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Original Article

Expression of cytochrome P450 enzymes and drug transporters is unaffected by the bioactive compound cyasterone from Cyathula officinalis Kuan

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Abstract: Objectives: The goal of this study was to assess potential herb-drug interactions of the bioactive compound cyasterone from *Cyathula officinalis Kuan.* The effects of cyasterone on cytochrome P450 enzymes (CYP450s) and drug transporter expression were investigated. Materials and Methods: HepG2 cells and C57BJ/6 mice were treated with cyasterone. mRNA and protein levels of cytochrome P450 enzymes and drug transporters were determined in HepG2 cells and mouse liver tissue using qRT-PCR and western blot methods, respectively. In addition, mRNA levels of nuclear receptors were determined using qRT-PCR. Results: Compared with controls, cyasterone did not induce or repress mRNA expression of cytochrome P450 enzymes (CYP3A4, CYP3A5, CYP1A2, CYP2C8, CYP2C19, CYP2D6 and CYP2E1) or drug transporters (P-GP, BCRP, BSEP, MRP2, MRP3, MRP6, SLCO1B1, SLCO1B3 and OCT1) (P>0.05) *in vitro*. Similarly, mRNA expression of nuclear receptors, such as PXR, FXR, and LXRα, which are regulated upstream of cytochrome P450 enzymes and drug transporters, were unaffected by cyasterone (P>0.05). *In vivo* studies confirmed that cyasterone did not influence mRNA expression of CYP3A11 or P-GP, nor protein expression of P-GP in mouse liver tissue (P>0.05). Conclusions: As cyasterone did not influence expression of cytochrome P450 enzymes or drug transporters, potential herb-drug interactions are unlikely to occur upon co-administration of cyasterone with other drugs that metabolized by cytochrome P450 enzymes or transported by drug transporters.

Keywords: Cyasterone, cytochrome P450 enzymes, drug transporters, herb-drug interactions

Introduction

Use of herbal medicines has been increasing worldwide [1-3], in part because they are generally considered safe owing to their natural source and long history of usage. However, increasing data shows that herbal medicines can induce drug interactions and endanger patients' health because, like synthetic drugs, they can also induce or inhibit activity of cytochrome P450 enzymes and drug transporters [4-6]. For example, St. John's wort (hypericum perforatum) and grapefruit juice have been reported to induce or inhibit the expression and activity of cytochrome P450 enzymes and drug transporters, which can change the efficacy and safety of anti-cancer drugs, anti-hypertension drugs, immunosuppressants, oral contraceptives, and lipid-lowering agents [7, 8]. Cyasterone, one of the main active components isolated from the dried root of Cyathula officinalis Kuan, is considered in Traditional Chinese Medicine to exhibit properties, such as activation of blood circulation to remove blood stasis, promotion of articular motion, and diuresis, and relief of stranguria. Pharmacological studies have also demonstrated that cyasterone has several pharmacological activities, including anti-cancer and anti-osteoporosis effects [9, 10]. However, it remains unclear whether cyasterone can cause herb-drug interactions like other active herbal components. Therefore, in this study, the modulatory effect of cyasterone on expression of cytochrome P450 enzymes and drug transporters *in vitro* and *in vivo* was investigated, and assessed the possibility of cyasterone-induced herb-drug interactions.

Methods

Reagents and animals

Cyasterone (No. 111804-201504) was purchased from National Institutes for Food and

Drug Control (Beijing, China). CCK8 test kit was purchased from Dojindo Laboratories (Kumamoto, Japan). P-glycoprotein and β-tubulin antibodies were purchased from Abcam Corporation (Cambridge, UK) and Yi Fei Xue Biotechnology Corporation (Nanjing, China), respectively. C57BL6/J mice were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China).

