Effect of tripterine on the pharmacokinetics of cyclosporine A and its mechanism in rats

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Abstract. Tripterine is a key active component in *Tripterygium wilfordii* polyglycosides tablets. Cyclosporine A (CsA) is an immunosuppressive drug that is widely used in organ transplantation. The combined use of *Tripterygium wilfordii* and CsA can reportedly enhance the immunosuppressive effects of Cyclosporine whilst reducing its toxicity. Therefore, in the present study, a detection method for CsA concentration in rat blood samples was developed using liquid chromatography-mass spectrometry (MS)/MS. Reverse transcription-quantitative (RT-q) PCR and western blotting (WB) were used to examine the impact of tripterine on the expression of a variety of drug-metabolizing enzymes, drug transporters and nuclear receptors. Compared with that in the control group, the maximum concentration (C_{max}) of CsA was found to be reduced across all tripterine dosage groups, where the area under the curve was significantly decreased in the 18 and 54 mg/kg groups. PCR and WB results indicated that tripterine inhibited the expression of cytochrome P450 (CYP) 3A1, CYP3A2, uridine diphosphate glucuronosyltransferase 1A1, organic anion-transporting polypeptide 1B2, p-glycoprotein 1, multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein, bile salt export pump (BSEP) and Na^+-taurocholate co-transporting polypeptide (NTCP). These results suggest that tripterine's inhibition of NTCP, BSEP and MRP2 in the liver may disrupt bile acid circulation. Additionally, tripterine's activation of farnesoid X receptor in the intestine may limit bile acid synthesis, resulting in reduced CsA uptake into the bloodstream and inhibiting its enterohepatic circulation, ultimately leading to a decrease in CsA blood concentration. In addition, the combination of *Tripterygium wilfordii* and CsA have the prospect of joint clinical application. The present study demonstrated the inhibitory effect of tripterine on the pharmacokinetics of CsA, which could be of significance for the combined use of these two drugs.

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Abbreviations: BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CAR, constitutive androstane receptor; CsA, Cyclosporine A; CYP, Cytochrome P450 proteins; DMEs, drug-metabolizing enzymes; DTs, drug transporters; FXR, farnesoid X receptor; MRP, multi-drug resistance protein; NRs, nuclear receptors; NTCP, sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptides; P-gp, P-glycoprotein; PXR, pregnane X receptor; UGT, UDP-glucuronosyltransferases

Key words: CsA, tripterine, DMEs, DTs, NRs

Introduction

Tripterygium wilfordii is a traditional Chinese medicinal herb that is commonly used for treating a number of conditions, such as arthritis, dermatitis, lupus erythematosus and eczema (1-4). Previous studies have also highlighted its potential for antitumor therapy and immune regulation (5-8). *Tripterygium wilfordii* contains >100 chemical compounds, which can be primarily categorized into alkaloids, terpenes and triterpenes (9). The Tripterygium polycoride tablet, a non-steroidal immunosuppressive agent (10), was developed and introduced for clinical use in China. This tablet has been shown to inhibit the secretion of IL-2 and the expression of IL-2 receptors on T lymphocytes (11), in addition to inducing lymphocyte apoptosis whilst downregulating T cell receptor signaling (12).

Tripterine, also known as celastrol, is an active compound that can be extracted from *Tripterygium wilfordii*. It is one of the key ingredients in the Chinese patent medicine Tripterygium tablet (with each tablet containing ~35 μg tripterine) (13). Numerous studies have demonstrated its immunomodulatory and antitumor properties (14-20). Additionally, tripterine has been shown to regulate the expression of various drug-metabolizing enzymes and drug transporters. Jin *et al* (21) previously examined the effects of tripterine on five subtypes of cytochrome P450 (CYP) enzymes (CYP1A2, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) in human liver microsomes, before finding that tripterine inhibited these enzymes to varying

extents. Similarly, Sun *et al* (22) reported that tripterine inhibited CYP1A2, CYP2C11, CYP2D6, CYP2E1 and CYP3A2, acting as a mixed-type inhibitor of CYP3A4 and a competitive inhibitor of CYP1A2 and CYP2C11. In addition, Zhang *et al* (23) demonstrated that celastrol (tripterine) is a potent inhibitor of uridine diphosphate glucuronosyltransferase (UGT) 1A6 and UGT2B7 (23). In another study, Zhao *et al* (24) found that tripterine could upregulate farnesoid X receptor (FXR) expression in the liver. Given its potential impact on the activity of various drug-metabolizing enzymes and transporters, tripterine has the potential to exert herb-drug interactions. Furthermore, tripterine has been shown to inhibit intestinal lipid absorption (25). Since bile acids and dietary fats serve a significant role in the oral bioavailability of cyclosporine A (CsA) (26,27), any changes in bile acid circulation and fat absorption induced by tripterine may notably influence the absorption and pharmacokinetics of orally administered drugs.

CsA is one of the most commonly used immunosuppressive agents in clinical practice, though it has a narrow therapeutic window. Numerous studies have suggested a potential for the combined use of *Tripterygium wilfordii* and CsA. Yang *et al* (28) reported that CsA combined with Tripterygium glycosides could prolong the mean survival time and decrease the rate of allograft rejection of the cardiac allografts. Additionally, Tripterygium glycosides have shown a synergistic effect with CsA for the treatment of immune rejection following organ transplantation (29-32). This synergy is considered to be associated with the immunomodulatory effects of Tripterygium glycosides.

CsA is a substrate of CYP3A, p-glycoprotein (P-gp), UGT1A, and multidrug resistance protein (MRP) 2 (33-36). Additionally, because CsA is a fat-soluble macromolecule, its oral bioavailability is significantly influenced by bile secretion and intestinal lipid absorption (37). Provided that Tripterine has been shown to affect drug-metabolizing enzymes and transporters, it is possible that it may alter the pharmacokinetics of CsA. However, to the best of our knowledge, the specific effects of Tripterine on CsA have not been reported to date.

Therefore, in the present study, the aim was to investigate the impact of Tripterine on the pharmacokinetics of CsA in rats. After 7 consecutive days of Tripterine pretreatment, CsA was orally administered, before the whole blood concentration of CsA was periodically measured. The transcriptional and protein expression levels of several bile-related transporters, drug transporters, metabolic enzymes and nuclear receptors were also assessed to explore the underlying mechanisms, using previous developed experimental methods (38).

Materials and methods

Materials. Tripterine, CsA and cyclosporine D (CsD) were purchased from Dalian Meilun Biology Technology Co., Ltd.

