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Original Article

Activation of P-glycoprotein and CYP 3A by Coptidis Rhizoma *in vivo*: Using cyclosporine as a probe substrate in rats

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ABSTRACT

Coptidis Rhizoma (CR), the rhizome of *Coptis chinensis* FRANCH, is a popular Chinese herb. CR contains plenty of isoquinoline alkaloids such as berberine, coptisine and palmatine. Cyclosporine (CSP), an important immunosuppressant with narrow therapeutic window, is employed as a probe substrate of P-glycoprotein (P-gp) and CYP3A4 in order to investigate the *in vivo* modulation effect of CR on P-gp and CYP3A4. Three groups of rats were orally administered CSP without and with single dose or repeated dosing of CR in a parallel design. Blood samples were collected at specific time points and the blood CSP concentration was determined by a specific monoclonal fluorescence polarization immunoassay. The results showed that a single dose (1.0 g/kg) and the 7th dose (1.0 g/kg) of CR significantly decreased the C_{max} of CSP by 56.9% and 70.4%, and reduced the $AUC_{0\text{-}540}$ by 56.4% and 68.7%, respectively. Cell study indicated that CR decoction, berberine, coptisine, palmatine all activated the efflux transport of P-gp. *Ex-vivo* study showed that the serum metabolites of CR activated CYP 3A4. In conclusion, through using CSP as an *in vivo* probe substrate, we have verified that oral intake of CR activated the functions of P-gp and CYP3A based on *in vivo* and *in vitro* studies.

1. Introduction

Coptidis Rhizoma (CR, the rhizome of *Coptis chinensis* FRANCH), is often prescribed for the treatments of intestinal infections, inflammation - related diseases, diabetes mellitus and cancers in Asian countries [1]. The major constituents of CR are isoquinoline alkaloids including berberine, coptisine and palmatine, which exhibited various beneficial effects such as anti-bacterial, anti-virus, anti-inflammatory and anti-cancer activities [2–5].

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P-glycoprotein (P-gp), an efflux pump of lipophilic and cationic drug, and cytochrome P450 enzymes (CYP450s) have been well known to play crucial roles in the oral bioavailability of various drugs and drug–drug interactions [6,7]. Recent studies have reported that berberine, the major isoquinoline alkaloid in CR, exhibited biphasic effects on P-gp activity, activation at lower concentration and inhibition at higher concentration [8,9]. In addition, CR and the isoquinoline alkaloids inhibited CYP450s such as CYP3A4, CYP2D6 and CYP1A2 [10]. It is reasonable to suspect that CR might alter the pharmacokinetics of various substrates of P-gp and/or CYPs. However till now, regarding the *in vivo* modulation of CR on P-gp and CYP450s, relevant animal or clinical evidence has not yet been reported.

Cyclosporine (CSP), a substrate of P-gp and CYP3A4, is one of the most important immunosuppressive agents but with narrow therapeutic range. CSP was prescribed for the prevention of allograft rejection after transplants and for the treatments of rheumatoid arthritis and psoriasis [11]. Therapeutic drug monitoring of CSP is usually performed for the avoidance of allograft rejection and adverse effects such as hepatotoxicity and nephrotoxicity [12]. Given that CR and the isoquinoline alkaloid modulated P-gp and CYPs, we hypothesized that CR might affect the blood levels of CSP. Therefore, this study employed CSP as an *in vivo* probe substrate of P-gp/CYP3A to investigate the virtual modulation of single dose and repeated dosing of CR on P-gp and CYP3A. Furthermore, cell study was used to evaluate the modulation effect of CR on P-gp, and ex-vivo approach was employed to verify the effect on CYP3A4.

2. Materials and methods

2.1. Materials and reagents

The dried crude drug of CR in the form of slices with 0.5 mm thickness was purchased from an herbal drugstore in Taichung, Taiwan and identified through visual inspection and microscopic examination by Dr. Yu-Chi Hou. A voucher specimen (CMU-1905-8) was deposited in China Medical University. Rhodamine 123, sodium dodecyl sulfate (SDS), 3-(4′,5′-dimethylthiazol-2′-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Triton X-100, daidzein (98%) and verapamil were purchased from Sigma (St. Louis, MO, USA). Palmatine (97%) was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA). Berberine (98%) was obtained from Tokyo Pure Chemical Industries, Ltd. (Tokyo, Japan). Coptisine (98%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rifampin was purchased from MP Biomedicals (California, USA). Dulbecco's Modified Eagle Medium (DMEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Nonessential amino acid, Hank's Buffered Salt Solution (HBSS) and trypsin/EDTA were purchased from Invitrogen (Grand Island, NY, USA). Cyclosporine (Neoral®, 100 mg/mL) was kindly provided by Novartis (Taiwan) Co. Ltd. Acetonitrile and methanol (LC grade) were purchased from ECHO Chemical Co. (Miaoli, Taiwan). TDx kit was supplied by Abbott Laboratories (Abbott Park, IL, USA). Other reagents were HPLC grade or analytical reagent grade. Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Preparation and characterization of CR decoction

The decoction was prepared by extracting CR with boiling water. Briefly, dried crude drugs of CR (25 g) were immersed in 500 mL of water for half an hour and then heated on a gas stove. After boiling, gentle heating was continued until the volume reduced to half volume and filtered while hot. The filtrate was concentrated to make 50 mL to afford a concentration of 0.5 g/mL and divided into aliquots, then frozen at −30 °C for later use.

