ORIGINAL RESEARCH

Effects of Saikosaponin D on CYP1A2 and CYP2D6 in HepaRG Cells

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Background: *Bupleurum* is one of the most important traditional Chinese medicines and an ingredient in many compound preparations. It is widely used together with other drugs in clinical practice, and thus there is great potential for drug–drug interactions. Saikosaponin D (SsD) is a major bioactive triterpenoid saponin extracted from *Bupleurum* with anti-inflammatory, anticancer, antioxidative, and antihepatic fibrosis effects. Effects of the main components of *Bupleurum* on cytochromes P450 (CYPs) need to be clarified in the clinical application of combination therapies of formulations containing SsD or *Bupleurum*.

Purpose: This study aimed to investigate the effects of SsD on the CYP1A2 and CYP2D6 mRNAs, protein expression, and relative enzyme activities in HepaRG cells.

Methods: HepaRG cells were cultured with SsD at concentrations of 0.5, 1, 5 and 10 μM for 72 hours. mRNA and protein expression of CYP1A2 and CYP2D6 were analyzed with real-time PCR and Western blot analysis. Relative enzyme activities were analyzed with HPLC based on consumption of the specific probe substrate.

Results: SsD significantly induced expression of mRNA and increased relative activity of CYP1A2 in HepaRG cells after the cells had been treated with SsD at concentrations of 1, 5 and 10 μM. SsD also induced protein expression of CYP1A2 at concentrations of 5 and 10 μM. SsD exhibited an inductive effect on CYP2D6 mRNA and protein expression, while increasing the relative activity of CYP2D6 at concentrations of 5 and 10 μM.

Conclusion: This study is the first to investigate the effect of SsD on CYP1A2 and CYP2D6 in HepaRG cells, and the results may provide some useful information on potential drug–drug interactions related to clinical preparations containing SsD or *Bupleurum*.

Keywords: saikosaponin D, HepaRG cells, drug–drug interactions

Introduction

Traditional Chinese medicines (TCMs) are widely administered concomitantly with Western therapeutic drugs for the treatment of major ailments, due to their easy availability, cost-effectiveness, and a general perception that they are safer than Western drugs. Their versatile prevention and treatment effects are combined with Western drugs for various ailments.[1,2] However, drug interactions between TCM and other drugs occur from time to time, due to the complex components of TCM. *Bupleurum* is a perennial herbaceous plant of the family Umbelliferae that is found in some regions of China (mainly in the provinces of Liaoning, Jiangsu, and Anhui) and other Asian countries (like Japan and South Korea), and has been used to treat fever, tumor, and inflammation diseases.[3] It is also one of the most typical TCM ingredients in a variety of clinical preparations approved by the Chinese National Medical Products Administration.[4] Saikosaponin D (SsD) is a major triterpenoid

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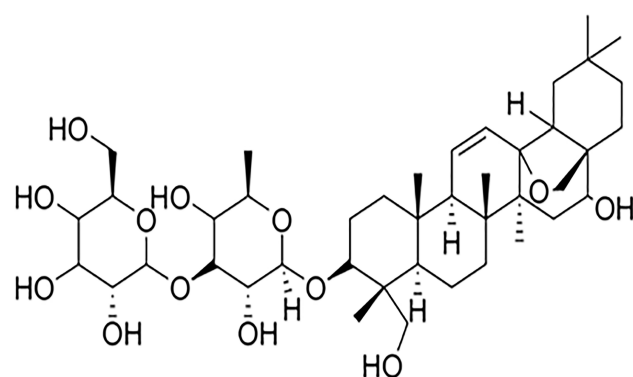Fax +86 851 2864 2334
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Figure 1 Chemical structure of SsD (molecular formula $C_{42} O_{13} H_{68}$).

saponin extracted from *Bupleurum*, and its chemical structure is shown in Figure 1. With its relatively high content and strongest activity in *Bupleurum*,[5] SsD has been proven by many studies to have antitumor,[6] anti-inflammatory,[7] anti–liver damage, and anti-infection effects.[4,8] As such, it is a candidate drug worthy of being developed into clinical preparations. The development of SsA–SsD compound liposomes[9] and SsD liposomes[10] has been reported. However, it is interesting that though SsD is the main bioactive component of *Bupleurum*, its effect on cytochromes P450 (CYPs) has rarely been investigated.