Animals. A total of 36 male Sprague-Dawley (SD) rats (weight, 180-220 g; age, 6-8 weeks) were purchased from the Laboratory Animal Research Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China) and were given access to a commercial rat chow diet and tap water. The animals were housed, two per cage and were maintained at 22±2˚C and 50-60% relative humidity, under a 12-h light/dark cycle. The experiments were initiated after acclimation under these conditions for ≥1 week. The experiments were performed in accordance with the 'Guiding Principles in the Use of Animals in Toxicology' adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. All animal studies were approved (approval no. 4352; date, 2023) by The Institutional Animal Care and Use Committee of Tongji Medical College Huazhong University of Science and Technology (Wuhan, China).

Pharmacokinetic studies in rats

Experiment one. In total, 24 rats were randomly divided into the following 4 groups (n=6): i) Control group, which was pretreated with 0.5% Carboxymethylcellulose sodium (CMC-Na); and ii) tripterine pretreated groups (6, 18 and 54 mg/kg, respectively). Tripterine were all prepared with the corresponding standard and 0.5% CMC-Na as suspension, which were shaken well before gavage. Rats in the control group were administered 0.5% CMC-Na by gavage. All groups of rats were administered by oral gavage for 7 consecutive days. The rats were fasted for ≥12 h (overnight) before the day of experiment and drank freely. On the experimental day (day 7), 30 min after the last dose of 0.5% CMC-Na or tripterine, CsA (10 mg/kg) was administered to the rats by oral gavage. Blood samples were collected at 1, 2, 4, 5, 6, 7, 8, 12, 24, 36, 48 and 72 h after the administration of CsA. All the blood samples were stored at -80˚C until analysis.

Experiment two. Rats were randomly divided into 4 groups consistent with experiment one, but there were only 3 rats in each group. All of these rats were administered by oral gavage for 7 days. The rats were fasted for ≥12 h (overnight) before the day of experiment and drank freely. After 2 h of intragastric administration on day 7, the rats were euthanized by injection of 100 mg/kg sodium pentobarbital. The liver, kidney and mucosa of small intestine were isolated, washed with saline and blotted dry. The samples were stored at -80˚C until further use.

Quantification of CsA by liquid chromatography-mass spectrometry (MS)/MS (LC-MS/MS). The method for the determination of CsA was based on a previously developed l LC-MS)/MS method (39). The linear concentration range of CsA was 5-4,000 ng/ml and the lower limit of quantification was 5 ng/ml. The extracted ion chromatogram diagram of CSA and CSD is shown in Fig. 1.

Sample preparation. A total of 100 μl of whole blood was absorbed into a 1.5-ml EP tube, vortexed for 5 min with 10 μl methanol, 300 μl water, 240 μl of 200 ng/ml CsD, and 60 μl of 0.4 mol/l ZnSO4. The mixture was centrifuged at 21,012.8 x g for 5 min, and the supernatant was collected for analysis.

HPLC-MS/MS. LC separation and MS detection were performed using the AB Sciex API 4000™ LC/MS/MS system. Chromatographic separation was performed on an ACE excel 5 C18 column (50x2.1 mm). The mobile phase consisted of 10 mmol/l ammonium acetate in aqueous solution (A) and Acetonitrile (B), (containing 0.1% formic acid), at a total flow rate of 0.4 ml/min. The gradient profile for the LC pumps under the final chromatography conditions were

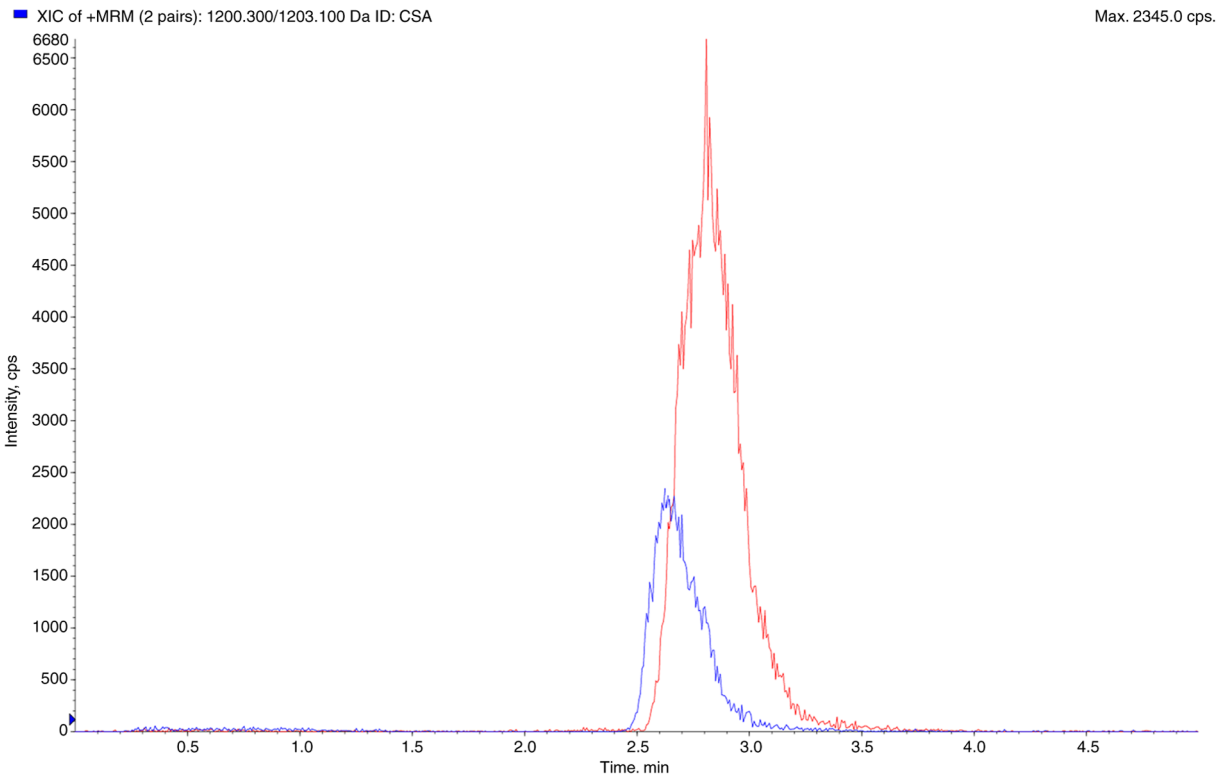

Figure 1. Extracted ion chromatogram of CsA and CsD. Cs, cyclosporine.

as follows: 0 min, 50:50; 0.1-3 min, 0:100; 3.1-5 min, 50:50 (A:B, v/v) (Fig. 1). The injection volume of all samples was 10 μl. The column temperature was set at 40˚C, and the sample tray temperature was maintained at 4˚C. For MS detection, the ESI source was operated in the negative ion mode. High purity nitrogen was used as the sheath (35 arb) and auxiliary (15 arb) gas and high-purity argon was used as the collision gas (1.5 mTorr). The parameters were as follows: spray voltage, 5.5 kV; capillary temperature, 300˚C; scan width for SRM, 0.5 m/z; scan time, 0.2 sec. The peak width settings for both Q1 and Q3 were 0.7 m/z. The SRM ion pair transitions and collision energy levels of each component are listed in the Table SI.

