Effects of total saikosaponins on CYP3A4 and CYP1A2 in HepaRG cells

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Abstract. Total saikosaponins (TSS) form a group of chemically and biologically active components that can be extracted from Bupleurum, with reported antidepressive, anti-inflammatory, antiviral, antiendotoxin, antitumor, anti-pulmonary fibrosis and anti-gastric ulcer effects. Bupleurum or TSS is frequently utilized in clinical practice alongside other medications (such as entecavir, lamivudine, compound paracetamol and amantadine hydrochloride capsules), leading to an increased risk of drug-drug interactions. The cytochrome P450 (CYP) family serves a critical role in the metabolism of numerous essential drugs (such as tamoxifen, ibuprofen and phenytoin), where the majority of drug interactions involve CYP-mediated metabolism. It is therefore essential to understand the effects of key components of Bupleurum on CYPs when administering combination therapies containing TSS or Bupleurum. The present study aimed to investigate the effects of TSS on the mRNA and protein expression of CYP3A4 and CYP1A2 in HepaRG cells. The effects of TSS on the survival of HepaRG cells was investigated using the Cell Counting Kit-8 (CCK-8) method. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) analysis were used to assess the effects of different concentrations of TSS (0, 5, 10 and 15 μg/ml) on CYP3A4 and CYP1A2 mRNA and protein expression in HepaRG cells. Based on the CCK-8 assay results, it was observed that the cell viability remained above 80% when treated with 1, 5, 10 and 15 μg/ml TSS. Although there was a statistically significant reduced cell viability at TSS concentrations of 10 and 15 μg/ml compared with the control group, the findings indicated that TSS did not exhibit notable cytotoxic effects at these concentrations. Furthermore, RT-qPCR results revealed that compared with those in the control group, TSS at concentrations of 10 and 15 μg/ml reduced CYP3A4 mRNA expression but increased CYP1A2 mRNA expression in HepaRG cells at concentrations of 15 μg/ml. WB analysis found that TSS at concentrations of 10 and 15 μg/ml downregulated CYP3A4 protein expression in HepaRG cells while increasing CYP1A2 protein expression at concentrations of 15 μg/ml. Results in the present study suggest that TSS can inhibit CYP3A4 mRNA and protein expression, but exerts opposite effects on their CYP1A2 counterparts. These findings suggest that it is necessary to consider drug interactions between clinical preparations containing TSS or Bupleurum and drugs metabolized by CYP3A4 and CYP1A2 to avoid potential adverse drug reactions in clinical practice.

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Introduction

Bupleurum is a widely used traditional Chinese medicine (1,2). Modern scientific research has revealed Bupleurum to possess various beneficial properties, including antitumor, anti-inflammatory, hepatoprotective, anti-fibrotic, sedative and antiepileptic effects (3). Consequently, Bupleurum-associated preparations have found extensive application in clinical practice.

With >100 ancient prescriptions and >549 formulated preparations containing Bupleurum currently available, such as Chaihu injections, Chaihushugan tablets, Xiaoyaowan

tablets, Chaihu oral solution and Chaihu granules, utilization of Bupleurum-associated preparations in clinical practice continues to expand in China and a number of other Asian countries such as Japan and South Korea (4).

Combinations of the Dachaihu decoction (a type of Chaihu preparation) with hypoglycaemic medicines (such as metformin, liraglutide and insulin) have been used for the treatment of type 2 diabetes mellitus (5), whereas combinations of the Chaihushugan decoction with antiepileptic drugs (such as sodium valproate, carbamazepine, lamotrigine, sodium valproate and phenobarbital) have been used for the treatment of epilepsy (6). In addition, the combination of Xiaochaihu decoction (a type of Chaihu preparation) with Entecavir has been used for the treatment of chronic viral hepatitis B (7). However, the increasing use of these combinations may result in potential drug interactions. It has been previously reported that the combined use of the Xiaochaihu decoction and cyclosporin A (CsA) can lead to an increase in the blood concentration of CsA resulting in toxic effects or therapeutic failure, though the specific mechanism remains unclear (8).

The cytochrome P450 (CYP) family serves a crucial role in the metabolism of a number of clinical drugs (9-11). Among the various CYPs, CYP3A4 accounts for 60% of the total protein of CYPs in the liver and is primarily responsible for metabolizing ~50% of all drugs currently used in the clinic, including antiviral drugs, calcium channel blockers, macrolides and benzodiazepines (12). By contrast, CYP1A2 constitutes ~13% of the total CYP content in the liver and participates in the metabolism of 9% of all drugs currently used in the clinic (13), including propranolol, chlorpromazine, phenytoin, mexiletine, propanolol, fluoxetine, verapamil and nitrendipine. Therefore, investigating the effects of drugs on CYP3A4 and CYP1A2 is likely to yield useful theoretical information and practical value.