The cytochrome P450 family mediates metabolism of the majority of important drugs,[11,12] and most drug interactions are related to CYPs.[13] At present, CYPs and its isoforms assays are currently used for the screening and metabolism research of new drugs candidates in Europe and the United States, and these are compulsory experiments to be performed for the development of new drug.[14]

In research by Gripon et al, HepaRG cells were isolated from the tissue of a female patient suffering from hepatocarcinoma in 2002. The cells were then differentiated into canaliculus-like and hepatocyte-like cells in the presence of 2% dimethyl sulfoxide (DMSO) and enzyme expression of CYP1A2, 2D6, 2B6, 2C9, and 2E1 examined for drug metabolism and drug interaction.[15] CYP1A2 in HepaRG cells can be induced by omeprazole,[16] and metoprolol can be used to evaluate the activity of CYP2D6 in HepaRG cells.[17] HepaRG cells are widely considered a suitable model for studying drug metabolism and drug–drug interactions due to their gene expression of cellular function consistent with primary human hepatocytes.[18,19,20] We aimed to investigate the effect of SsD on mRNA and protein expression, as well as the enzyme activity of CYP1A2 and CYP2D6, in HepaRG cells. We believe that the results of

this study will provide more evidence for drug interactions related to clinical formulations containing SsD or *Bupleurum*.

Methods
Reagents and Cells
HepaRG cell lines were supplied by Guandao Biological Engineering (Shanghai, China). RPMI 1640 medium and FBS were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trypsin–EDTA 0.25% solution and 1% antibiotics (100 × streptomycin–penicillin) were purchased from Biosharp (Shanghai, China) and HyClone (Logan, UT, USA), respectively. SsD (S114050, 20 mg, ≥98% purity), phenacetin, and dextromethorphan hydrobromide were purchased from Aladdin Bio-Chem Technology (Shanghai, China). MTT, PBS, and DMSO were provided by Solarbio Technology (Beijing, China). GAPDH rabbit polyclonal antibody, CYP1A2-specific rabbit polyclonal antibody, HRP-conjugated AffiniPure goat antirabbit IgG (H+L), and CYP2D6 rabbit polyclonal antibody were obtained from Proteintech (Wuhan, China).

Cell Culture
Undifferentiated HepaRG cells were cultured in RPMI 1640 medium containing 10% FBS, 1% antibiotics (100 × streptomycin–penicillin), and 50 μM hydrocortisone sodium hemisuccinate (basal growth medium) and incubated at a constant 37°C, 5% CO_2, and 95% humidity for 2 weeks. Then, they were incubated in the same culture medium supplemented with 2% DMSO (differentiation medium) for 2 weeks,[15] and then were differentiated into canaliculus-like and hepatocyte-like cells expressing liver-specific functions. Next, differentiated HepaRG cells were seeded at a density of $4.5×10^5$ cells/cm^2 in 96-well plates or six-well plates for follow-up research.[15,21] The medium was renewed every 2–3 days.

In Vitro Cell-Viability Assays
Evaluation of SsD activity in HepaRG cells was undertaken to find a suitable range of concentration for studying drug–drug interaction. Differentiated HepaRG cells were seeded in 96-well plates, incubated for 24 hours, treated with different concentrations of SsD (0.1, 1, 10, 20, 30, and 40 μM) and incubated for 72 hours at 37°C. Meanwhile, untreated cells incubated in medium and the medium without cells were used as the control and the blank, respectively. After incubation, 20 μL MTT solution (5 mg/mL) prepared in

RPMI 1640 medium (without FBS, antibiotics, or hydrocortisone sodium hemisuccinate) was added to each well. After 4 hours' incubation, the MTT containing medium was removed and DMSO (150 μL/well) added to dissolve the formazan crystals. The absorbance of each well was detected with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 490 nm.