Calibration curves. CSA was dissolved in methanol and prepared into a 1 mg/ml stock solution. The stock solution was diluted in methanol gradient to working standard solutions of 40,000, 20,000, 10,000, 5,000, 1,600, 400, 100 and 50 ng/ml. For analyte identification and quantification, calibration standards were prepared by spiking 10 μl of working standard solutions into 100 μl of blank rat blood at final concentrations of 5-4,000 ng/ml. The solutions for calibration curves were pretreated according to the method in 1.1. Regression line of calibration curve was y=0.000411x+0.0294, R^2=0.9946.

Pharmacokinetic analysis. The blood concentration data were analyzed using the non-compartmental method using Drug and Statistics software (version 3.2.8; Shanghai BioGuider Medicinal Technology Co. Ltd.; http://www.gooddrug.net/). The peak blood concentration (C_{max}) and time to reach C_{max} (T_{max}) of CsA were acquired directly from the concentration-time curve. The elimination rate constant (K_{el}) was calculated using the log-linear regression of the phase-eliminated data. The area under the plasma concentration-time curve (AUC_{0-t}) from time 0 to the time of last measured concentration (C_{last}) was calculated using the linear trapezoidal rule. The AUC 0 to infinity ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by Clast/K_{el}. The terminal half-life ($T_{1/2}$) was calculated using the formula 0.693/K_{el}. The mean residence time was calculated using the curve area under the first moment vs. time curve/AUC. Apparent clearance was calculated using the formula Dose/$AUC_{0-\infty}$ and the apparent volume of distribution was calculated using the formula CL/K_{el}.

Measurement of mRNA expression. Reverse transcription-quantitative PCR was used to quantify the mRNA expression of breast cancer resistance protein (*BCRP*), bile salt export pump (*BSEP*), constitutive androstane receptor (*CAR*), *CYP3A1*, *CYP3A2*, *FXR*, *MRP2*, Na^+-taurocholate co-transporting polypeptide (*NTCP*), organic anion-transporting polypeptide 1B2 (*OATP1B2*), *P-gp*, pregnane X receptor (*PXR*) and *UCT1A1* in the liver, the mRNA expression of *BCRP, CAR, CYP3A1, CYP3A2, FXR, MRP2, P-gp, PXR* and *UGT1A1* in the small intestine and the mRNA expression of *BCRP, MRP2* and *P-gp* in the kidney. In total, ~100 mg tissue was received and thoroughly ground in 1 ml pre-chilled TRIzol (Thermo Fisher Scientific, Inc.). RNA was extracted with 250 μl chloroform, precipitated with isopropanol and washed with 75% ethanol. According to the manufacturer's instructions, RNA was reverse transcribed to cDNA using the PrimeScript RT reagent kit with gDNA Eraser (cat. no. RR047A; Takara Biotechnology Co., Ltd.). qPCR was performed using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) and a StepOne Real-Time PCR System (Thermo Fisher Scientific,

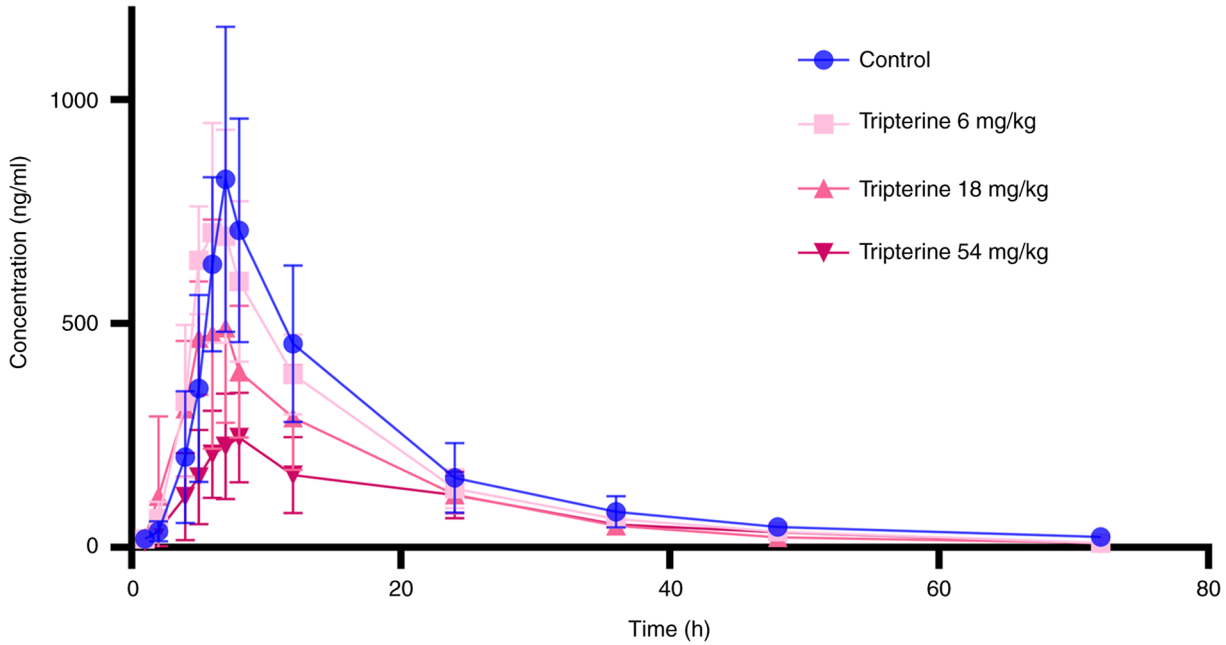

Figure 2. Mean blood concentration-time profiles of CsA. The concentration of CsA was determined after oral administration (10 mg/kg) pre-treated with saline (control) or tripterine (6, 18 and 54 mg/kg) to rats. Values are expressed as the mean ± SD (n=6). CsA, cyclosporine A.

Inc.). The reaction procedure involved first pre-denaturing at 95˚C for 1 min, followed by 40 cycles of 15 sec at 95˚C, 20 sec at 58˚C, 45 sec at 72˚C and finally the temperature rose from 60 to 95˚C at a rate of 1˚C per 20 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative mRNA expression levels (40). The sequences of all the primers used for PCR were provided in Table SII.