The HepaRG cell line was first developed by Gripon *et al* (14) in 2002 whilst investigating hepatitis B virus-infected human liver cancer cell line. In the presence of 2% DMSO, HepaRG cells can differentiate into canaliculus-like and hepatocyte-like cells that can functionally express CYP1A2, CYP2D6, CYP2C9, CYP2E1 and CYP3A4 to resemble human liver cells (14-16). Therefore, HepaRG cells have been proposed to be a viable model for studying drug metabolism and interactions *in vitro* as a substitute for human primary liver cells (15-17). Previous studies have established a HepaRG-based model to investigate drug metabolism and interactions. Specifically, studies on the effects of saikosaponin (SS)-D on the mRNA and protein expression levels of CYP1A2, CYP2D6 and CYP3A4, as well as the relative enzyme activities in HepaRG cells have been conducted (18,19).

Bupleurum-associated preparations exert pharmacological effects through multiple targets and pathways in the body, but the specific active components responsible for regulating CYP expression remain to be fully determined. Total saikosaponins (TSS) are triterpene saponins that are extracted solely from Bupleurum and consist of >100 different types, including SSA, SSB, SSC and SSD (20,21). A number of pharmacological effects of Bupleuri Radix have been directly associated with TSS (22). Several previous studies have examined TSS metabolism in rats, identifying cetyl-SSE, saikogenin A, saikogenin G, SSA, SSB2 and saikogenin C as major metabolites (22-24).

Therefore, the present study used HepaRG cells as a model to investigate the impact of TSS on the mRNA and protein expression of CYP3A4 and CYP1A2, aiming to elucidate the underlying basis for potential drug interactions involving preparations containing TSS or Bupleurum. It is hoped that these findings will provide a theoretical basis for rational combination therapy and further research on drug metabolism involving TSS or Bupleurum.

Materials and methods

Reagents and cells. Undifferentiated HepaRG cells were purchased from Shanghai Binsui Biotechnology Co., Ltd. (cat. no. C554). TSS (purity, ≥70%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. Omeprazole and rifampicin were acquired from Shanghai Aladdin Biochemical Technology Co., Ltd. Other reagents used were as follows: TB Green® Premix EX Taq™ II (cat. no. RR820A; Takara Biotechnology Co., Ltd.), PrimeScript™ IV 1st strand cDNA Synthesis Mix (cat. no. 6215A; Takara Biotechnology Co., Ltd.), CYP3A4 rabbit polyclonal antibody (cat. no. DF3586; Affinity Biosciences), CYP1A2 rabbit polyclonal antibody (cat. no. AF5312; Affinity Biosciences), GAPDH rabbit polyclonal antibody (cat. no. 10494-1-AP; ProteinTech Group, Inc.), HRP-conjugated Affinipure Goat Anti-Rabbit IgG (cat. no. SA00001-2; ProteinTech Group, Inc.), BCA protein assay kit (cat. no. CW0014S; CoWin Biosciences) and Cell Counting Kit-8 (CCK8; cat. no. SC119-01; Seven Innovation (Beijing) Biotechnology Co., Ltd.).

Cell culture and morphology. Undifferentiated HepaRG cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% antibiotics (100x streptomycin-penicillin) and 50 μM hydrocortisone sodium hemisuccinate (basal growth medium; Shanghai Aladdin Biochemical Technology Co., Ltd.), incubated in a constant temperature incubator at 37˚C, 5% CO_2 and 95% humidity for 2 weeks. Subsequently, the same culture medium supplemented with 2% DMSO (differentiation medium) was added to the cells for a further 2 weeks at 37˚C (14). Images of differentiated and undifferentiated cells were captured using an inverted light microscope (Olympus CKX53; Olympus Corporation). Differentiated HepaRG cells were seeded into 96-well plates or 6-well plates for follow-up experiments (14,25). The differentiation medium was renewed every 2-3 days.