Real-Time Polymerase Chain–Reaction Quantification

Differentiated HepaRG cells were seeded in six-well plates, incubated for 24 hours, treated with different concentrations of SsD (0, 0.5, 1, 5 and 10 μM [this range was chosen based on MTT results to achieve cell viability for research needs]) and incubated for 72 hours at 37°C. Total RNA was acquired from HepaRG cells using RNAiso Plus reagent according to the manufacturer's instructions, and total RNA concentration was quantified by absorbance at a wavelength of 260 nm. Total RNA (500 ng) was reverse-transcribed using the PrimeScript RT reagent kit (perfect real-time), and cDNA obtained from reverse transcription was diluted ten times with RNase-free distilled water. TB Green Premix Ex Taq II (TIi RNaseH Plus) was used for quantification. Primers sequences of related genes are shown in Table 1.

Western Blot Assays

The effect of SSd on the protein expression of CYPs in HepaRG cells was determined by Western blot analysis. Differentiated HepaRG cells were seeded, incubated and treated as per the the previous section. Total protein was extracted from HepaRG cells using RIPA cell-lysis buffer containing protease and phosphatase inhibitors on the basis of instructions.

After protein concentration had been measured using the BCA assay, proteins were loaded on sodium dodecyl sulfate–polyacrylamide gels, transferred onto a polyvinylidene fluoride membranes, and blocked with 5% skimmed milk for 2 hours (phosphorylated protein was incubated with 5% BSA). The primary antibodies GAPDH (1:5,000), CYP1A2 (1:2,000), and CYP2D6 (1:1,000) were incubated at 4°C overnight. The secondary antibody HRP-conjugated AffiniPure goat antirabbit IgG (H+L, 1:5,000) was incubated at room temperature for 1 hour. Membranes were washed three times with TBST. Chemiluminescent detection was performed using ECL reagents, and Image Lab Analysis software was used for quantitative analysis of the blots.

Detection of Enzyme Activity

Relative enzyme activity was reflected in the percentage of phenacetin (CYP1A2) and dextromethorphan hydrobromide (CYP2D6) consumption. Previous studies have frequently used 50 μM phenacetin and dextromethorphan for CYP1A2 and CYP2D6 enzyme-activity assays.[22,23] As such, differentiated HepaRG cells were treated the same as outlined in the "Real-time polymerase chain–reaction quantification" section. Later, serum-free medium containing 50 μM phenacetin and dextromethorphan hydrobromide was added and incubated for 12 hours at 37°C. After that, reactions were stopped on ice and cell supernatant (medium) samples stored at −80°C before determining concentrations of phenacetin and dextromethorphan hydrobromide by HPLC. The HPLC method used was adapted and optimized from a previously established method.[24,25] Briefly, chromatographic analysis was performed using an HPLC-DAD system (Agilent 1260 Infinity), and chromatographic separation was achieved on a 150×4.6 mm, 5 μm particle Agilent Extend C18 column at 25°C.

Statistical Analysis

SPSS 20.0 was used for statistical analyses, and results are expressed as means ± SD (n=3). Differences were analyzed with one-way ANOVA or unpaired Student's t-test, and $P<0.05$ was considered statistically significant.

Results
Cell-Viability Tests

The effect of SsD on the viability of HepaRG cells was tested mainly to determine the appropriate concentration without significant toxicity to the cells for subsequent drug-interaction studies. The results of in vitro cell-viability assays shown

Table 1 Primer Sequences for Real-time RT-PCR

Accession number	Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
NM_000761	CYP1A2	ATGCTCAGCCTCGTGAAGAAC	GTTAGGCAGGTAGCGAAGGAT
NG_008376	CYP2D6	ACCAGGCTCACATGCCCTA	TTCGATGTCACGGGATGTCAT
NM_002046	GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

in Figure 2 suggested that SsD had no significant effects on cell viability at a dose <10 μM. SsD concentrations of ≤10 μM were used for subsequent drug-interaction studies.

Effects of SsD on Expression Levels of CYP mRNA

Real-time PCR assays were implemented to detect whether SsD were able to induce or inhibit CYPs expression at the mRNA level in HepaRG cells with the treatment of SsD. As illustrated in Figure 3, compared with the control, SsD at concentrations of 1, 5, and 10 μM significantly induced CYP1A2 mRNA expression in a concentration-dependent manner (Figure 3A), while CYP2D6

was observed to be markedly induced by SsD at concentrations of 5 and 10 μM (Figure 3B).