Measurement of protein expression. The protein expression of BCRP (1:1,000; cat. no. ab207732), BSEP (1:3,000; cat. no. ab217532), CAR (1:1,000; cat. no. ab186869), CYP3A1 (1:5,000; cat. no. ab22733), CYP3A2 (1:5,000; cat. no. ab195627), FXR (1:1,000; cat. no. ab228949), MRP2 (1:5,000; cat. no. ab203397), NTCP (1:5,000; cat. no. ab131084), OATP1B2 (1:500; ab15442), P-gp (1:6,000; cat. no. ab170904), PXR (1:1,000; cat. no. ab118336) and UCT1A1 (1:6,000; cat. no. ab194697; all from Abcam) in the liver, the protein expression of BCRP, CAR, CYP3A1, CYP3A2, FXR, MRP2, P-gp, PXR and UGT1A1 in the small intestine and the protein expression of BCRP, MRP2 and P-gp in the kidney of rats were analyzed by western blotting. After the total protein in the tissues was extracted using a RIPA lysis buffer (cat. no. G2002; Wuhan Servicebio Co., Ltd.) containing PMSF (cat. no. G2008; Wuhan Servicebio Co., Ltd.), and the protein concentration was measured by BCA, uniformly diluted to 20 μg/ml, and 10 μl was loaded per well. The protein samples were separated by 8-20% SDS-PAGE and then transferred onto PVDF membranes (MilliporeSigma). The PVDF membranes were added to the blocking solution (5% w/v skim milk) for 1 h at room temperature and then incubated with the diluted primary antibodies overnight at 4˚C. After the membranes were washed three times with TBST (TBS with 10% Tween), the membranes were incubated with the diluted HRP-conjugated secondary antibodies goat anti-rabbit IgG H&L (1:10,000; cat. no. ab205718,) and goat anti-mouse IgG H&L (1:10,000; cat. no. ab205719; both from Abcam) for 30 min at room temperature. The freshly prepared ECL mixed solution (BeyoECL Moon; purchased from Beyotime Institute of Biotechnology) was added dropwise to the protein side of the membranes and exposed in a dark room. The exposure conditions were adjusted according to different light intensities and then development and fixing were performed. Finally, the film was scanned and archived, before the AlphaEaseFC software (Version 3.0; ProteinSimple) processing system was used to analyze the optical density of the target band.

Statistical analysis. Experimental data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference by one-way ANOVA followed by Tukey's HSD test as a post hoc analysis.

Results

Effect of tripterine on the pharmacokinetics of CsA. Changes in the pharmacokinetic parameters of CsA under the influence of tripterine are revealed in Fig. 2 and Table I. Tripterine exerted a dose-dependent inhibitory effect on the blood concentration of CsA (Fig. 2). Compared with those in the control group, AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} of CsA were all found to be reduced at all doses of tripterine administration groups. Specifically, the AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} of CsA were decreased by 52.52 ($P<0.05$), 37.53 ($P<0.05$) and 66.74% ($P<0.05$), respectively, whereas the CLz/F of CsA was increased by 105.44% ($P<0.05$) in the 54 mg/kg dose group. Additionally, when the dosage of tripterine was 18 mg/kg, the $AUC_{0-\infty}$ of CsA decreased by 33.34% ($P<0.05$). There was no statistically significant difference in the other data among the administration groups and the control group.

Table I. Pharmacokinetic parameters of CsA after intragastric administration of CsA (10 mg/kg) in rats pre-treated with saline (control) or tripterine (n=6).

Parameters	Control	Triperine 6 mg/kg	Tripterine 18 mg/kg	Tripterine 54 mg/kg
AUC (0-t) (μg/l x h)	11,534.533±3,905.446	10,312.868±1,738.357	7,907.875±2,450.828	5,476.198±1,663.259[a]
AUC (0-∞) (μg/l x h)	1,2014.629±3,724.995	10,473.354±1,714.618	8,008.644±2470.771[a]	5,705.554±1,624.031[a]
Mean residence time (0-t) (h)	18.16±2.19	16.38±1.14	16.502±1.167	22.112±2.956
t1/2z (h)	18.995±8.132	12.4±2.1	11.77±2.95	15.86±7.006
Tmax (h)	7.167±0.408	6.167±1.169	6.167±1.472	7±1.265
Vz/F (l/kg)	28.537±24.462	17.799±5.386	23.422±10.932	47.16±38.51[a]
CLz/F (l/h/kg)	0.919±0.347	0.98±0.19	1.362±0.446	1.888±0.593[a]
Cmax (μg/l)	827±331.162	794.833±249.397	594.83±222.34	275±114[a]

[a]P<0.05 vs. control. CsA, cyclosporine A; AUC, area under the curve.

Relative mRNA expression levels of drug-metabolizing enzymes (DMEs), drug transporters (DTs) and nuclear receptors (NRs) in the liver, kidney and small intestine after the treatment of tripterine. The mRNA levels of DMEs, DTs and NRs were determined by RT-qPCR (Fig. 3).

mRNA expression levels of DMEs, DTs and NRs in the liver. As demonstrated in Fig. 3A, the mRNA expression of CYP3A1, CYP3A2, UGT1A1, OATP1B2, P-gp, MRP2, BCRP, BSEP, NTCP, CAR, PXR and FXR in the liver was measured. The mRNA expression of DMEs and DTs was found to be decreased, whilst the expression of NRs was increased, compared with those in the control group. The mRNA expression levels of CYP3A1 in the three tripterine pre-treated groups (6, 18 and 54 mg/kg) were decreased by 24.92, 46.62 and 57.54%, respectively. The decrease in the mRNA expression level of CYP3A2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 29.26, 39.42 and 79.25%, respectively. In addition, the mRNA expression level of UGT1A1 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 41.82, 62.6 and 84.04%, respectively. The mRNA expression level of OATP1B2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 45.78, 66.67 and 71.46%, respectively. The reductions of the mRNA expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups was by 42.81, 70.09 and 80.59%, respectively. The mRNA expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 44.63, 67.61 and 70.54%, respectively. For MRP2, the expression of mRNA was reduced by 52.03, 71.52 and 83.08% in the 6, 18 and 54 mg/kg tripterine pre-treated groups, respectively. BSEP mRNA expression was decreased by 20.49, 58.16 and 57.39%, respectively, in the 6, 18 and 54 mg/kg pre-treatment groups. The mRNA expression level of NTCP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 38.46, 46.04 and 66.12%, respectively. The mRNA expression level of CAR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was increased by 113.5, 287.7 and 367.68%, respectively. The mRNA expression level of PXR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was raised by 74.04, 221.33 and 235.20%, respectively. The increase of FXR mRNA expression in the 6, 18 and 54 mg/kg dose groups was 145.4, 272.55 and 275.28%, respectively.