Cell viability assay. A toxicity analysis of TSS on HepaRG cells was performed to determine a suitable range of concentrations for the study regarding drug-drug interactions. Differentiated HepaRG cells were seeded into 96-well plates at a density of $4.5x10^5$ cell/cm^2 and incubated for 12 h at 37˚C before being treated with different concentrations of TSS dissolved in 0.1% DMSO (0, 1, 5, 10, 15, 20, 30 and 40 μg/ml) for 72 h at 37˚C with 5% CO_2. Untreated cells that were incubated in the medium served as a control and the medium served as a blank. Subsequently, 10 μl CCK8 solution (5 mg/ml) prepared in RPMI 1640 medium (without FBS, antibiotics and hydrocortisone sodium hemisuccinate) was added into each well. After 2 h of incubation at 37˚C, the CCK8-containing medium

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward (5'-3')	Reverse (5'-3')
CYP3A4	CTTCATCCAATGGACTGCATAAA	TCCCAAGTATAACACTCTACACACACA
CYP1A2	ATGCTCAGCCTCGTGAAGAAC	GTTAGGCAGGTAGCGAAGGAT
CYP2B6	TTCCTACTGCTTCCGTCTATC	GTGCAGAATCCCACAGCTCA
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

CYP, cytochrome P450.

was removed. The absorbance of each well was detected by an enzyme-labelled instrument (iMARK; Bio-Rad Laboratories, Inc.) at a wavelength of 490 nm.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To characterize the differentiation of HepaRG cells, differentiated HepaRG cells were seeded into 6-well plates at a density of $4.5x10^5$ cell/cm^2 and incubated for 24 h at 37˚C, whereas undifferentiated cells were used as a control. To investigate the effects of TSS on the mRNA expression levels of CYP1A2 and CYP3A4, differentiated HepaRG cells were treated with 50 μM rifampicin, 50 μM omeprazole or different concentrations of TSS [0, 5, 10 or 15 μg/ml (this range was selected based on the CCK8 results in order to achieve cell viability)] and incubated for 72 h at 37˚C. Total RNA was extracted from HepaRG cells using the RNAiso Plus reagent (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol and the total RNA concentration was quantified using a spectrophotometer (A260/A280). Total RNA (500 ng) was reverse transcribed using the PrimeScript™ IV 1st strand cDNA Synthesis Mix (cat. no. 6215A; Takara Biotechnology Co., Ltd.), and the reverse transcription conditions were: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for 10 min. The complementary DNA (cDNA) obtained from reverse transcription was diluted 10 times with RNase-free distilled water. The TB Green® Premix EX Taq™ II (TIi RNaseH Plus) (cat. no. RR820A; Takara Biotechnology Co., Ltd.) was used for quantitation with the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.). The PCR amplification procedure was performed as follows: Sample volume, 25 μl; initial denaturation, 95˚C for 30 sec; PCR amplification (40 cycles), consisting of denaturation (95˚C for 5 sec) and annealing/extension (60˚C for 30 sec). Analysis of each specimen for each target was repeated three times. Gene transcription was analysed with the $2^{-\Delta\Delta Cq}$ method (26) using GAPDH expression as a reference. The primer sequences used are presented in Table I.

Western blot analysis. The effects of TSS on the protein expression levels of CYP3A4 and CYP1A2 in HepaRG cells were detected using western blot analysis. The differentiated HepaRG cells were seeded in 6 well plates, incubated and treated as aforementioned. Total protein was extracted from HepaRG cells using RIPA cell lysis buffer containing proteinase inhibitor (Beijing Solarbio Science & Technology Co., Ltd.). The protein concentration was measured using a BCA assay kit (cat. no. CW0014S; CoWin Biosciences). Protein samples (40 μg) were loaded per lane and separated on 10% sodium dodecyl sulphate-polyacrylamide gels with electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies at 4˚C overnight and were then incubated with an HRP-conjugated secondary antibody at room temperature for 1 h. The membranes were washed three times with TBST, which contains 0.1% Tween20. Chemiluminescent detection was performed using electrochemiluminescence reagents (Nanjing KeyGen Biotech Co., Ltd.) with a ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc.).

The primary and secondary antibodies were used at the following dilutions: Antibodies for GAPDH (1:5,000), CYP3A4 (1:1,000), CYP1A2 (1:2,000) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (1:5,000). The protein expression levels were quantified by comparing against the GAPDH bands using ImageJ Software v1.6.0 (National Institutes of Health).

