Inhibitory effects of celastrol on rat liver cytochrome P450 1A2, 2C11, 2D6, 2E1 and 3A2 activity

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ARTICLE INFO

Article history:
Received 13 August 2013
Accepted in revised form 11 October 2013
Available online 19 October 2013

Keywords:
Celastrol
Cytochrome P450 (CYP)
Competitive inhibitor
Mixed-type inhibitor

ABSTRACT

The present study was the first time to investigate the effects of celastrol, derived from Tripterygium wilfordii Hook F. ("Thunder of God Vine"), a traditional Chinese medicine plant, on the metabolism of model probe substrates of CYP isoforms, CYP1A2, CYP2C11, CYP2D6, CYP2E1 and CYP3A2, which are important in the metabolism of a variety of xenobiotics. The effects of celastrol on CYP1A2 (phenacetin O-deethylase), CYP2C11 (tolbutamide 4-hydroxylase), CYP2D6 (dextromethorphan O-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylase) and CYP3A2 (testosterone 6β-hydroxylase) activities were investigated using rat liver microsomes. HPLC-DAD was used to measure the model substrates and metabolites. Inhibition of rat CYP isoforms (IC50) by celastrol in potency order was CYP2C11 (10.2 μM) > CYP3A2 (23.2 μM) > CYP1A2 (52.8 μM) > CYP2E1 (74.2 μM) > CYP2D6 (76.4 μM). Enzyme kinetic studies showed that the celastrol was not only a competitive inhibitor of CYP1A2 and 2C11, but also a mixed-type inhibitor of CYP3A2, with Ki of 39.2 μM, 7.05 μM and 14.2 μM, respectively. The data indicate that celastrol inhibited the metabolism of CYP1A2, 2C and 3A substrates in rat liver in vitro with a different mode of inhibition. These in vitro studies of celastrol with CYP isoforms may be helpful for the development and application of celastrol as a promising anti-cancer agent. Further systematic studies in humans in vitro and in vivo are needed to identify the interactions of celastrol with cytochrome P450s.

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1. Introduction

The plant Tripterygium wilfordii Hook F. ("Thunder of God Vine"), as a traditional Chinese medicine, is used to treat chronic inflammation and autoimmune diseases, and has clinical efficacy in rheumatoid arthritis [1]. In recent years, celastrol (Fig. 1), a triterpenoid isolated from Tripterygium wilfordii Hook F., was reported to possess a number of pharmacological properties such as anti-inflammatory [2] antioxidant [3] and anti-cancer activities [4–7]. Our previous studies have also shown that celastrol effectively inhibited tumor angiogenesis and tumor growth by targeting the AKT/mTOR/P70S6K signaling pathway [7]. Therefore, celastrol is considered as a promising anti-cancer candidate for drug development.

Cytochrome P450 (CYP) are important Phase I enzymes in the biotransformation of xenobiotics. Most drugs undergo deactivation by CYP, either directly or by facilitated excretion from the body. Also, many substances are bioactivated by CYP to form their active compounds. The relative abundance and significance of individual CYP enzyme in human drug metabolism include CYP1A, CYP2C, CYP2D and CYP3A isoform families. Induction or inhibition of the CYP enzymes, after exposure to different drugs and chemicals, is directly linked to a number of drug-induced toxicity and drug interactions leading to treatment failure [8]. Furthermore, CYP-associated metabolic studies in vitro have been considered cost-effective for predicting the potential clinical drug–drug interaction, which is one of the major attritions in drug development and recommended by the US Food and Drug Administration (FDA). Recently it was reported that celastrol inhibited the Phase II drug-metabolic enzymes UGT1A6 and UGT2B7 activities [9].
However, the effects of celastrol on CYP enzymes remain unclear.

The aim of this study is to investigate the effects of celastrol on the metabolism of model probe substrates of specific CYP isoforms in rats, including CYP1A2, CYP2C11, CYP2D6, CYP2E1 and CYP3A2 isoform. In this study, the effects of celastrol on the above-mentioned CYPs activities were investigated in vitro to determine the potential of celastrol in affecting CYP mediated phase I metabolism in rats, employing phenacetin (CYP1A2), tolbutamide (CYP2C11), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1) and testosterone (CYP3A2) as the probe substrates. At the same time enzyme kinetic studies were also conducted to determine the inhibition mode of celastrol on CYPs by using the model CYP probe substrates in the presence of various concentrations of celastrol.

