007 nM [11]. In human tissues examined, CYP1B1 is differentially expressed between tissues, with the highest levels in a wide variety of tumors including those arising from hormone-responsive tissues, in contrast to typically very low or even undetectable amounts in normal extra-hepatic tissues [12,13]. Regarding CYP1B1 expression in normal or cancer tissues, the inhibition of hydroxylation of E2 by CYP1B1 has been postulated to be important for the estrogen related carcinogenesis such as endometrial cancer, breast cancer. The present study of fisetin is based on the recently completed studies, where it has been shown to modulate different pathways activated in various cancer cells [14–16]. However, it is not known whether fisetin possesses inhibition of CYP1B1 activity. Compared with flavone (Fig. S1), our study investigated the inhibitory activity of fisetin against CYP1B1 using estrogen E2 as substrate in vitro, and the inhibition kinetics was studied in vitro models. This study reveals one of fisetin anticancer mechanisms and helps to reveal the relationship between the structure of flavonoids and the inhibition CYP1B1 for discovering new drugs in cancer therapy and prevention.

Abbreviations: E2, 17β-estradiol; 2-OH-E2, 2-hydroxy-17β-estradiol; 4-OH-E2, 4-hydroxy-17β-estradiol; CYP1B1, cytochrome P450 (CYP) 1B1; Δ6-E1, Δ6-estrone; IC50, 50% inhibition.

* Corresponding author.
E-mail address: mengxin52309@163.com (X. Meng).

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2. Experimental

2.1. Chemicals

HPLC grade fisetin and flavone were purchased from Extrasynthese (Genay, Cedex, France). Δ^6-estrone (Δ^6-E1), E2, 2-OHE2 and 4-OHE2 were obtained from Sigma Chemical Co. (St. Louis, MO). β-$NADP^+$, glucose-6-phosphate and Glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Ltd., Osaka, Japan), recombinant human CYP1B1 supersomes (Gentest Corporation via BD Biosciences, San Jose, CA), All the other chemicals and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Metabolism of E2 by CYP 1B1 in vitro

The metabolism for CYP1B1-catalyzed E2 was measured at increasing concentrations of E2 (0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 μM), respectively. The reaction mixtures were preincubated at 37 °C for 5 min, which contain Tis-HCl buffer (pH 7.4, 37 °C) (50 mM), $MgCl_2$ (5 mM), ascorbic acid (0.5 mM), β-$NADP^+$ (0.5 mM), G6P (5 mM), EDTA (50 μM), G6PDH (1 U ml^{-1}) [17]. Reactions were initiated by addition of recombinant microsomes CYP1B1 (5, 10, 20 pM). Incubations were performed in a shaking water bath at 37 °C for 20 or 30 min, respectively. The reactions were terminated by heating in a water bath at 95 °C for 2 min and immediately cooled in ice-water.

2.3. Inhibitory effect of fisetin on E2 metabolism

Briefly, the reaction mixtures, described above but with fisetin (0, 0.005, 0.05, 0.5, 5 μM) or flavone (0, 0.005, 0.05, 0.5, 5 μM), were incubated at 37 °C for 30 min. The concentration of E_2 was 20 μM, with 10 pM recombinant microsomes CYP1B1. The method of metabolism for CYP1B1-catalyzed E2 was as above.

2.4. Enzyme inhibition kinetics

The flavonoid compounds concentration was set at IC50. The enzyme kinetics for CYP1B1 was measured at increasing concentrations of E2 (0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 μM), respectively [18]. Incubations were performed for 30 min. The method of metabolism for CYP1B1-catalyzed E2 was described as above. Then samples were analyzed by HPLC. For the inhibition kinetics studies, V_{max} and K_m values were determined by the nonlinear regression curve fit using the Michaelis–Menten equation by GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

2.5. Extraction and HPLC analysis

500 pM Δ^6-E1 was added into the samples as internal standard. The reaction mixture was extracted with 5 ml of methanol. After centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to glass vials. The mixtures were extracted 3 times. Then ascorbic acid (25 nmol) was added to the supernatant and evaporated to dryness at 37 °C under a light stream of nitrogen. Samples were dissolved in 50 μl methanol.