Statistical analysis. SPSS 29.0 (IBM Corp.) was used for statistical analysis of all data, and the results are presented as the mean ± standard deviation (n=3) as indicated in corresponding figure legends. The Shapiro-Wilk normality test was used to test distribution normality and the Levene test to assess variance homogeneity. The comparison between groups were conducted using one-way analysis of variance (ANOVA). When variances were found to be homogeneous, multiple comparisons between groups were evaluated using the Bonferroni post hoc test. In instances where the assessed variances exhibited non-normality and/or unequal variances, non-parametric statistical analysis via the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was utilized. For comparison between two groups, the unpaired Student's t-test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of differentiated HepaRG cells. Microscopic examination of undifferentiated HepaRG cells revealed the presence of numerous characteristic granular epithelial cell colonies encircled by flat, well-cytoplasmic cholangiocyte-like cells, as depicted in Fig. 1A-a. By contrast, differentiated HepaRG cells exhibited distinct features under phase contrast microscopy, with hepatocyte-like cells displaying a dense and indefinite cytoplasm, epithelial-like cells with flat and

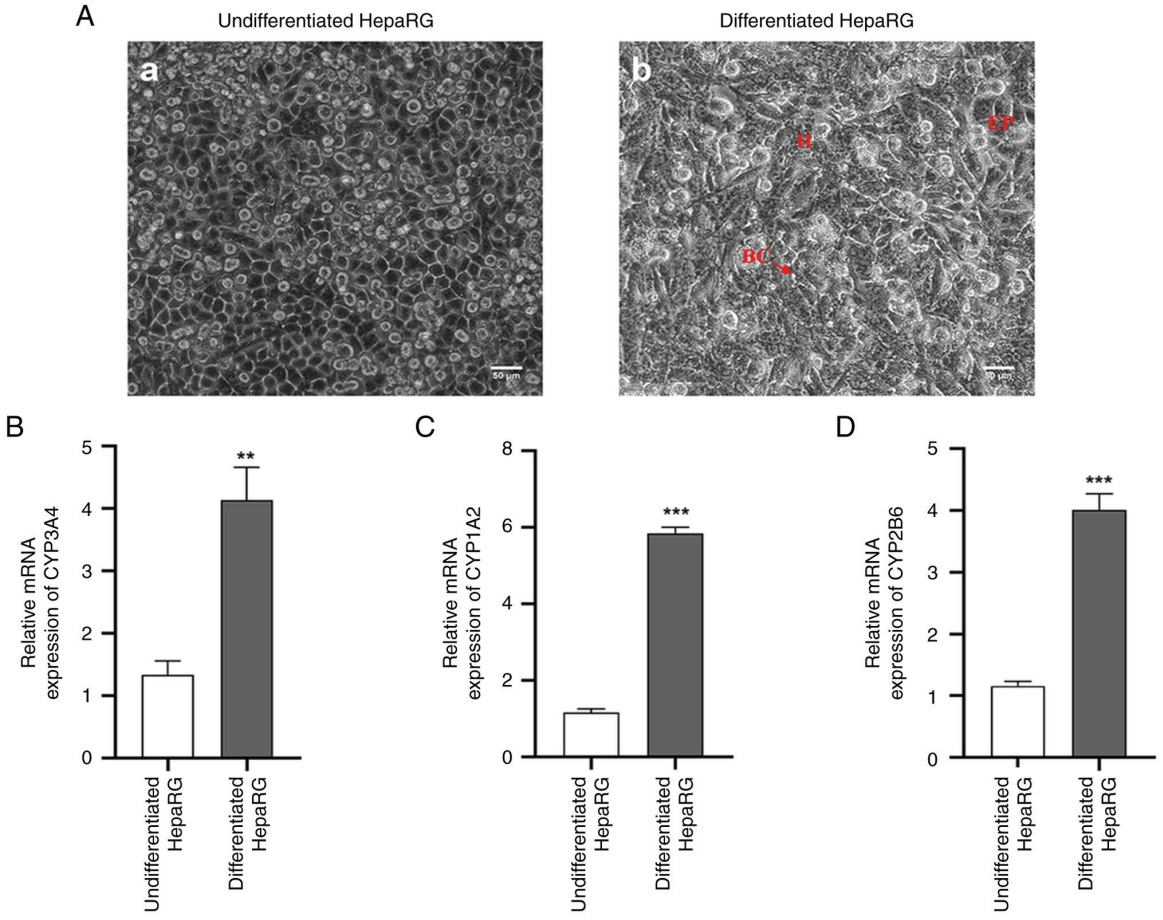

Figure 1. Identification of differentiated HepaRG cells. (A-a) Phase contrast micrograph of undifferentiated HepaRG cells, which indicated the formation of a large number of typical granular epithelial cell colonies surrounded by flat and well-cytoplasmic cholangiocyte-like cells. (A-b) Phase contrast micrograph of differentiated HepaRG cells. Hepatocyte-like cells with dense and indefinite cytoplasm (indicated by 'H'), epithelial-like cells with flat and well-defined cytoplasm (indicated by 'EP'), and bile canalicular structures (indicated by 'BC' and an arrow) can be observed. Expression