Cell viability assay

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere with 5% CO_2 at 37°C. Cell viability was measured by CCK8 colorimetric assay as previously described [11, 12]. Briefly, cells were seeded into 96-well plates at a density of 5×10^4 cells per well. Different concentrations of cyasterone were added when plates were seeded. After incubation for 48 hours, 10 μL of CCK-8 reagent was added to each well and incubated at 37°C for an additional 2.5 hours. Absorbance was measured at 450 nm using a spectrophotometer.

Cell treatment

Cyasterone was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 20 mM and stored at -20°C. HepG2 cells were grown to sub-confluence in 75-cm^2 flasks and washed twice with PBS prior to treatment with 20 μM cyasterone in fresh medium for 48 hours. Mock treatments with an identical volume of DMSO were used as controls.

Animal treatment

Twelve female C57BJ/6 mice (18-20 g) were housed in a temperature-controlled room with 12-hour light-dark cycles, and free access to rodent chow and water. After a 1-week acclimation period, mice were randomly assigned into two groups (n=6 per group): control and cyasterone treatment. Mice in the control group were orally administered 0.5% carboxymethyl cellulose sodium, while mice in the cyasterone group were orally administered to 20-mg/kg cyasterone (cyasterone was dissolved in 0.5% carboxymethyl cellulose sodium at a concentration of 2.5 mg/mL, temporary preparation before use). After 7 days of treatment, animals were anesthetized and then sacrificed by cervical dislocation. Liver tissues were excised and frozen immediately in liquid nitrogen and stored at -80°C. All animal care and experimental protocols were approved by People's Hospital of Leshan.

Real-time quantitative PCR (qRT-PCR)

Total RNA was isolated from HepG2 cells and mouse liver tissues using Trizol reagent (Takara, Dalian, China). cDNA synthesis was performed using a PrimeScript™ RT reagent kit (Takara, Shiga, Japan). mRNA expression of cytochrome P450 enzymes and drug transporters was determined by qRT-PCR with primer sets listed in **Table 1**. GADPH was used as an internal control for qRT-PCR.

Western blot

Total protein from mouse liver tissues was extracted in RIPA lysis buffer. Protein concentrations were determined using the BCA method. Equal amounts of protein were electrophoresed by 8% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. After blocking in 5% nonfat milk dissolved in TBST for 1 hour at room temperature, membranes were incubated with a diluted solution of primary antibodies against P-GP (1:1000) and β-tubulin (1:5000). After incubation with primary antibodies at 4°C overnight, membranes were probed with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Blots were subsequently washed with TBST and incubated with enhanced chemiluminescence reagents (Millipore, Bedford, MA). Protein bands were analyzed using a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA).

Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Dunnett's test was performed for cell viability analysis, while unpaired t-tests were performed for other analyses using Prism 6.0 (GraphPad Software, La Jolla, CA). All statistical tests were two-tailed; a *P*-value of <0.05 was considered statistically significant.

Results

Effect of cyasterone on HepG2 cell viability

Cyasterone treatment led to a significant decrease in HepG2 cell viability (**Figure 1**). When