For characterization, CR decoction (300 μL) was mixed with 700 μL of MeOH. After vortexed and centrifuged, the supernatant (140 μL) was mixed with 60 μL of daidzein (100 μg/mL as internal standard) and 20 μL was subject to HPLC analysis. The system was equipped with a Shimadzu SIL-10AD VP automatic sample injector, a Shimadzu SPD-10AVP Detector and a Shimadzu LC-10AT VP pumps. Reversed-phase separation was carried out using an Apollo C18 column (4.6 × 250 mm, 5 μm) and equipped with a guard column (4.6 × 50 mm, 5 μm) (Alltech Associates Inc., USA). The mobile phase consisted of acetonitrile and 0.1% phosphoric acid (25:75) and the flow rate was 1.0 mL/min. The detection wavelength was set at 270 nm.

2.3. Drug administration and blood collection

The animal study was carried out in strict accordance with the recommendations by "The Guidebook for the Care and Use of Laboratory Animals" published by the Chinese Society for the Laboratory Animal Science, Taiwan. The Institutional Animal Care and Use Committee (IACUC), China Medical University approved this animal protocol. All blood samplings were conducted under anesthesia with 2–3% isoflurane to minimize the suffering and distress of rats.

Male Sprague–Dawley rats were supplied by National Laboratory Animal Center (Taipei, Taiwan) and kept in the Animal Center of China Medical University (Taichung, Taiwan). Rats weighing 280–430 g were fasted for 12 h before drug administration, and food was withheld for another 3 h. Drug administration was conducted in a parallel design. A dose of 1.0 g/kg of CR comparable to human dose was chosen for this study according to FDA guideline for "Conversion of Animal Doses to Human Equivalent Doses" because the daily dose of CR was around 10 g in clinical practice. In the first group, six control rats received 2.0 mL/kg of water, equal to the volume of CR decoction, at 0.5 h before CSP (2.5 mg/1.0 mL/kg). In the second group, CR decoction (1.0 g/2.0 mL/kg) was orally given to five rats at 0.5 h before CSP (2.5 mg/1.0 mL/kg) via gastric gavage. In the third group, five rats were given CR decoction (1.0 g/2.0 mL/kg) twice daily and the 7th dose was administered at 0.5 h before CSP (2.5 mg/1.0 mL/kg). Rats were anesthetized with 2–3% isoflurane before blood sampling. After dosing of CSP, the withdrawn blood samples (0.3 mL each) were collected into small plastic vials containing EDTA at 20, 40, 60, 180, 300 and 540 min and assayed within 24 h. Water was supplied to rats via gastric gavage at specific time during experiment.

2.4. Quantitation of blood CSP concentration

CSP concentration in blood was measured by a specific monoclonal fluorescence polarization immunoassay (Abbott, Abbott Park, III, USA) [13]. The calibration range was 0.0–1500.0 ng/mL and the lower limit of quantitation (LLOQ) was 25.0 ng/mL. Validation of the calibration curve was conducted by testing three controls with three concentration ranges (L: 120.0–180.0 ng/mL; M: 340.0–460.0 ng/mL; H: 680.0–920.0 ng/mL) before sample assay. If the validation failed, a new calibration curve was then constructed. The coefficients of variation and the relative error were all less than 4% for intra-day and inter-day analysis. The recoveries were 97.8–109.2%.

2.5. Cell line and culture conditions

LS 180, human colon adenocarcinoma cell line, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). DMEM medium supplemented with 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel), 0.1 mM nonessential amino acid, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 292 μg/mL of glutamine were used for cell maintenance and cells were incubated at 37 °C containing 5% CO_2. The medium was changed every other day and cells were subcultured when 80%–90% confluency was reached. The passages used in this study were between 56 and 65.

2.6. Cell viability assay

The effects of tested drugs on the viability of LS 180 cells were evaluated by MTT assay [14]. After seeding the cells into a 96-well plate for overnight incubation, tested drugs including CR (200 μg/mL), berberine (10 μM), coptisine (10 μM), palmatine (10 μM), verapamil (100 μM) and rifampin (20 μM) were added and incubated for 72 h, then 15 μL of MTT (5 mg/mL) was added and incubated for additional 4 h. Acid-SDS (10%) solution was added to lyse the cell and then the cell viability determined at 570 nm by a microplate reader (BioTex, Highland Park, Winooski, VT, USA).