levels of (B) CYP3A4, (C) CYP1A2 and (D) CYP2B6 mRNA in HepaRG cells. Scale bars, 50 μm. **P<0.01 and ***P<0.001 vs. undifferentiated HepaRG cells. For comparison between two groups, the unpaired Student's t-test was used to test the statistical significance (B, C and D). H, hepatocyte-like cells; EP, epithelial-like cells; BC, bile canalicular structures; CYP, cytochrome P450.

well-defined cytoplasm, and the presence of bile canalicular structures, as illustrated in Fig. 1A-b. The mRNA expression levels of CYP3A4, CYP1A2 and CYP2B6 were significantly increased in the differentiated HepaRG cells compared with those in the undifferentiated cells (Fig. 1B-D).

Cell viability analysis. Results of the *in vitro* cell viability assay indicated that the HepaRG cell viability was >80% with TSS concentrations in the range of 0-15 μg/ml, indicating no cytotoxic effect (Fig. 2). This suggested that TSS concentrations <15 μg/ml could be used for the subsequent drug interaction experiments.

Effects of TSS on the expression levels of CYP3A4 and CYP1A2 mRNA. As presented in Fig. 3, compared with those in the control (0 μg/ml TSS), 50 μM rifampicin and 50 μM omeprazole significantly increased CYP3A4 and CYP1A2 mRNA expression, respectively. However, 10 and 15 μg/ml TSS significantly reduced the mRNA expression levels of CYP3A4 in a dose-dependent manner compared with those in the control group (Fig. 3A). By contrast, CYP1A2 expression was observed to be significantly increased by 15 μg/ml TSS compared with that in the control group (Fig. 3B).

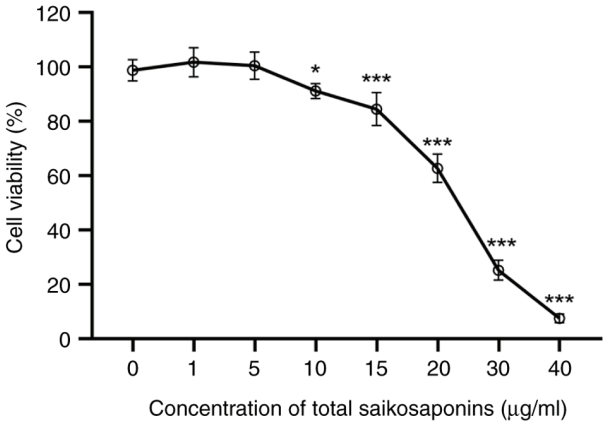

Figure 2. Cytotoxicity assays of total saikosaponins after 72 h of incubation with HepaRG cells. Data are presented as the mean ± SD (n=3). *P<0.05 and ***P<0.001 vs. 0 μg/ml. One-way analysis of variance was used to test the statistical significance.

Effects of TSS on the expression levels of CYP3A4 and CYP1A2 protein. Protein expression levels of CYP3A4 and