Table 1. qRT-PCR primer sequences

Gene	Forward Primer	Backward Primer
Human CYP1A2	AACAAGGGACACAACGCTGAAT	GGAAGAGAAACAAGGGCTGAGT
Human CYP2C8	GCAGGAAAAGGACAACCAAA	GTGTAAGGCATGTGGCTCCT
Human CYP2C19	ACAGATAGTGAAATTTGGAC	TTCATGCCTTTCTCAGCAGG
Human CYP2D6	CTAAGGGAACGACACTCATCAC	CTCACCAGGAAAGCAAAGACAC
Human CYP2E1	GTGATGCACGGCTACAAGG	GGGTGGTCAGGGAAAACCG
Human CYP3A4	CACAGATCCCCCTGAAATTAAGCTTA	AAAATTCAGGCTCCACTTACGGTG
Human CYP3A5	ATCGAAGGTCTTTAGGCCCAG	CTTCCCGCCTCAAGTTTCTC
Human MRP2	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
Human MRP3	GTCCGCAGAATGGACTTGAT	TCACCACTTGGGGATCATTT
Human MRP6	TGTCGCTCTTTGGAAAATCC	AGGAACACTGCGAAGCTCAT
Human P-GP	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTCGGCATTA
Human BCRP	CAGGTGGAGGCAAATCTTCGT	ACCCTGTTAATCCGTTCGTTTT
Human BSEP	TTGGCTGATGTTTGTGGGAAG	CCAAAAATGAGTAGCACGCCT
Human SLCO1B1	TCTCTATGAGATGTCACTGGAT	TGAACACCGTTGGAATTGC
Human SLCO1B3	CAACCCAACGAGAGTCCTTAGG	GTCCAGTCATTGGCTTTGCA
Human OCT1	TAATGGACCACATCGCTCAA	AGCCCCTGATAGAGCACAGA
Human PXR	GCCCATGCTGAAATTCCACTA	GCCGATTGCATTCAATGTAGGA
Human FXR	GACTTTGGACCATGAAGACCAG	GCCCAGACGGAAGTTTCTTATT
Human LXRα	ACACCTACATGCGTCGCAAG	GACGAGCTTCTCGATCATGCC
Human β-actin	GGATGCAGAAGGAGATCACTG	CGATCCACACGGAGTACTT
Mice CYP3A11	GGCCCAGTGGGGATAATGAG	TTCCACTGGTGAATGTGGGG
Mice P-GP	AGGCCGCTGCTTCCATCTTCTGA	CATCACCACCTCACGTGCCACCT
Mice GADPH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

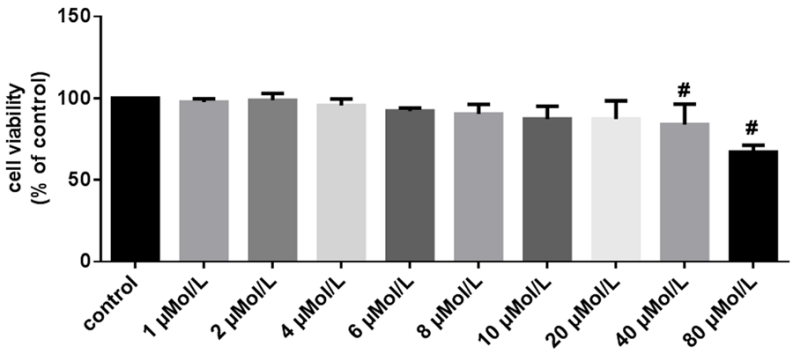

Figure 1. Influence of various cyasterone concentrations on cell viability of HepG2 cells. Data represent mean ± SD of triplicate values. #*P*<0.05 (One-way ANOVA, Dunnett's multiple comparisons test, two-tailed *P* values).

the concentration of cyasterone reached 40 μM, a significant decrease in cell viability (less than 85%) was observed (*P*<0.05). However, pre-treatment with 20 μM cyasterone had no effect on cell viability (>85%). Therefore, concentrations of 20 μM of cyasterone were selected to further investigate effects on cytochrome P450 enzymes and drug transporter expression in HepG2 cells.

Effect of cyasterone on cytochrome P450 enzyme and drug transporter gene expression in vitro

Compared with controls, mRNA expression levels of CYP450 enzymes CYP1A2, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were unchanged in HepG2 cells (*P*>0.05, **Figure 2**). In addition, mRNA expression levels of drug transporters MRP2, MRP3, MRP6, P-GP, BCRP, BSEP, SLCO1B1, SLCO1B3, and OCT1 were unchanged in HepG2 cells (*P*>0.05, **Figure 3**).