2. Materials and methods

2.1. Chemicals and reagents

Phenacetin, paracetamol, metacetamol, tolbutamide, chlorpropamide, furafylline, dextromethorphan, celastrol, 6β-hydroxytestosterone, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), β-nicotinamide adenine dinucleotide phosphate (NADP), and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-hydroxytolbutamide, 6-hydroxychlorzoxazone and dextromorphan were obtained from Toronto Research Chemical (North York, Canada). Testosterone and ketoconazole were purchased from Tokyo Chemical Industry Co. (Shanghai, China). Chlorzoxazone was purchased from Alfa Aesar (Massachusetts, USA). A 50 mmol/L stock solution of celastrol in dimethyl sulfoxide (DMSO) was prepared and frozen at −20 °C in small aliquots until needed. Acetonitrile and methanol (all HPLC grade) were purchased from Merck (Darmstadt, Germany). Acetic acid glacial (HPLC-grade) was purchased from TEDJA. Ethyl acetate (HPLC grade) was purchased from Fisher chemicals (Leicester, UK). Distilled water was purified in a Milliapore system Milli Q.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch of China. The animals were kept in animal holding room under standard conditions with 12 hr light—dark cycles, with free access to rodent cubes and tap water. Animals were maintained according to the National Institutes of Health standards established in the ‘Guidelines for the Care and Use of Experimental Animals’, and all the experimental protocols were approved by the Animal Investigation Committee of the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University.

2.3. Preparation of rat liver microsomes

In this study, male Sprague–Dawley rats (200–250 g) were fasted overnight and killed by cervical dislocation before removal of the liver. The liver was excised, rinsed with ice-cold saline (0.9% NaCl w/v), weighed and homogenized in a 0.05 mM Tris/KCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 × g at 4 °C for 30 min. The supernatant was then centrifuged at 105,000 × g at 4 °C for 60 min. The pellet was reconstituted with 0.05 mM Tris/KCl buffer (pH 7.4) and stored at −150 °C until use. The protein concentration of the liver microsomes was determined by a protein quantitative assay using bicinchoninic acid [10].

2.4. Assays of CYP1A2 activity in rat liver microsomes

CYP1A2 activity was assessed by formation of paracetamol from phenacetin by the method reported previously [11]. The oxidative metabolism of phenacetin was measured in a system consisting of an NADPH-generating system and microsomes according to the method specified below. The incubation mixture (final volume of 500 μL in 0.05 M Tris/HCl buffer, pH 7.4) consisted of an NADPH-regenerating system (1 mM NADP, 10 mM G6P, 0.4 units/mL G6PDH, 5 mM magnesium chloride and 2 mg/mL rat liver microsomes). For inhibition study, 100 μM phenacetin was used. For kinetic studies, phenacetin concentrations ranged from 25 to 100 μM. The concentrations of celastrol used were from 1.25 to 100 μM. Furafylline, a selective CYP1A2 inhibitor, was used as positive control. The tubes were incubated in Eppendorf Thermomixer at 800 rev/min, 37 °C. The reaction was initiated by adding protein to incubation mixture. After 120 min, incubations were terminated by adding 500 μL ice-cold acetonitrile. The tubes were then centrifuged in microcentrifuge at 13,000 × g for 12 min to precipitate protein. The supernatant was collected and 50 μL was added as internal standard. The whole mixture was then extracted with 500 μL ice-water, and then 50 μL was used for HPLC analysis. HPLC analyses of paracetamol, metacetamol (internal standard) and phenacetin were performed on an Agilent 1260 series instrument with DAD detection at 249 nm. An Agilent reverse phase C18 column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm) with a C18
guard column in a gradient mobile phase containing acetonitrile and water (0.1% acetic acid) at a room temperature and flow rate of 1.0 mL/min. Condition for elution was as follows: 0–5 min, 5% acetonitrile; 5–10 min, 5% to 60% acetonitrile; 10–13 min, 60% to 5% acetonitrile; 13–15 min, 5% acetonitrile. Under the experimental conditions, paracetamol, metacatol (internal standard), and phenacetin were eluted at 7.4, 9.2, and 11.1 min, respectively.