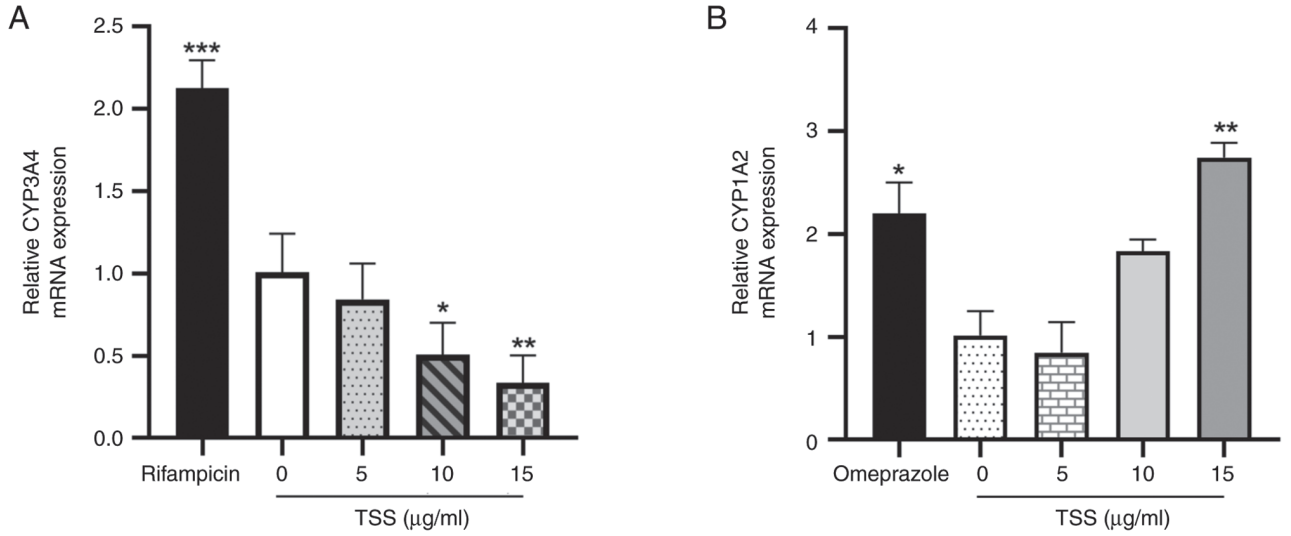

Figure 3. Effects of TSS on the mRNA expression levels of CYP3A4 and CYP1A2 in HepaRG cells. Expression levels of (A) CYP3A4 and (B) CYP1A2 mRNA in HepaRG cells treated with different concentrations of TSS (0, 5, 10 and 15 μg/ml) for 72 h. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μg/ml TSS. TSS, total saikosaponins; CYP, cytochrome P450. One-way analysis of variance was used to test the statistical significance (A). Non-parametric statistical analysis via the Kruskal-Wallis test with Dunn's multiple comparisons post hoc test was performed (B).