Effect of cyasterone on cytochrome P450 enzyme and drug transporter expression in vivo

To further validate the influence of cyasterone on expression of cytochrome P450 enzymes and drug transporters, CYP3A11 (human CYP-

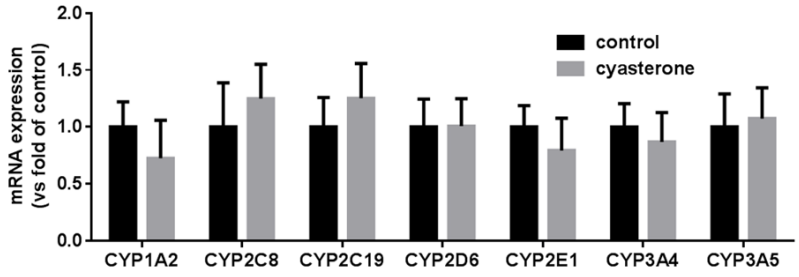

Figure 2. Influence of cyasterone on mRNA expression of cytochrome P450 enzymes in HepG2 cells. Data represent mean ± SD of triplicate values. Control: black column; cyasterone: gray column.

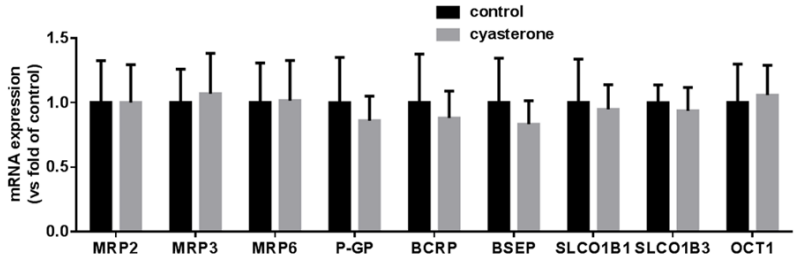

Figure 3. Influence of cyasterone on mRNA expression of drug transporters in HepG2 cells. Data represent mean ± SD of triplicate values. Control: black column; cyasterone: gray column.

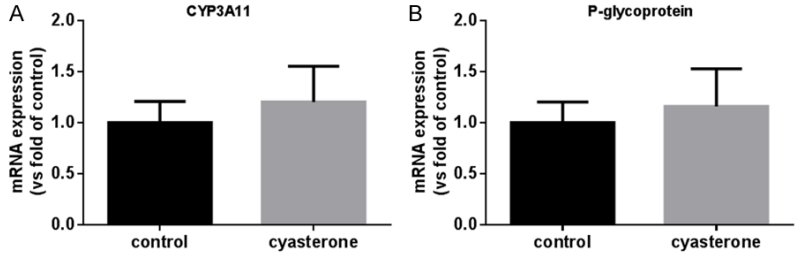

Figure 4. Influence of cyasterone on mRNA expression of CYP3A11 and P-GP in mouse liver. A: CYP3A11; B: P-glycoprotein. Data represent mean ± SD of triplicate values. Control: black column; cyasterone: gray column.

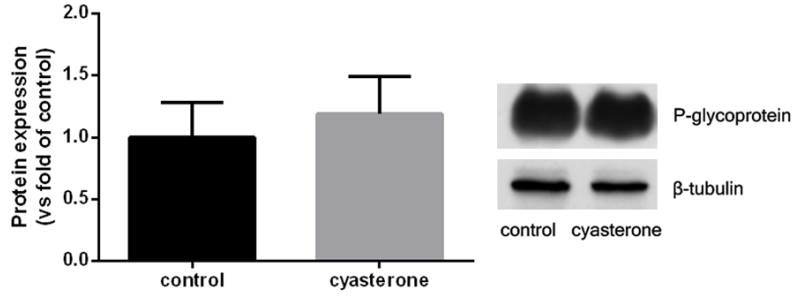

Figure 5. Influence of cyasterone on protein expression of P-GP in mouse liver. Data represent mean ± SD of triplicate values. Control: black column; cyasterone: gray column.

3A4 gene analogue) and P-glycoprotein expression were evaluated in cyasterone-treated mouse livers. mRNA levels of CYP-3A11 and P-glycoprotein were unchanged in the cyasterone group compared with the control group (P>0.05, **Figure 4**). A similar tendency was also observed for P-glycoprotein protein expression (P>0.05, **Figure 5**).