2.5. Assays of CYP2C11 activity in rat liver microsomes

CYP2C11 activity was assessed by formation of 4-hydroxytolbutamide from tolbutilamid [12]. Liver microsomes (1 mg/mL) were incubated with 0.05 M Tris/HCl buffer (pH 7.4) with NADPH-regenerating system (1 mM NADP, 5 mM G6P, 2 units/mL G6PDH, and 5 mM magnesium chloride). The incubation mixture was pre-incubated for 5 min at 37 °C before adding NADPH and 90 min incubation was initiated by NADPH in an Eppendorf Thermomixer at 37 °C thermomixer, at 800 rev/min. Tolbutamide (50 μM) was used in inhibition studies and in enzyme kinetic studies (25 to 100 μM). The final concentrations of celastrol ranged from 2.5 to 25 μM. Sulfaphenazole (5–50 μM), a selective rat CYP2C11 inhibitor, was used as a positive control. The HPLC method for tolbutamide and 4-hydroxytolbutamide was as previously described [12]. Chlorpropamide (500 μg/mL, 20 μL) was used as the internal standard for extraction and HPLC analysis.

2.6. Assays of CYP2D6 activity in rat liver microsomes

CYP2D6 activity was assessed by the formation of dextromethorphan from the CYP2D6 probe dextromethorphan. The incubation mixture (final volume 500 μL, in 0.05 M Tris/HCl buffer, 5 mM MgCl2, pH 7.4) consisted of an NADPH-regenerating system (1 mM NADP, 5 mM G6P, 2 units/mL G6PDH, and 5 mM magnesium chloride). The incubation mixture was pre-incubated for 5 min at 37 °C before adding NADPH and 90 min incubation was initiated by NADPH in an Eppendorf Thermomixer at 37 °C thermomixer, at 800 rev/min. Tolbutamide (50 μM) was used in inhibition studies and in enzyme kinetic studies (25 to 100 μM). The final concentrations of celastrol ranged from 2.5 to 25 μM. Sulfaphenazole (5–50 μM), a selective rat CYP2C11 inhibitor, was used as a positive control. The HPLC method for tolbutamide and 4-hydroxytolbutamide was as previously described [12]. Chlorpropamide (500 μg/mL, 20 μL) was used as the internal standard for extraction and HPLC analysis.

2.7. Assays of CYP2E1 activity in rat liver microsomes

CYP2E1 activity was assessed by the formation of 6-hydroxychloroxazone from the CYP2E1 probe chloroxazone [13]. Rat liver microsomes (0.5 mg) were incubated with incubation buffer (0.05 M Tris/HCl buffer containing 5 mM magnesium chloride, pH 7.4). NADPH-regeneration system (1 mM NADP, 5 mM G6P, 0.4 units/mL G6PDH) and 100 μM probe substrate chloroxazone in a total volume of 500 μL. The incubation mixture was pre-incubated for 5 min at 37 °C before adding NADP. 60 min incubation was initiated by NADP at 37 °C thermomixer at 800 rev/min. The reaction was terminated by adding 500 μL ice-cold acetonitrile. The mixture was centrifuged and the supernatant was extracted with 500 μL ethyl acetate. The organic layer was dried under a gentle stream of nitrogen. The residue was reconstituted with 200 μL mobile phase (87% potassium phosphate buffer, 13% acetonitrile) and 20 μL was injected for HPLC analysis. Phenacetin (500 μg/mL, 50 μL) was used as the internal standard for extraction and HPLC analysis. The incubation mixture was separated through an Aligent ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) at flow rate of 1 mL/min with a gradient elution (A: potassium phosphate buffer (0.01 M, pH 3.4); B: acetonitrile) as follows: 0–5 min, 18% B; 5–9 min, 18% to 35% B; 9–14 min, 35% to 18% B; 14–15 min, 18% B. Aligent Technologies 1260 series HPLC system with a DAD detector set at 287 nm was used for analysis.