Effects of SsD on Protein Expression of CYPs

Protein expression of CYPs in HepaRG cells treated with SsD for 72 hours is shown in Figure 4. The results demonstrated that treatment of HepaRG cells with SsD significantly upregulated protein-expression levels of CYP1A2 and CYP2D6 at concentrations of 5 and 10 μM compared with the control.

Effects of SsD on Relative Activity of CYPs

As demonstrated in Figure 5, the effect of SsD on the relative enzyme activity of CYP1A2 and CYP2D6 in HepaRG cells was consistent with the trend of mRNA and protein expression. In brief, it significantly induced relative activity of CYP1A2 and CYP2D6 at 1, 5, 10 μM and 5 and 10 μM, respectively compared with the control.

Discussion

It has been reported that CYPs are involved in >90% of clinical drug metabolism[26] and are one of the major indicators in predicting drug–drug interactions recommended by the FDA.[27] Directly and indirectly, TCM affects the efficacy and safety of other drugs through drug–drug interactions because a large number of active ingredients in TCM are metabolic substrates of CYPs.[28] *Bupleurum* is
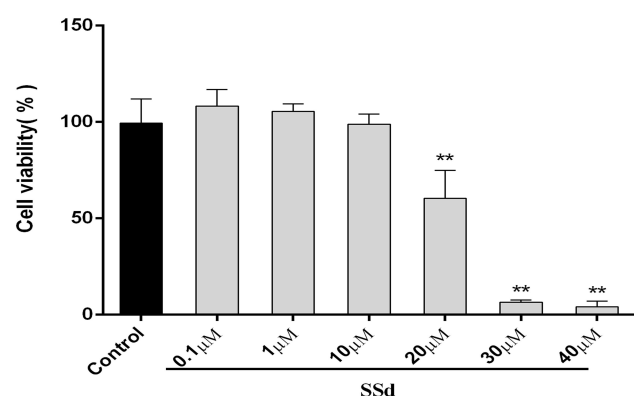

Figure 2 Cytotoxicity assays of SsD after 72 hours of incubation in HepaRG cells. Data expressed as means ± SD (n=3). **$p<0.01$ compared with control.
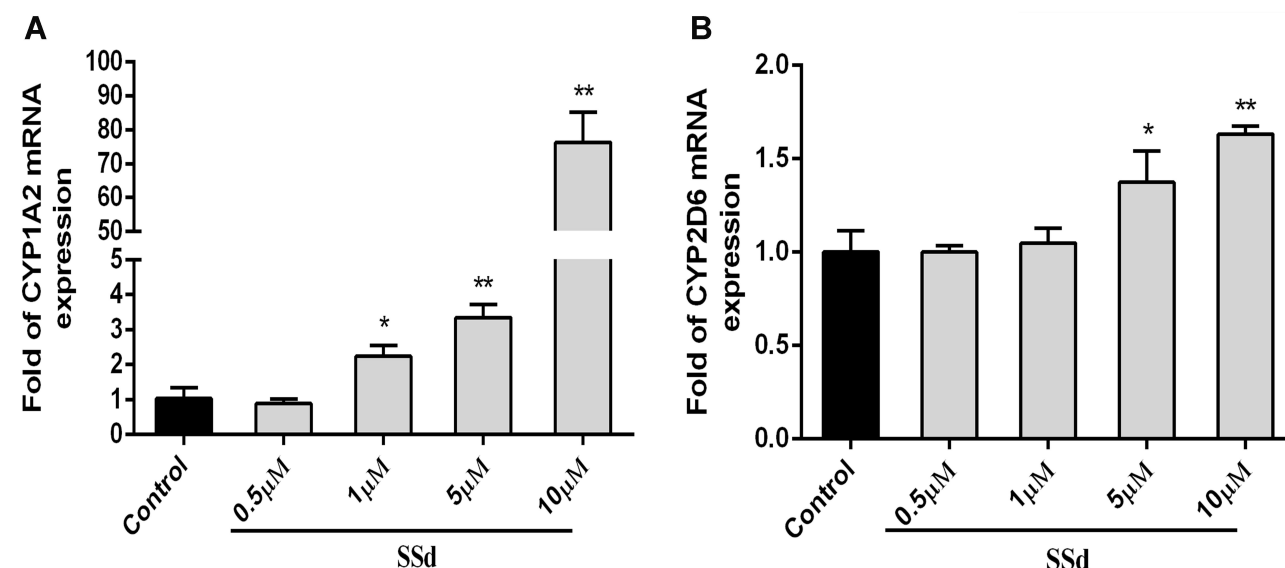