The conditions of HPLC were as follows: Briefly, the samples (5 μl) were injected into an Mightisil RP-18 GP 250–3.0 (5 μm) HPLC column (KANTO CHEMICAL Co., INC.) for separation followed by detection by an ESA 5010 analytical cell (E1 = +300 mV, E2 = +900 mV) (ESA Coulochem II, ESA, Inc., Chelmsford, MA). A guard cell (ESA 5020) was placed in line before the injection loop and set at a potential of +500 mV. The mobile phase, consisted of 45% $NH_4H_2PO_4$ buffer (pH = 3, 37 °C) and 55% methanol, delivered at a flow rate of 0.5 ml min^{-1} [19].

2.6. Statistical analysis

Results were expressed as mean ± standard deviation (SD) for experiments performed at least in quadruplicate. Statistical significance of differences was evaluated using two-tailed unpaired ANOVA followed by Dunnett's multiple comparison tests.

3. Results

3.1. Metabolism of E2 by CYP 1B1 in vitro

Initial experiments involved E2 kinetics assessment by measuring the rate of 4-OHE2 production. As shown in Fig. S2, at the concentration of 5, 10, 20 pM CYP1B1 followed Michaelis-Menten kinetics, which were incubated for 30 min. Apparent kinetic parameters for CYP1B1 were 0.298 ± 0.023, 0.309 ± 0.018, 0.926 ± 0.029 μM (K_m) and 2.333 ± 0.133, 1.759 ± 0.165, 2.310 ± 0.142 pmol/min/pmol of enzyme (V_{max}). The activity of CYP1B1 decreased with increasing concentration. At the concentration of 10 pM CYP1B1 followed Michaelis-Menten kinetics, which were incubated for 20, 30 min. Apparent Kinetic parameters were 1.859 ± 0.158, 0.309 ± 0.018 μM (K_m) and 2.477 ± 0.1784, 1.759 ± 0.065 pmol/min/pmol of enzyme (V_{max}). The results showed that the activity of CYP1B1 decreased with prolonged incubation time.

3.2. Inhibitory effects of fisetin and flavone on E2 by CYP1B1

Fisetin and flavone were investigated for their inhibition of CYP1B1 activity in vitro using estrogen E2 as substrate. Percentage of CYP1B1 activity was plotted against flavonoid concentration and IC50 values were estimated by sigmoidal dose–response curves (Fig. 1). Flavone exhibited extremely potent inhibitory effect on CYP1B1 activity with IC50 of 1.238 ± 0.197 μM. Compared with flavone, the 3,3′,4′,7-tetrahydroxyflavone fisetin with IC50 of 0.774 ± 0.113 μM showed slightly stronger inhibitory activity against CYP1B1.

3.3. Enzyme inhibition kinetics

A kinetic study of formation of 4-OHE2 from E2 catalyzed by human recombinant CYP1B1 was analyzed in the presence of fisetin (0.5 μM) and flavone (1 μM) using Michaelis–Menten kinetics model (Fig. 2). The concentration of flavonoid compounds was set at 50% inhibitory concentration. The V_{max} and K_i values were 1.950 ± 0.157 pmol/μg protein/min and 4.925 ± 0.689 nM for fisetin and 2.277 ± 0.231 pmol/μg protein/min and 9.148 ± 2.150 nM for flavone, respectively. By the Lineweaver–Burk Curve analysis (Fig. 3), both of fisetin and flavone displayed mixed inhibition, which shows a line intersect the original enzyme-substrate plot in the second quadrant.

4. Discussion

In the present study, compared with flavone, we demonstrated the inhibitory effect of fisetin (3,3′,4′,7-tetrahydroxyflavone) on CYP1B1 using estrogen E2 as substrate in vitro models. Fisetin is found in strawberry, apple, persimmon, grape, onion, cucumber and displays a variety of biological effects including antioxidant, anti-inflammatory, anti-carcinogenic and in vitro anti-angiogenesis [20]. CYP1B1 exhibits catalytic activity for the NADPH-dependent oxidative metabolism of E2 to yield 4-OHE2. This activity of CYP1B1 is attributed to the presence of a hydrogen bond between E2 and CYP1B1 [21]. If the catechol metabolite (4-OHE2) is not eliminated by the process of conjugation (methylation, glu-

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