2.8. Assays of CYP3A2 activity in rat liver microsomes

CYP3A activity was assessed by formation of 6β-hydroxytestosterone from testosterone by the method reported previously [8,14]. The oxidative metabolism of testosterone was measured in a system consisting of an NADPH-generating system and microsomes according to the method specified below. The incubation mixture (final volume of 500 μL) consisted of an NADPH-regenerating system (1 mM NADP, 10 mM G6P, 0.4 units/mL G6PDH, 5 mM magnesium chloride and 1 mg/mL rat liver microsomes, in 0.05 M Tris/HCl buffer, pH 7.4). Testosterone (100 μM) was used in inhibition study and in enzyme kinetic studies (50–400 μM). The concentrations of celastrol used were from 1.25 to 100 μM. Ketoconazole (0.5–10 μM), a CYP3A-selective inhibitor, was used as positive control. The analysis for testosterone and its metabolites was performed on a Waters reverse C18 column (Symmetry 4.6 × 150 mm, 5 μm) with Phenomenex Security Guard column in a gradient mobile phase containing acetonitrile and water (0.1% acetic acid) at room temperature and flow rate of 1.0 mL/min. Condition for elution was as follows: 0–3 min, 25% acetonitrile; 3–8 min, 25% to 50%; 8–13 min, 50% acetonitrile; 13–15 min, 50% to 25% acetonitrile. 6β-hydroxytestosterone and testosterone were detected at 245 nm. The retention time of 6β-hydroxytestosterone, corticosterone (internal standard) and testosterone was at 6.4, 9.4 and 11.7 min, respectively.

2.9. Statistical analysis

Enzyme kinetic and inhibition parameters estimation as well as statistical analyses were performed using GraphPad Prism 5.0 (GraphPad software Inc., CA, USA). Graphical inspection from different plots was used to determine the inhibition constant (K_i) values and modes of celastrol to different CYP enzymes. These plots included the Lineweaver–Burk plot (obtained by reciprocal of reaction velocities versus reciprocal of substrate concentrations), the secondary plot of Lineweaver–Burk plot for K_i (obtained by the slopes of the regression lines in the Lineweaver–Burk plot versus inhibitor concentrations), the secondary plot of Lineweaver–Burk plot for kcat (obtained by...
y-intercepts of the regression lines in the Lineweaver–Burk plot versus inhibitor concentrations). When $K_i \neq \alpha K_i$ ($\alpha \neq 1$), the mixed inhibition (competitive and non-competitive) type is observed [15]. IC$\text{SO}_0$ values (concentration of inhibitor to cause 50% inhibition of original enzyme activity) were determined by GraFit where appropriate using the following equation:

$$V = \frac{V_0}{1 + (I/IC_{SO_0})^2}$$

where $V_0$ is uninhibited velocity, $V$ is observed velocity, $S$ is slope factor and $I$ is inhibitor concentration. All data were expressed as the mean ± SEM. One-way analysis of variance was used to estimate the significance of differences. $p$-Values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Inhibition of metabolism of model CYP1A2, 2C11, 2D6, 2E1 and 3A2 probe substrates by celastrol in rat liver microsomes

To investigate whether the celastrol affects the catalytic activity of CYP enzymes, the probe reaction assays were conducted with varied concentrations of celastrol. Specific inhibitors of CYP1A2, 2C, 2D6, 2E1 and 3A were used as positive controls. Results showed that celastrol inhibited rat CYP1A2, 2C11, 2D6, 2E1 and 3A2 activities in a concentration-dependent manner (Fig. 2). Inhibition of rat CYP isoforms (IC$\text{SO}_0$) by celastrol in potency order was CYP2C11 (10.2 μM) > CYP3A2 (23.2 μM) > CYP1A2 (52.8 μM) > CYP2E1 (74.2 μM) > CYP2D6 (76.4 μM). At the same time celastrol concentration-dependently decreased the ratios of the metabolite and probe substrate of each CYP isoform (Fig. 3).