mRNA expression levels of DMEs, DTs and NRs in the intestine. As revealed in Fig. 3B, the mRNA expression of CYP3A1, CYP3A2, UGT1A1, P-gp, MRP2, BCRP, CAR, PXR and FXR in the intestine was measured. The mRNA expression level of CYP3A1 in three tripterine pretreated groups (6, 18 and 54 mg/kg) was decreased by 45.07, 64.55 and 71.89%, respectively. The decrease in the mRNA expression level of CYP3A2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 40.48, 60.33 and 67.96%, respectively. The mRNA expression level of UGT1A1 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 41.82, 62.60 and 84.04%, respectively. The reductions of the mRNA expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups was by 42.81, 70.09 and 80.59%. The mRNA expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 46.4, 73.69 and 77.22%, respectively. In terms of MRP2, the expression of its mRNA was reduced by 37.03, 58.88 and 80.06% in the 6, 18 and 54 mg/kg tripterine pre-treated groups. The mRNA expression level of CAR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was increased by 110.16, 230.32 and 270.74% respectively. The mRNA expression level of PXR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was raised by 72.16, 120.29 and 220.24%. The increase of FXR mRNA expression in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 71.02%, 163.56 and 184.31%, respectively.

mRNA expression levels of DTs in the kidney. As shown in Fig. 3C, the mRNA expression of P-gp, MRP2 and BCRP was measured in the kidney. The mRNA expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 38.6, 58.7 and 79.73%, respectively. The mRNA expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 37.52, 59.82 and 76.82%, respectively. The mRNA expression level of MRP2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 39.5, 67.92 and 80.93%, respectively.

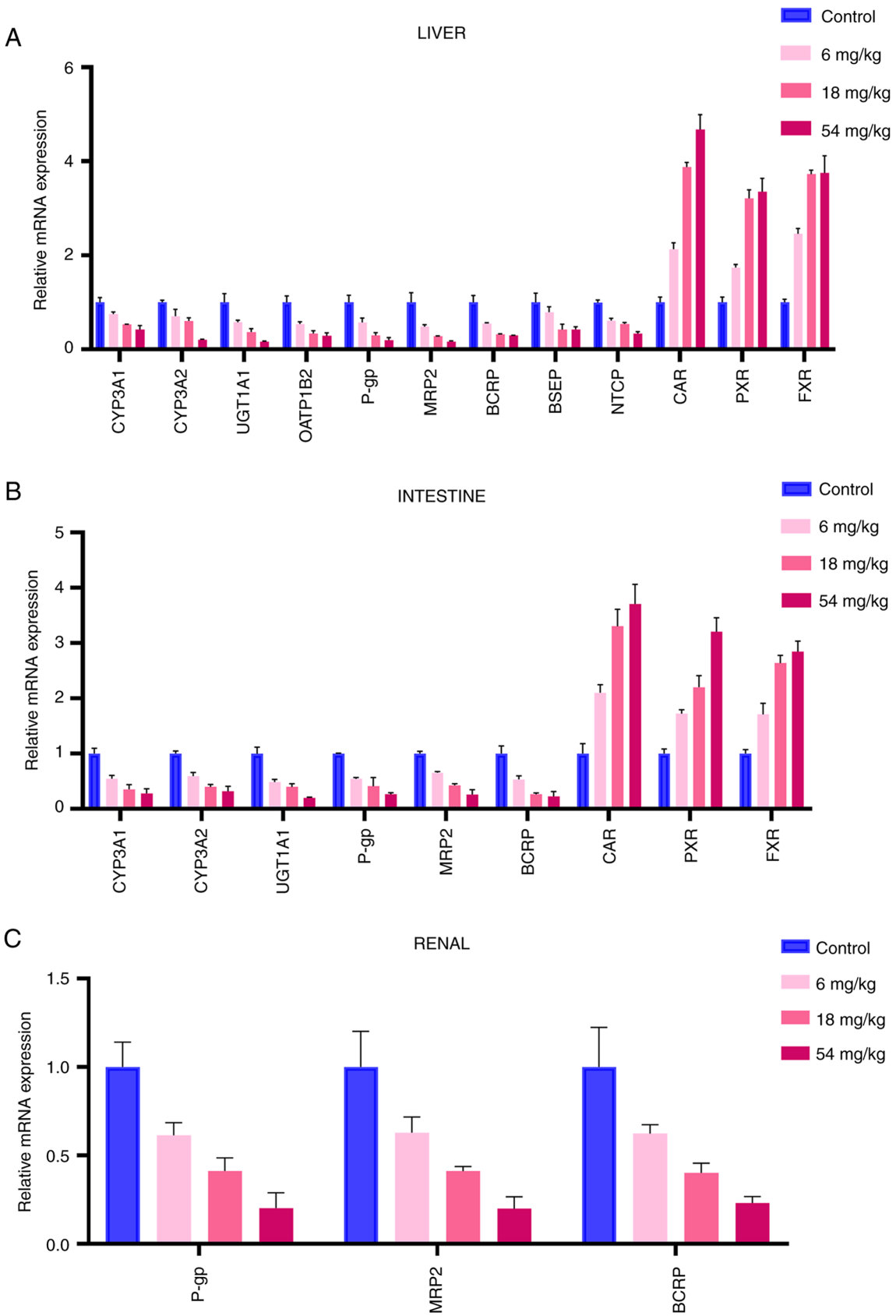

Figure 3. Effect of tripterine on the mRNA expression levels. (A) Effect of tripterine on the mRNA expression levels of CYP3A1, CYP3A2, UGT1A1, OATP1B2, P-gp, BCRP, MRP2, BSEP, NTCP, CAR, PXR and FXR in the liver. (B) Effect of tripterine on the mRNA expression levels of CYP3A1, CYP3A2, UGT1A1, P-gp, BCRP, MRP2, CAR, PXR and FXR in the intestine. (C) Effect of tripterine on the mRNA expression levels of P-gp, BCRP and MRP2 in the renal tissues. Relative mRNA expression levels in rats in the control and different doses of tripterine groups were measured by reverse transcription-quantitative PCR and calculated as comparative levels over control using the $2^{-\Delta\Delta Cq}$ method. Vertical bars represent the mean ± SD (n=3). CYP, Cytochrome P450 proteins; UGT, UDP-glucuronosyltransferases; OATP, organic anion transporting polypeptides; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multi-drug resistance protein; BSEP, bile salt export pump; NTCP, sodium taurocholate co-transporting polypeptide; CAR, constitutive androstane receptor; PXR, pregnane X receptor; FXR, farnesoid X receptor.

Protein expression levels of DMEs, DTs and NRs in the liver, kidney and small intestine after the treatment of tripterine. The protein expression of DMEs, DTs and NRs were next determined by western blotting. Densitometric analysis results are revealed in Fig. 4. The western blotting images of proteins in liver, kidney and small intestine are presented in Fig. 5.