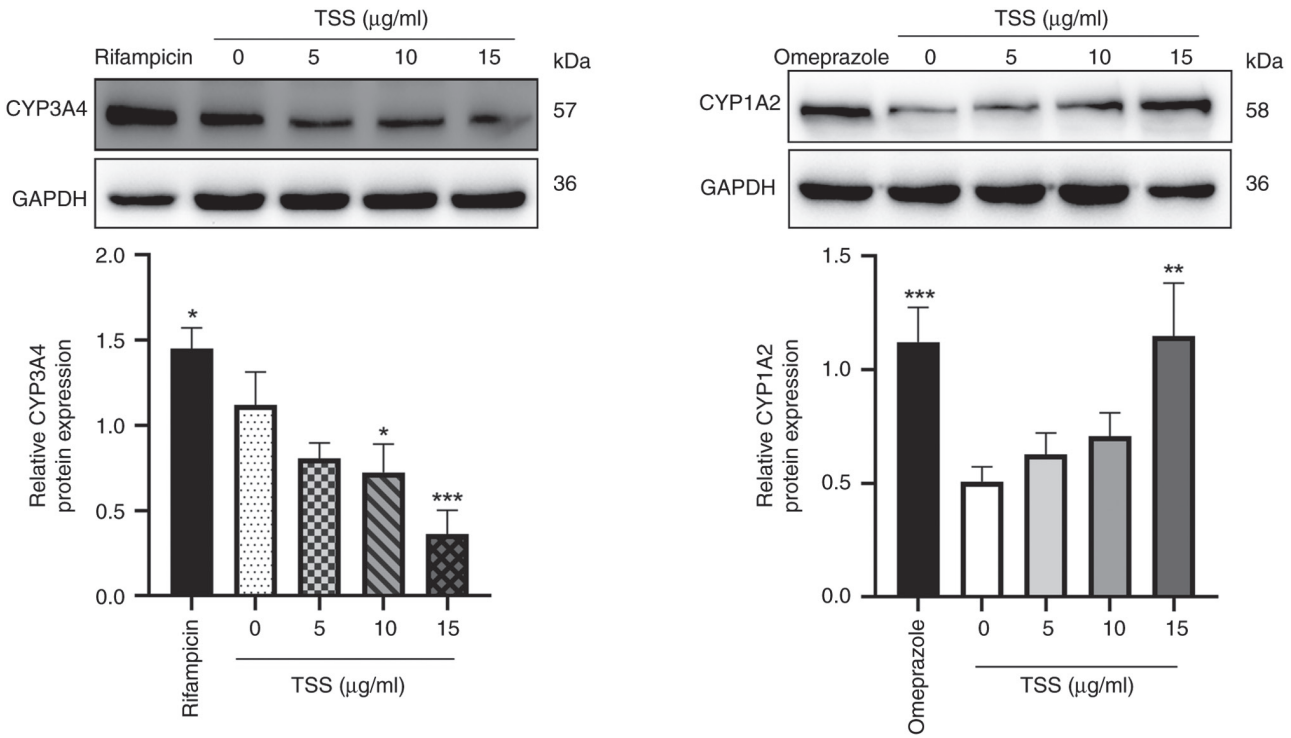

Figure 4. Effects of TSS on the protein expression levels of CYP3A4 and CYP1A2 in HepaRG cells. Protein expression levels of CYP3A4 and CYP1A2 in HepaRG cells treated with different concentrations of TSS (0, 5, 10 and 15 μg/ml) for 72 h. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μg/ml TSS. TSS, total saikosaponins; CYP, cytochrome P450. One-way analysis of variance was used to test the statistical significance.

CYP1A2 in HepaRG cells treated with TSS for 72 h were next measured (Fig. 4). Compared with those in the control group (0 μg/ml TSS), 50 μM rifampicin and 50 μM omeprazole significantly increased the CYP3A4 and CYP1A2 protein expression levels, respectively. In addition, 10 and 15 μg/ml TSS significantly reduced the protein expression levels of CYP3A4 in a dose-dependent manner compared with those in the control group (Fig. 4A). However, CYP1A2 expression levels were observed to be significantly increased by 15 μg/ml TSS compared with those in the control group (Fig. 4B).

Discussion

Pharmacokinetic drug-drug interactions, resulting from the induction or inhibition of CYP enzymes, are commonly known concerns in clinical practice as these interactions can potentially

lead to changes in the drug concentration in the blood, which may result in adverse reactions or therapeutic failure (27,28). Screening and metabolism studies of novel drug candidates in Europe and the United States mandate CYP assays, which are also mandatory for new drug applications (29). Similarly, China's Guiding Principles for Non-Clinical Pharmacokinetic Studies of Chemical Drug stipulates that novel prospective drugs should be evaluated for their effects on CYPs induction or inhibition (30-32). Therefore, drug interactions involving CYPs have become a topic of interest for research in the field of clinical pharmacy.

Traditionally, rodents and primates have been used as experimental models for studying drug metabolism and drug interactions. However, species differences can affect the relevance of these models for human metabolism (33-35). The liver is the main organ for drug metabolism (36). Primary human hepatocytes (PHH) have been considered to be the gold standard for *in vitro* drug metabolism research. However, the practicality and reliability of PHH as a research model remain limited due to difficulties in cell culture, early phenotypic changes, short maintenance time of metabolic enzyme activity and donor-to-donor variations (13,37). The human liver cancer cell line HepG2 has been proposed to be an alternative *in vitro* drug metabolism model. However, HepG2 cells express relatively low levels of human liver-specific functions such as drug-metabolizing enzymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) despite being readily available, limiting their application in drug metabolism and interaction research (37). Another cell line, HuH7 (38), suffer from similar limitations (12,39-41). By contrast, the HepaRG cell line, which was originally isolated from a patient with a chronic hepatitis C viral infection (11), has shown promising characteristics for *in vitro* drug metabolism research. When cultured with DMSO, HepaRG cells can differentiate into hepatocyte-like cells that express various enzymes, including CYPs. These differentiated cells maintain liver-specific functional properties and have been proposed to be an appropriate model for drug metabolism research (12,42,43). They have been used in studies associated with drug metabolism, drug interactions and toxicology (44-47). Furthermore, HepaRG cells can be induced to increase the expression levels of CYP1A2, CYP2B6 and CYP3A4 enzymes by specific inducers, such as omeprazole, phenobarbital and rifampicin (48).

In the present study, TSS, the main active component of Bupleurum, was investigated. TSS has been used in clinical practice due to its various reported biological activities, including antidepressive, anti-inflammatory, antiviral, antiendotoxin, antitumor, anti-pulmonary fibrosis and anti-gastric ulcer effects (49-51). TSS has also gained attention over the past decade for its neuroprotective and nephroprotective effects (52,53). Given its extensive use and potential interactions with drugs metabolized by CYPs, TSS is an active ingredient worthy of attention in drug development. However, despite TSS being the main bioactive component of Bupleurum, its effects on CYPs have, to the best of our knowledge, only been investigated twice (31,54). A previous study reported the downregulation of CYP3A4 protein expression by TSS in mice, supporting the potential inhibitory effects of TSS on CYP3A4 (54). Additionally, TSS has been found to exert inhibitory effects on CYP3A11 while inducing CYP1A2 in the liver and intestines of mice (31). In addition, the effects of Xiaochaihu decoction extract (containing TSS) on the mRNA and protein expression levels of CYP1A2, CYP3A1, CYP2D6 and CYP1B1 in rats have been examined (55). However, the effects of TSS alone on CYPs still requires further investigation.