3.2. Enzyme inhibition kinetic analysis

To further characterize the inhibition of CYP1A2, 2C11 and 3A2 activities by celastrol, enzyme inhibition kinetic experiments were carried out. From the primary Lineweaver–Burk plot linear transformation of reciprocal of enzyme reaction velocities versus reciprocal of substrate concentrations, Figs. 4A and 5A showed the straight lines intersected on the common point on the ordinate axis, which indicated competitive inhibition of celastrol on CYP1A2 and 2C11. However, as shown in Fig. 6A, the straight lines intersected on the negative x-axis, which indicated a mixed inhibition of celastrol on CYP3A2 activity. The $K_i$ values of celastrol on CYP1A2, 2C11 and 3A2 were obtained from the secondary Lineweaver–Burk plot for $K_i$ with values of 39.2 μM, 7.05 μM and 14.2 μM, respectively (Figs. 4B, 5B and 6B). From the secondary plot of Lineweaver–Burk plot for $\alpha K_i$, value of celastrol on CYP3A2 was 9.53 μM (Fig. 6C). Since $\alpha$ value of CYP3A2 ($\alpha = 1.49$) was unequal to 1, the mixed type inhibition of celastrol on CYP3A2 was obtained, which was also consistent with the results in above mentioned graphical inspection.

4. Discussion

Studying the inhibition of CYP enzymes is necessary to evaluate drug–drug interaction in the drug development since inhibition of CYP enzymes is one of the most common causes of harmful drug–drug interaction and has led to the withdrawal of several marketed drugs in the past few decades [16]. To our knowledge, this study is the first time to investigate the effects of celastrol on the metabolism of probe substrates of several CYP isoforms including CYP1A2, 2C11, 2D6, 2E1 and 3A2.

CYP1A2 is one of the major P450 enzymes in the human liver which accounts for approximately 13% of the total content of this enzyme group [17]. CYP1A2 plays an important role in the metabolism of several clinically used drugs including theophylline, clozapine, and tacrine, and foodborne procarcinogens such as polycyclic aromatic hydrocarbons or imidazoquinoline derivatives [18,19]. Murine and human CYP1A2 showed about 72% amino acid sequence homology with common catalytic activity [20]. Our previous study also found that the Sprague–Dawley rat liver microsomes were useful for assessing drug–drug interaction in the hepatic first-pass metabolism responsible for CYP1A2 at the early drug-discovery stage [21]. The current study showed that celastrol competitively inhibited rat liver microal CYP1A2 activity with the $K_i$ and IC$\text{SO}_0$ values of 39.2 μM and 52.8 μM, respectively. Therefore, celastrol was a weak CYP1A2 inhibitor and the potential drug–drug interaction with CYP1A2 would be low.

The CYP2C subfamily represents an important group of isozymes for drug metabolism in human, as these constitute about 16% of the total hepatic P450 complement, and is known to be responsible for the metabolism of roughly 15% of drug oxidations [22]. Human CYP2C9 is a major CYP enzyme involved in the metabolism of a wide range of therapeutic agents, including non-steroidal anti-inflammatory drugs, oral anticoagulants and oral hypoglycaemic agents [22]. More than 100 currently used drugs are known substrates of CYP2C9 which corresponds to 10–20% of commonly prescribed drugs, such as meloxicam, suprofen, tolbutamide, and S-warfarin. In addition, CYP2C9 also contributes to the metabolism of fatty acids, prostanoids and steroid hormones [23]. The rat forms of the human CYP2C9 equivalent CYP2C isoforms include CYP2C6
and CYP2C11, with CYP2C11 being a homologue of the human CYP2C9 with 77% homology [12]. In the male SD rats, the CYP2C11 isoform is more important since it is the more abundantly expressed CYP, in a way equivalent to CYP3A4 in humans. Recently, many herbs and nature compounds isolated from herbs have been identified as inhibitors of CYP2C enzyme. For example, tanshinones from Danshen competitively inhibited rat CYP2C11 activity, which is also mainly responsible for S-warfarin hydroxylase [24]. In this study, it is clear from the in vitro inhibition kinetic studies in rat liver microsomes that celastrol can act as competitive inhibitor to rat CYP2C11 with a $K_i$ of $7.05\mu M$. The inhibitory effect of celastrol on formation of 4-hydroxytolbutamide was comparable to sulfaphenazole, a selective rat CYP2C11 inhibitor ($K_i = 30.8\mu M$). Therefore, celastrol was a potent CYP2C11 inhibitor. Due to species difference in CYP2C isoform expression, some further human in vitro and in vivo studies should be needed to determine the potential drug–drug interaction with CYP2C enzymes.