Protein expression levels of DMEs, DTs and NRs in the liver. As shown in Fig. 4A, the protein expression of CYP3A1, CYP3A2, UGT1A1, OATP1B2, P-gp, MRP2, BCRP, BSEP, NTCP, CAR, PXR and FXR in the liver was measured. The protein expression of DMEs and DTs were found to be decreased, whilst that of NRs was increased, compared with those in the control group. The protein expression level of CYP3A1 in the three tripterine pre-treated groups (6, 18 and 54 mg/kg) was decreased by 49.25, 91.47 and 86.88%, respectively. The decrease in the protein expression level of CYP3A2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 29.26, 39.42 and 79.25%, respectively. The protein expression level of UGT1A1 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 30.7, 77.83 and 68.07%, respectively. The protein expression level of OATP1B2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 41.84, 74.97 and 69.8%, respectively. The reductions of the protein expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups were by 49.64, 80.85 and 85.82%, respectively. The protein expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 40, 52.18 and 72.5%, respectively. Regarding MRP2, the expression of its protein was reduced by 68.43, 91.6 and 92.62% in the 6, 18 and 54 mg/kg tripterine pre-treated groups. BSEP protein expression was decreased by 58.98, 92.1 and 88.9%, respectively, in the 6, 18 and 54 mg/kg tripterine pre-treated groups. The protein expression level of NTCP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 51.70, 89.50 and 86.83%, respectively. The protein expression level of CAR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was increased by 249.55, 790.28 and 764.19%, respectively. The protein expression level of PXR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was raised by 57.28, 465.38 and 1,231.11%, respectively. The increase of FXR protein expression in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 167.03, 454.8 and 449.81%, respectively.

Protein expression levels of DMEs, DTs and NRs in the intestine. As revealed in Fig. 4B, the protein expression of CYP3A1, CYP3A2, UGT1A1, P-gp, MRP2, BCRP, CAR, PXR and FXR in the intestine was measured. The protein expression level of CYP3A1 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was found to be decreased by 62.9, 90.53 and 90.34%, respectively. The decrease of the protein expression level of CYP3A2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 56.07, 82.61 and 86.31%, respectively. The protein expression level of UGT1A1 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 55.66, 87 and 88.26%, respectively. The reduction of the protein expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 31.78, 66.81 and 69.24%, respectively. The protein expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 46.96, 76.92 and 73.24%, respectively. For MRP2, the expression of protein was reduced by 60.64, 81.78 and 81.36% in the 6, 18 and 54 mg/kg tripterine pre-treated groups, respectively. The protein expression level of CAR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was increased by 268.82, 688.72 and 665.55%, respectively. The protein expression level of PXR in the 6, 18 and 54 mg/kg tripterine pre-treated groups was elevated by 397.40, 1,055.17 and 1,167.56%, respectively. The increase in FXR protein expression in the 6, 18 and 54 mg/kg tripterine pre-treated groups was 94.91, 158.49 and 215.79%, respectively.

Protein expression levels of DTs in the kidney. As demonstrated in Fig. 4C, the protein expression of P-gp, MRP2 and BCRP in the kidney tissues was measured. The protein expression level of P-gp in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 32.47, 74.65 and 64.99%, respectively. The protein expression level of BCRP in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 47.61, 69.27 and 70.29%, respectively. The protein expression level of MRP2 in the 6, 18 and 54 mg/kg tripterine pre-treated groups was decreased by 52.41, 76.12 and 77.17%, respectively.

Discussion

Tripterygium wilfordii is a traditional Chinese medicine with a wide range of pharmacological activities that has been garnering significant attention over the past two decades (8,32,40). By contrast, CsA is one of the most commonly used long-term immunosuppressive agents in clinical practice and has been shown in numerous animal studies to have enhanced immunosuppressive effects when combined with *Tripterygium wilfordii* after transplantation whilst reducing its toxic effects. However, as a natural herb, *Tripterygium wilfordii* contains a complex mixture of compounds, making it challenging to elucidate its underlying mechanisms precisely. To address this, tripterine, one of the primary active components of *Tripterygium wilfordii*, was focused upon to investigate whether it could regulate the pharmacokinetics of CsA. To the best of our knowledge, the present study was the first to demonstrate the dose-dependent inhibitory effect of tripterine on the pharmacokinetics of CsA in rats. These results suggest that pre-treatment with tripterine can significantly reduce the bioavailability of CsA in rats.

In the present study, two important phase-I metabolic enzymes, CYP3A1 and CYP3A2 were selected, along with the widely distributed phase-II metabolic enzyme UGT1A1 as the targets of investigation. CYP3A1 and CYP3A2 are primarily expressed in the liver and small intestinal epithelial cells of rats, where they metabolize ~70% CsA. Hou *et al* (41) previously reported that the bioavailability of CsA was decreased when P-gp and CYP3A4 were activated by glycyrrhizin. Based on this finding, it was hypothesized that inhibiting CYP3A enzymes would likely increase the bioavailability of CsA (36). However, the present results showed that the expression of both CYP3A1 and CYP3A2 was reduced, where the blood concentration of CsA was also decreased, following tripterine administration. This suggests that, in addition to its effects on drug-metabolizing enzymes and transporters, tripterine likely regulated the pharmacokinetics of CsA through other mechanisms.