Effect of cyasterone on nuclear receptors in HepG2 cells

Previous studies reported modulation of cytochrome P450 enzyme and drug transporter expression by various nuclear receptors. Thus, whether cyasterone interferes with nuclear receptors in HepG2 cells was investigated. Compared with controls, pregnane X receptor (PXR), farnesoid X receptor (FXR), and liver X receptor alpha (LXRα) were not influenced by cyasterone (P>0.05, **Figure 6**).

Discussion

Herbs are becoming increasingly popular worldwide as complementary alternative medicines for preventing and treating diseases. However, accumulating evidence demonstrates that herbs can change the efficacy and safety of clinically prescribed drugs by altering the expression and activity of cytochrome P450 enzymes and drug transporters [4, 5]. Thus, understanding the influence of herbal components on drug metabolism and transport would allow better assess-

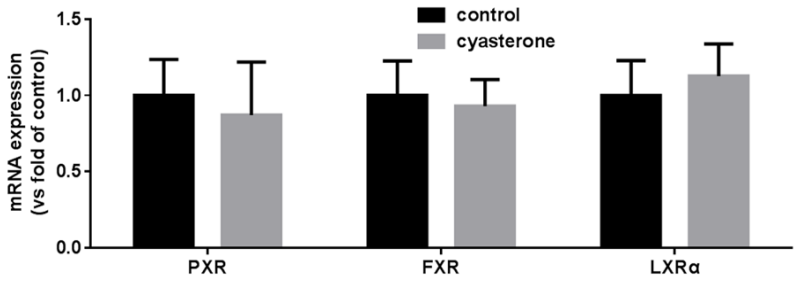

Figure 6. Influence of cyasterone on mRNA expression of nuclear receptors in HepG2 cells. Data represent mean ± SD of triplicate values. Control: black column; cyasterone: gray column.

ment of the potential risks of concurrent use of herbs and pharmaceuticals.

To avoid the influence of cyasterone-induced cytotoxicity on cytochrome P450 enzyme and drug transporter expression *in vitro*, the effect of cyasterone on HepG2 cell viability was first investigated. Pretreatment with 20 μM cyasterone elicited no cytotoxicity of HepG2 cells (>85% viability). Therefore, 20 μM cyasterone was used in subsequent experiments.

To the best of our knowledge, this is the first report that cyasterone does not influence expression of cytochrome P450 enzymes CYP1A2, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5, nor drug transporters MRP2, MRP3, MRP6, P-GP, BCRP, BSEP, SLCO1B1, SLCO1B3, or OCT1 *in vitro*. Similarly, cyasterone did not influence expression of CYP3A11 or P-GP *in vivo*. Additionally, the absence of changes in RNA levels corresponded to an absence of changes in P-GP protein levels. These results show that cyasterone does not modulate the expression of cytochrome P450 enzymes or drug transporters, unlike many other herbal components. For example, Praeruptorin C and D can significantly upregulate CYP3A4 expression [13, 14].

It has been established that gene expression of cytochrome P450 enzymes and drug transporters are regulated by nuclear receptors, such as PXR, CAR, and LXRα [15-17]. For example, ginkgolide B upregulated CYP3A4 and MDR1 expression by activating PXR signaling [18], whereas baicalein-induced CYP3A4 and MDR1 expression by activating CAR and PXR signaling [19]. Therefore, the modulatory effects of cyasterone on common nuclear receptors was further examined. Consequently, expression of nuclear receptors PXR, CAR, and LXRα were found to be unchanged by cyasterone, further demonstrating that cyasterone does not influence the expression of cytochrome P450 enzymes and drug transporters.

Conclusions

Cyasterone did not influence the expression of cytochrome P450 enzymes or drug transporters, suggesting that potential herb-drug interactions are unlikely when cyasterone is co-administered with other drugs metabolized by cytochrome P450 enzymes or transported by drug transporters.

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Disclosure of conflict of interest

None.

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