A previous study has reported an increase in the blood concentration of CsA when combined with Xiaochaihu decoction, but the specific substance and pathway involved in this mechanism remains unknown (8). In our previous studies, it was revealed that one of the saponins isolated from Bupleurum, SSD, can inhibit the activity of the CYP3A4 enzyme in HepaRG cells whilst increasing CYP1A2 enzymes (18,19). In the present study, it was observed that compared with those in the control group, TSS downregulated the mRNA and protein expression levels of the CYP3A4 enzyme in HepaRG cells but upregulated the mRNA and protein expression levels of CYP1A2. CsA is a substrate of the drug metabolism enzyme CYP3A4 (56). Therefore, it could be hypothesized that the increase in the blood concentration of CsA caused by the Xiaochaihu decoction may be due to the inhibition of CYP3A4 by TSS, leading to an impaired CsA metabolism.

Consistent with the findings of the present study, another previous study has also reported the downregulation of CYP3A4 protein expression by TSS in mice, supporting the potential inhibitory effect of TSS on CYP3A4 (54). Additionally, TSS has been found to exert an inhibitory effect on CYP3A11 (which corresponds to human CYP3A4) whilst inducing CYP1A2 in the liver and intestines of mice (31). Our previous study on the effect of the Xiaochaihu decoction in mouse found that it induced the mRNA and protein expression levels of both CYP1A2 and CYP3A1 (the mouse homolog of human CYP3A4) (55). This discrepancy may be attributed to species differences and the interactions of other components in the Xiaochaihu decoction. At present, it is challenging to determine the concentrations and the active forms of all components in TSS. Due to the metabolism of TSS, it can be transformed into specific metabolites *in vivo* by various transformation factors, such as the intestinal microflora, gastrointestinal enzymes and gastric acids, making it difficult to determine the actual pharmacological substances after TSS enters the body. CYPs can be influenced by both endogenous and exogenous substances such as anticoagulants, antidiabetics and antimicrobials (36), leading to modifications in enzyme quantity and activity (57). In the present study, the effects of TSS on CYP3A4 and CYP1A2 protein and mRNA levels were investigated, without assessing enzyme activity. The lack of experiments investigating enzyme activity was a limitation of the present study.

The results of the present study suggest that TSS may inhibit CYP3A4 whilst inducing CYP1A2 expression. Further research is warranted to investigate the bioavailability and gastrointestinal absorption of each SS, whilst also exploring the impact of TSS on the enzyme activity of CYP3A4 and CYP1A2. In addition, the effects of other substances in the Xiaochaihu decoction on CYP enzymes and the clinical implications of TSS-associated interactions with drugs metabolized by CYPs are worthy of further investigation. These aspects are important for a more comprehensive understanding of the implications of the present findings.

In the present study, established protocols and guidelines for using rifampicin as a positive control inducer for CYP3A4

and omeprazole as a positive control inducer for CYP1A2 were followed. Rifampicin is recommended by the U.S. Food and Drug Administration (FDA) as a reference compound for evaluating the CYP3A4 induction potential and, as a well-known potent inducer of CYP3A4, it is used as a positive control inducer for studying the expression of CYP3A4 *in vitro* (58,59). Similarly, omeprazole is a known inducer of CYP1A2, and it is used as a positive control inducer for studying the expression of CYP1A2 *in vitro*, which is also recommended by the FDA as a standard reference compound for assessing the CYP1A2 induction potential (58,59). By using these compounds, the present study aimed to ensure the reliability and comparability of the present results with previous studies.

In conclusion, utilizing HepaRG cells as a model for drug metabolism enzymes revealed that compared with those in the control group, TSS reduced the mRNA and protein expression levels of CYP3A4 whilst increasing those of CYP1A2. These findings highlight the potential for drug interactions when TSS or Bupleurum associated preparations are used in combination with drugs metabolized by CYP3A4 and CYP1A2 enzymes in clinical practice. Particular attention should be given to drug interactions to optimize therapeutic effects and minimize potential adverse reactions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FT, YT and HL conceived and designed the experiments. HL, LH and YL performed the experiments. HL, YT, FM and JT analyzed the data. FT, HL and YT confirm the authenticity of all the raw data. FT, HL and YT prepared the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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