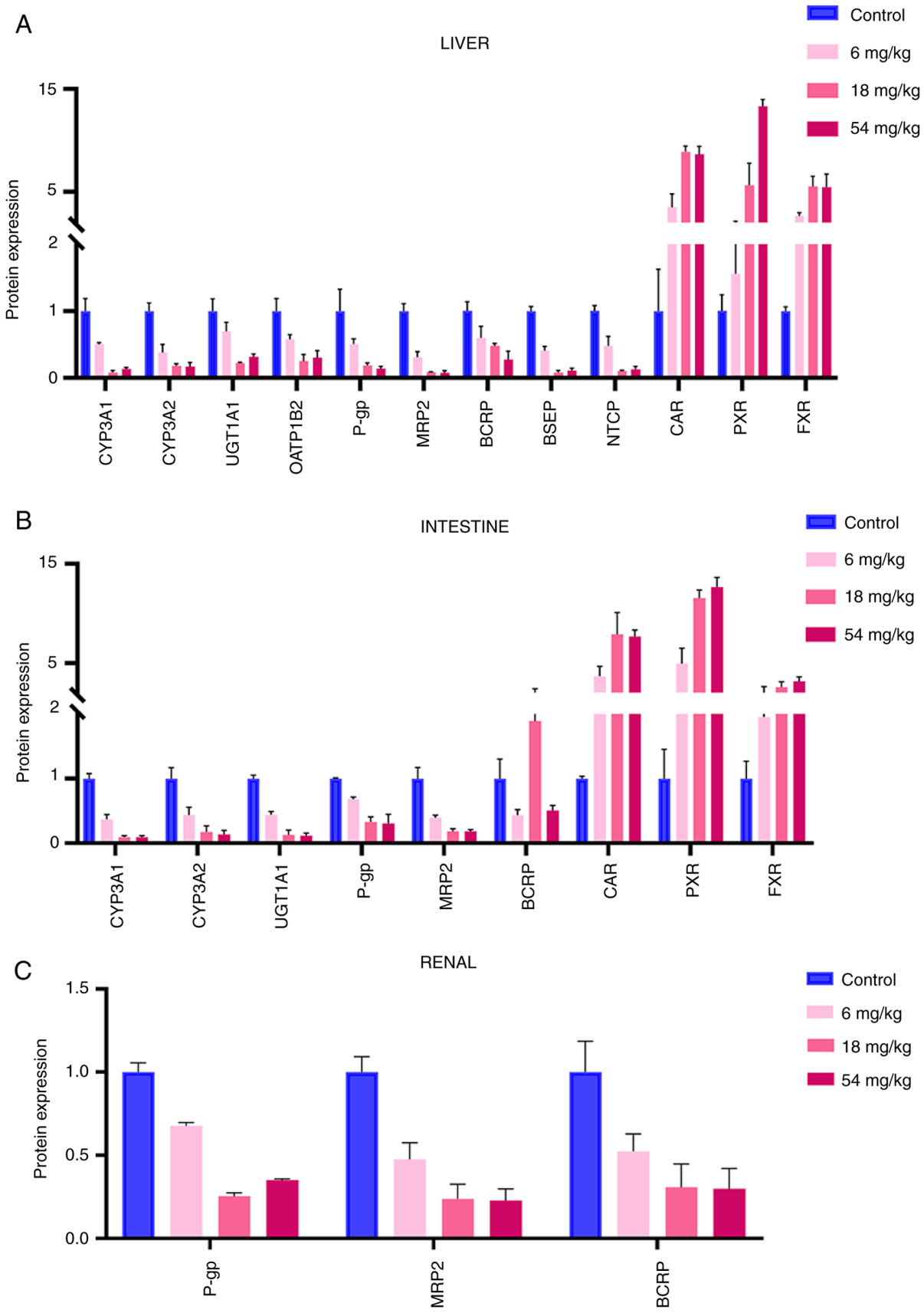

Figure 4. Effect of tripterine on protein expression levels in various tissues. (A) Effect of tripterine on the protein expression levels of CYP3A1, CYP3A2, UGT1A1, OATP1B2, P-gp, BCRP, MRP2, BSEP, NTCP, CAR, PXR and FXR in the liver. (B) Effect of tripterine on the protein expression levels of CYP3A1, CYP3A2, UGT1A1, P-gp, BCRP, MRP2, CAR, PXR and FXR in the intestine. (C) Effect of tripterine on the protein expression levels of P-gp, BCRP and MRP2 in the renal tissues. The protein expression levels in rats in the control and different doses of tripterine groups were assessed by western blotting. Vertical bars represent the mean ± SD (n=3). CYP, Cytochrome P450 proteins; UGT, UDP-glucuronosyltransferases; OATP, organic anion transporting polypeptides; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multi-drug resistance protein; BSEP, bile salt export pump; NTCP, sodium taurocholate co-transporting polypeptide; CAR, constitutive androstane receptor; PXR, pregnane X receptor; FXR, farnesoid X receptor.