Figure 3 Effects of SsD on mRNA expression of CYP1A2 and CYP2D6 in HepaRG cells. Relative mRNA expression of (**A**) CYP1A2 and (**B**) CYP2D6 in HepaRG cells treated with series concentrations of SsD (0.5, 1, 5, and 10 μM) for 72 hours. Results presented as means ± SD (n = 3). *$p<0.05$, **$p <0.01$ compared with blank control.
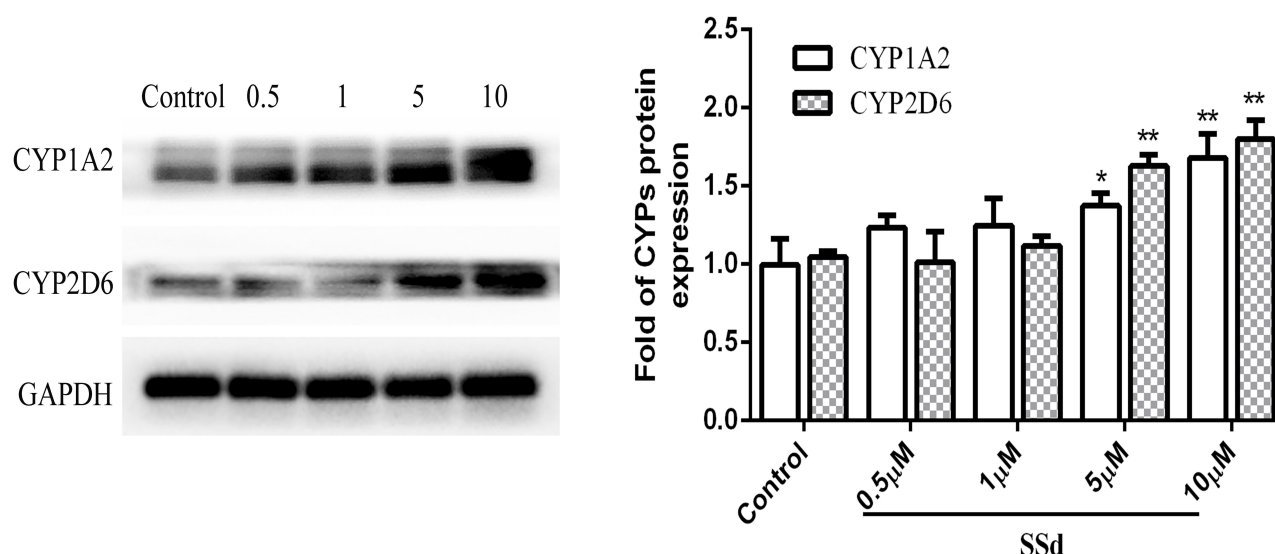
Figure 4 Effects of SsD on protein expression of CYP1A2 and CYP2D6 in HepaRG cells. Protein expression of CYP1A2 and CYP2D6 in HepaRG cells treated with different concentrations of SsD (0.5, 1, 5, and 10 µM) for 72 hours. Results presented as means ± SD (n=3), *$p<0.05$, **$p<0.01$ compared with blank control.
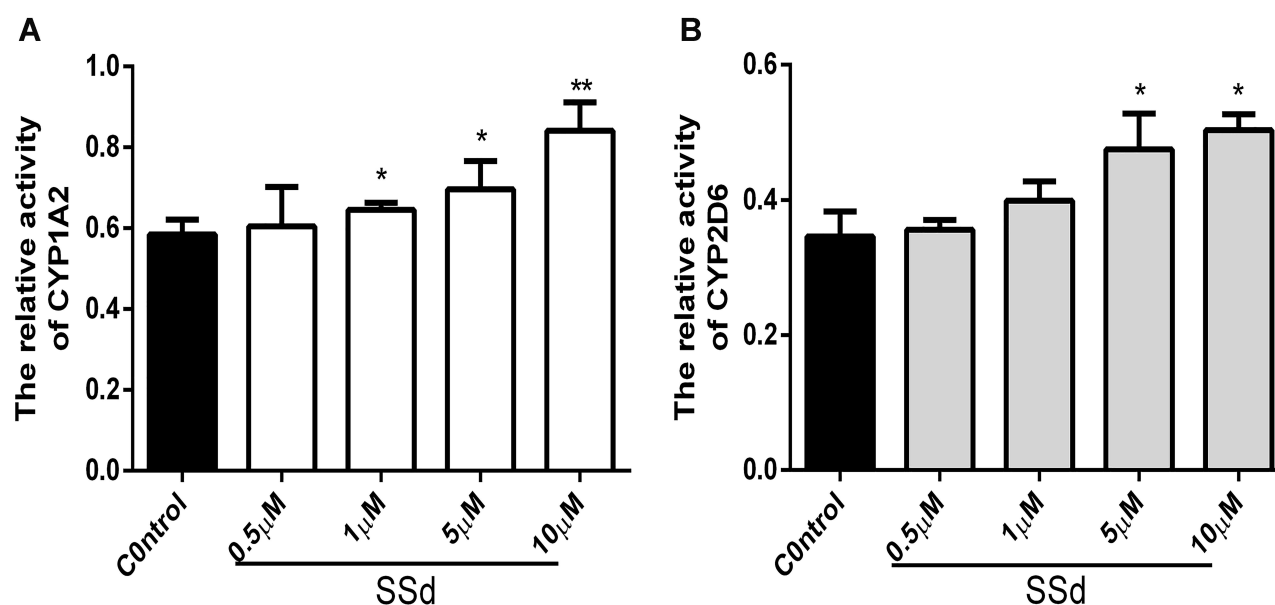