The CYP3A subfamily is one of the dominant CYP enzymes in both the liver and extra-hepatic tissues such as intestine and plays an important role in the oxidation of xenobiotics and contributes to the biotransformations of about 60% of currently used therapeutic drugs [25]. Human CYP3A4 is one of the most abundant drug-metabolizing P450 isoforms in human liver microsomes which accounts for approximately 40% of the total P450 [26]. In fact characterization of the CYP3A4 isoform responsible for the metabolism of drugs and herbal constituents is important for the identification of potential drug–drug or herb–drug interactions in humans [27]. The present study showed that celastrol had inhibitory effects in vitro on CYP3A2 isoform, which is the major contributor to testosterone $6\beta$-hydroxylation in male rats, with the $K_i$ and $IC_{50}$ values of 0.5

Fig. 3. Effects of celastrol on metabolite/probe substrate ratios mediated by CYP1A2 (A), CYP2C11 (B), CYP2D6 (C), CYP2E1 (D) and CYP3A2 (E) in rat liver microsomes ($n = 6$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicated significant difference compared to control.
The inhibition of CYP3A2 by celastrol seemed to be a mixed type inhibition because the intersection of Lineweaver–Burk plots of celastrol was on the negative x-axis and α Ki value of celastrol on CYP3A2 (9.53 μM) was unequal to Ki value (14.2 μM, α = 1.49). In mixed inhibition, the inhibitor celastrol can bind to the enzyme CYP3A2 at the same time as the enzyme’s substrate. However, the binding of celastrol may affect the binding of the CYP3A2 substrate, and vice versa. This type of inhibition can be reduced, but not overcome by increasing concentrations of substrate. Although it is possible for celastrol to bind in the active site of CYP3A2, this mixed-type of inhibition generally results from an allosteric effect where the celastrol binds to a different site on CYP3A2. Celastrol binding to this allosteric site may change the conformation of CYP3A2 (i.e., tertiary structure or three-dimensional shape), so that the affinity of the substrate for the active site is reduced. Moreover, since celastrol has a quinol structure, inhibition should occur either by iron chelation or by nucleophilic trapping. However, this mechanistic alternative to the inhibition of cytochrome P450 is still not sure. Therefore, further systematic study is needed to identify the potential relationship of celastrol with CYP3A enzyme in humans. The effects of celastrol on the metabolism of model probe substrates of CYP2D6 and CYP2E1 were also carried out in this study. CYP2D6 and 2E1 were slightly inhibited by celastrol with IC50 at 76.4 μM and 74.2 μM, respectively. Thus, celastrol would appear to have a low potential to cause drug–drug interaction with CYP2D6 and CYP2E1.

The results of this study showed celastrol may affect in vitro the metabolism of drugs which are substrates of the CYP isoforms, especially CYP2C and 3A. Previous in vivo studies reported the plasma Cmax values of celastrol in rats treated with an intravenous dose (100 μg/kg) and an oral dose (1000 μg/kg) were less than 40 μg/L [28]. Therefore, the rat plasma concentrations were much lower than the IC50 and Ki values determined in this study because celastrol administered orally in the rat was poorly absorbed into the systemic circulation. However, the poor absorption of celastrol could be greatly improved when celastrol-containing “Thunder of God Vine” tablets orally administered, and thereby the oral bioavailability of celastrol was significantly increased [28]. Furthermore, due to species difference in ADME and CYP expressions, further system
study in vivo is needed to identify the interactions of celastrol with CYP isofrom in humans.

5. Conclusions

The data in the present study demonstrated that celastrol inhibited the metabolism of CYP1A2, 2C11 and 3A2 substrates in rat liver in vitro with different mode of inhibition. These in vitro studies of celastrol with CYP isofroms may be helpful for the development and application of celastrol as a promising anti-cancer agent. However, the drug–drug interaction potential should be confirmed by further in vivo studies. Further systematic studies in humans in vitro and in vivo are also needed to identify the interactions of celastrol with cytochrome P450.

Conflict of interest statement

None declared.

Acknowledgments

The work described in this paper was supported by grants from National Natural Science Foundation of China (81301908), and the Science and Technology Commission of Shanghai Municipality (11DZ2260300, 12XD1406100 and 13ZR1412600).

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