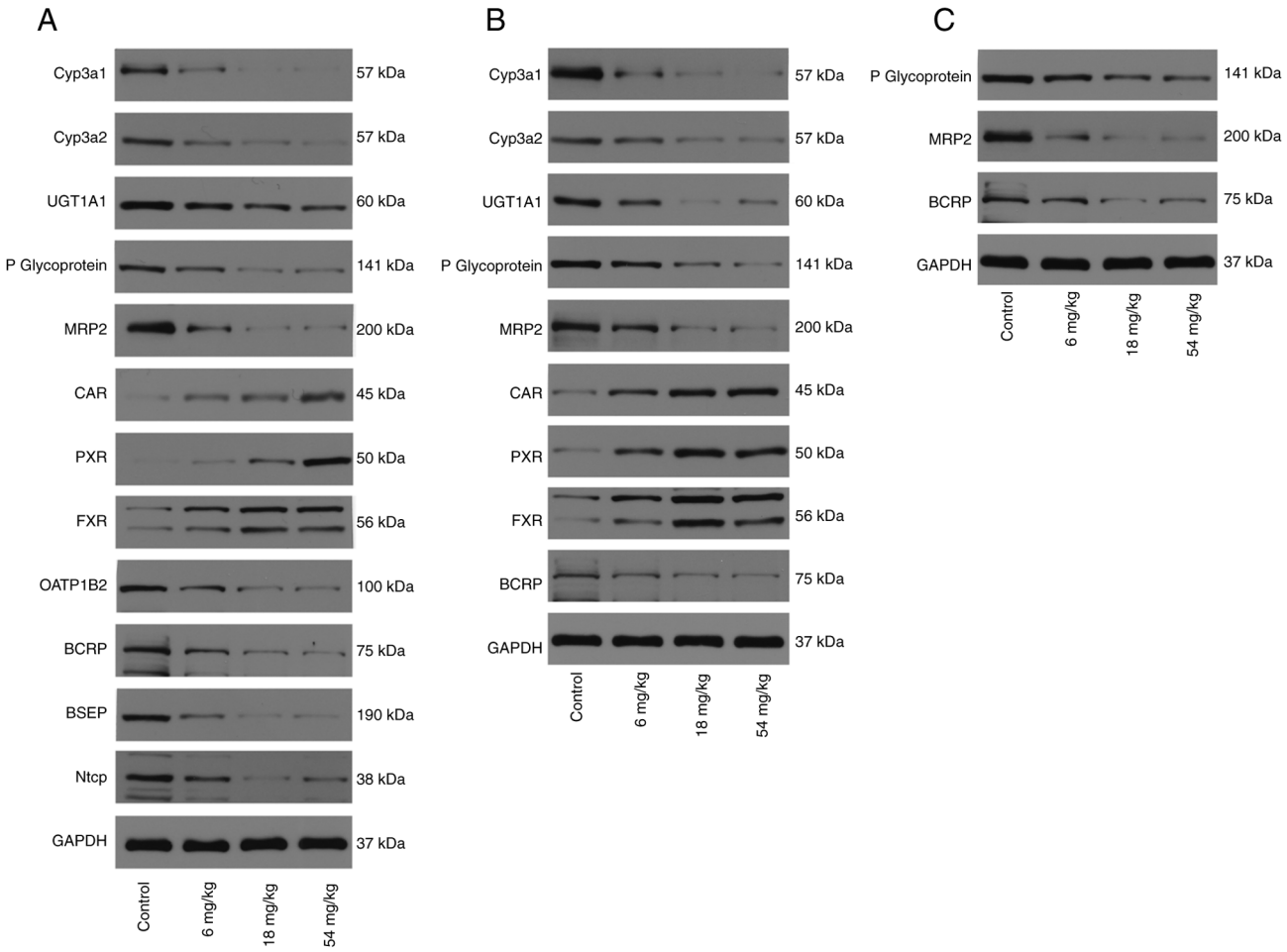

Figure 5. Western blotting images of drug-metabolizing enzymes, drug transporters and nuclear receptors in rat samples from each pre-treatment group. Representative western blotting images in (A) liver, (B) in small intestine and (C) in kidney. CYP, Cytochrome P450 proteins; UGT, UDP-glucuronosyltransferases; OATP, organic anion transporting polypeptides; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP, multi-drug resistance protein; BSEP, bile salt export pump; NTCP, sodium taurocholate co-transporting polypeptide; CAR, constitutive androstane receptor; PXR, pregnane X receptor; FXR, farnesoid X receptor.

OATP1B2 is typically expressed on the hepatocyte sinusoidal membrane, with human homologs OATP1B1 and OATP1B3 being closely related. They serve a role in the transport of linear and cyclic peptides to the liver from the basal side of rat hepatocytes (42,43). Both OATP1B2 and NTCP are influx transporters that transport specific molecules from the blood into the hepatic cytosol and are predominantly found on the basolateral membrane of hepatocytes (44). By contrast, P-gp, MRP2, BCRP and BSEP are efflux transporters located on the canalicular membrane of hepatocytes. These transporters facilitate the excretion of drugs and metabolites from the hepatic cytosol into bile (44). In the intestine, P-gp, MRP2 and BCRP, located on the apical side of enterocytes, can pump drugs back into the gut lumen, thereby limiting the oral absorption of CsA (45). In the kidney, P-gp, MRP2 and BCRP are found on the basolateral side of proximal tubular cells, where they pump drugs and their metabolites into the urine (46). CsA has been previously recognized to be a broad-spectrum modulator for multi-drug resistance proteins, such as P-gp, BCRP, MRP2 and OATP1B2 (47-50). CAR, PXR and FXR are nuclear receptors that can regulate the expression of various DMEs and DTs in a negative feedback manner, including CYP3As, MRP2 and P-gp (51). The present study identified that tripterine inhibited the expression of both DMEs (CYP3A1 and CYP3A2) and DTs (OATP1B2, P-gp, BCRP and MRP2), likely by activating CAR, PXR and FXR. However, whilst inhibition of P-gp and BCRP would typically be expected to increase the plasma concentration and decrease the clearance of their substrates (such as CsA), results from the present study suggest a more complex interaction at play.

In addition to the effects on DMEs and DTs, bile acid metabolism serves a crucial role in the pharmacokinetics of CsA. Previous studies have shown that the concentration of CsA significantly decreases after bile duct ligation, suggesting the importance of bile acids in its absorption and metabolism (52,53). The present study demonstrated that tripterine had a profound effect on bile-related transporters. Specifically, tripterine significantly inhibited the expression of MRP2, NTCP and BSEP. NTCP primarily mediates the uptake of bile salts into hepatocytes, whilst BSEP and MRP2 are responsible for secreting mono-anionic and di-anionic conjugated bile salts into bile (54,55). By inhibiting the expression of NTCP, BSEP and MRP2 in the liver, tripterine appeared to disrupt bile acid circulation. Furthermore, tripterine was previously found to upregulate

FXR expression in the intestine, inhibiting bile acid synthesis through the FXR/FGF19 pathway (56). This suggests that tripterine cannot only reduce bile acid synthesis but also regulate bile acid metabolism. Given that the oral absorption of CsA is known to depend on bile secretion (8,26,57), the emulsification of bile acids aids in the absorption of CsA, a macromolecular lipid-soluble drug, in the intestine (58). Therefore, findings from the present study suggest that tripterine can decrease the oral bioavailability of CsA by inhibiting bile acid metabolism and disrupting the normal bile acid circulation process.

However, in the present study, only whole blood drug concentration was detected to explore the pharmacokinetics of CsA, but its intestinal and kidney drug concentration was not detected, which is a limitation of the present study. The main components of Tripterygium glycosides include Tripterygium triptolide, tripterine and triptolide ketone. Different from Wuzhi capsule, which exerts immunosuppressive effects by increasing the blood concentration of CsA, multiple active components of Tripterygium glycosides possess immunosuppressive properties, although its underlying mechanisms remain elusive (59). Therefore, it is highly likely that tripterine and CsA can exert immunosuppression through different mechanisms, whereby the change in plasma CsA concentration is not the only indicator that can be used to evaluate their immune synergistic effects. The blood concentration of CsA is not only closely associated with its immunosuppressive efficacy but also to its toxicity. The primary objective of the present study was to evaluate whether the combination of tripterine and CsA can alter the pharmacokinetic profile of the latter and to explore the underlying mechanisms, if any. Associated pharmacodynamic or toxicity studies will be required in subsequent studies. At present, the specific mechanism of the association between the efficacy of CsA and its blood concentration is lacking. Through the correlation analysis of population pharmacokinetics, blood concentration was found to be the dependent variable that had a positive correlation with the efficacy of CsA. Further research will be needed to discover novel easy-to-detect biochemical markers that can more specifically represent the efficacy of CsA.

In conclusion, tripterine was found to inhibit the expression of both DMEs and DTs, in addition to bile acid-related transporters, which profoundly impacts bile acid circulation and gastrointestinal lipid absorption. Given the significant positive correlation between CsA absorption and lipid absorption, the primary mechanism through which tripterine can reduce the oral bioavailability of CsA appears to be its interference with lipid absorption and bile acid metabolism. This inhibition disrupts the normal absorption process of CsA, leading to a decrease in its bioavailability. In addition, tripterine can dose-dependently inhibit the oral bioavailability of CsA, which may be associated with inhibition of the expression of bile acid transporters in liver, activation of FXR in the intestine and the regulation of bile acid metabolism.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SS, YL and RZ participated in research design and supervision. PG and PL conducted experiments and confirm the authenticity of all the raw data. JZ finished data analysis and visualization of data. JZ and RZ wrote or contributed to the writing of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal studies were approved (approval no. 4352; date, 2023) by The Institutional Animal Care and Use Committee of Tongji Medical College Huazhong University of Science and Technology (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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