Figure 5 Effects of SsD on relative activity of CYP1A2 and CYP2D6 in HepaRG cells. Effect of SsD on relative activity of CYP1A2 (**A**) and CYP2D6 (**B**) in HepaRG cells treated with phenacetin (50 µM) and dextromethorphan hydrobromide (50 µM) for 12 hours after different concentrations of SsD (0.5, 1, 5, and 10 µM) had been incubated for 72 hours. Results presented as means ± SD (n=3). *$p<0.05$, **$p<0.01$ compared with blank control.

the dry root of *Bupleurum chinense* (Apiaceae) or *Bupleurum scorzonerifolium* according to the Chinese Pharmacopoeia, and has been used clinically for >2,000 years to treat fever, hepatitis, and inflammation in China, Japan, South Korea, and other Asian countries.[29] So far, 43 kinds of saikosaponins have been isolated and identified from *Bupleurum*, which has the highest content of SsA, SsC, and SsD, among which SsD is considered the

most bioactive component,[8] with multiple pharmacological effects of anti-inflammation, antioxidation, and liver protection.[30] Formulations containing *Bupleurum* have been widely used in clinical practice, but their effects on CYPs have not been generally studied.

Though rodents and monkeys have been used as experimental models to study drug metabolism for many years, the application of animal models in this area is

limited. The reason for the limited drug-metabolism information from animal experimental models may be the limited reference value of the results due to species differences, as well as concerns related to animal protection and ethics. As such, the application of animal models, such as dogs and monkeys, are increasingly restricted in medical research.[31] Although various models for the study of drug metabolism or drug–drug interactions have been developed, these models, including animals, primary human hepatocytes, and HepG2 and Huh7 cells have various limitations. Specifically, there are species differences from humans when animal experimental models are applied in this area, frequently resulting in failure to predict drug–drug interactions.[32] HepG2 and Huh7 cells only express relatively low functions of human liver–specific CYPs.[21,33,34] Primary human hepatocytes are considered the gold standard of in vitro for drug-metabolism studies, but difficulty in culture, poor stability of functions in culture, and differences among donors limit their application as an ideal model for in vitro drug-metabolism studies,[35,36] while the HepaRG cell line can exhibit CYP activities (CYP3A4, 1A2, 2D6, 2C9, 2E1) free of the disadvantages of difficulty in culture and unstable functional expression in primary human hepatocytes.[37] As such, HepaRG cell is a better and promising model for in vitro drug-metabolism and drug–drug interaction research. In the present study, HepaRG cells were used to determine the effect of SsD on mRNA and protein expression of major CYPs to understand drug interactions related to clinical formulations containing SsD or *Bupleurum*.

Notably, we found that SsD significantly induced mRNA and protein expression of CYP1A2 and CYP2D6 in HepaRG cells in a concentration-dependent manner (Figures 3 and 4). Their relative enzyme activity was also induced, as shown in Figure 5. A previous study demonstrated *Bupleurum* and vinegar-baked *Bupleurum* had strong induction effects on CYP2D6 in rats,[38] while our results indicated that SsD plays an important role in the induction effect of CYP2D6, which may be one of the imperative factors for *Bupleurum* and vinegar-baked Bupleurum in that it can induce the CYP2D6 enzyme. For CYP1A2, SsD has been reported to have a slight inhibition effect as a compromise to its induction effect in rat-liver microsomes,[39] while our study indicated that SsD can significantly induce CYP1A2, probably owing to the fact that when the activity of CYP enzymes was measured through microsomal in vitro incubation, the actual concentration of substrate available to them depends

on processes missing in subcellular models, covering transport mechanisms, cytosolic enzymes, and intracellular protein binding, while intact cells can better simulate the disposal environment of drugs in the liver.[40] Disadvantages of the liver microsomal drug-metabolism model in vitro include but are not limited to destruction of the complete structure during the preparation process and aspecific reactions being more likely to occur in the in vitro incubation system and the lack of a complete enzyme-reaction system being required for metabolism, which needs to have the right amount of NADPH added. Some drug-metabolizing enzymes are removed during the preparation process, such as the metabolic enzymes located in the cytoplasm. Also, screening of CYP inducers cannot be done in microsomes, as it requires a cellular system fully capable of expressing CYP genes.[34,40] Further research is required. *Bupleurum* contains a complex mixture of components (such as flavonoids, phenyl propanol derivatives, triterpenoid saponins, and volatile oils), so to highlight the necessity of determining which ingredients are responsible for the effect on 1A2 and 2D6 enzymes is indispensable.

It is interesting that the mRNA level of CYP1A2 (~80-fold, Figure 3A) was not absolutely consistent with the protein level (~1.6-fold, Figure 4). The lower posttranscription level may be due to degradation of the CYP1A2 protein.[36] Protein ubiquitination is an imperative pathway in a variety of degradation of proteins and performs an indispensable role in posttranslational modification.[41] Additionally, the ubiquitin–26S proteasome pathway is the most important and highly selective protein-degradation pathway. It has been demonstrated that CYP3A1 and CYP3A2 can be ubiquitinated and then degraded by proteasomes.[42]

In clinical practice, 10% of clinical drugs are metabolized by CYP1A2, including propranolol, clomipramine, phenacetin, mexiletine, propanol, fluamine, verapamil, and nifedipine.[43,44] Though CYP2D6 accounts for only 2%–4% of the total CYPs in the liver, it is involved in 20%–25% of drug metabolism,[45,46] including in antidepressants, antiarrhythmics, antipsychotics, β-blockers, and analgesics,[47] which may cause the blood concentration of these drugs to decrease when combined with formulations containing SsD or *Bupleurum*. Therefore, the mechanisms need more investigation. To some extent, the results of this study are of great significance for the prediction of CYP-mediated drug metabolic interactions and clinically relevant drug interactions of preparations containing SsD or *Bupleurum* early in the drug-discovery process. This study focused only on one of the most

important ingredients in *Bupleurum*, and other components may also have certain effects on CYPs. As such, further studies need to be carried out on other major ingredients of *Bupleurum* to provide more comprehensive and reliable evidence for clinical medication combinations related to *Bupleurum*.

Conclusion

Our results indicated that SsD has induction effects on CYP1A2 and CYP2D6 in HepaRG cells. When drugs metabolized by CYP1A2 and CYP2D6 are coadministered with formulations containing SsD or *Bupleurum* in clinical practice, blood concentrations and effects of these drugs should be observed carefully to avoid or make use of the potential drug interactions.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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