2.7. Acute effects of CR and isoquinolines on the function of P-gp

The transport assay of rhodamine 123 was performed by using LS 180 cells to measure the effect of each isoquinoline alkaloid and CR on the efflux activity of P-gp [15–21]. Briefly, after seeding the cells in a 96-well plate and incubated for overnight, rhodamine 123 was added and incubated for 1 h. Then, tested agents including CR (100 and 200 μg/mL), berberine (2.5, 5.0, 10 μM), coptisine (2.5, 5.0, 10 μM) and palmatine (2.5, 5.0, 10 μM), which were nontoxic concentrations based on cell viability assay, were added to individual wells and incubated at 37 °C. Verapamil (100 μM) was used as a positive control of P-gp inhibitor. After 4-h incubation, the cells were washed twice with ice-cold PBS, and 100 μL of 0.1% triton X-100 was added to lyse the cells. The fluorescence of the lysate was measured with excitation at 485 nm and emission at 528 nm. The relative intracellular accumulation of rhodamine 123 was calculated by comparison with that of control and corrected with protein contents.

2.8. Chronic effect of CR on the function of P-gp

The transport assay of rhodamine 123 by using LS 180 cells was modified from previous studies to evaluate the chronic effect of CR on the efflux transport of P-gp [15–21]. Briefly, after seeding the cells in a 96-well plate, tested agents (100 and 200 μg/mL of CR) and rifampin (20 μM, a positive control of P-gp inducer) were added to individual well and incubated for 72 h at 37 °C. After 72-h incubation, the cells were washed twice with ice-cold PBS, and rhodamine 123 was added and incubated for 1 h. Then, the cells were washed with ice-cold PBS, incubated with HBSS buffer for another 4 h and other procedures were the same as previously prescribed.

2.9. Preparation of the serum metabolites of CR (CRM)

In order to mimic the real molecules interacting with CYP3A4 in enterocytes or hepatocytes, the serum metabolites of CR in rats was prepared. Briefly, after fasted overnight, rats were orally administered two doses of 2.0 g/kg of CR with 30-min interval. Blood was collected at 30 min after the second dose. After centrifuging at 10,000 g for 15 min, the serum was vortexed with 4-fold volume of methanol. After centrifugation, the supernatant was concentrated in a rotatory evaporator under vacuum to dryness. To the residue, an appropriate volume of water was added to afford a solution with 10-fold serum concentration, which was divided into aliquots and stored at −80 °C for later use. In addition, blank serum was collected from rats without receiving CR and processed likewise to prepare the blank serum control as the background for CRM.

2.10. Effect of CRM on the function of CYP3A4

For evaluating the effect of CRM on the activity of CYP3A, Vivid® CYP450 screening kits (Invitrogen, Carlsbad, CA, USA) were used. All the procedures were performed according the manual provided by the manufacturer. Briefly, the test agents including CRM (1/4-, 1/2- and 1-fold serum concentrations), ketoconazole (10 μM, spiked in blank serum as a positive control of CYP3A4 inhibitor) or blank serum control were incubated with CYP450 recombinant BACULOSOMES®, glucose-6-phosphate and glucose-6-phosphate dehydrogenase in 96-well black plate at room temperature for 20 min, then a specific CYP3A4 substrate (Vivid® BOMR) and $NADP^+$ were added and incubated at room temperature for another 30 min. After incubation, the fluorescence was measured with excitation at 530 nm and emission at 590 nm.

2.11. Data analysis

The pharmacokinetic parameters of CSP were calculated using noncompartment model with the aid of WinNonlin® (version 1.1, SCI software, Statistical Consulting, Inc., Apex, NC). The peak blood concentration (C_{max}) and time to reach peak blood concentration (T_{max}) were obtained from experimental data. The area under the serum concentration–time

curve (AUC_{0-t}) was calculated using trapezoidal rule to the last point. The statistical software SPSS was used for analyzing the differences among treatments by using unpaired Student's *t*-test for *in vivo* and *in vitro* studies. Statistical significance level was set at $p < 0.05$.

3. Results

3.1. Characterization of CR decoction

Fig. 1 shows the HPLC chromatogram of berberine, coptisine and palmatine in CR decoction, which were satisfactorily resolved within 30 min by an isocratic elution. Quantitation results showed that the concentrations of berberine, coptisine and palmatine in CR decoction were 175.5, 44.5 and 47.0 μM, respectively. The validation of this analytical method confirmed that all coefficients of variation (CVs) and the relative errors of intraday and interday analyses were below 8.1% and 9.5%, respectively. The LLOQs were 3.8, 1.3 and 1.3 μg/mL, and the lower limit of detections (LODs) were 0.01, 0.01 and 0.01 μg/mL for berberine, coptisine and palmatine, respectively.

3.2. Effects of single dose and repeated dosing of CR on CSP pharmacokinetics in rats

The blood concentration profiles of CSP in rats administered CSP with and without single dose and the 7th dose of 1.0 g/kg of CR are shown in Fig. 2. The pharmacokinetic parameters are listed in Table 1. The results showed that coadministration with single dose of CR significantly decreased the C_{max} and AUC_{0-t} of CSP by 56.9% and 56.4%, respectively. In addition, coadministration with the 7th dose of 1.0 g/kg of CR significantly decreased the C_{max} and AUC_{0-t} of CSP by 70.4% and 68.7%, respectively. Other parameters including T_{max}, $T_{1/2}$ and MRT were not affected.

3.3. Cell viability assay

MTT assay indicated that 200 μg/mL of CR, 10 μM of berberine, coptisine and palmatine, 100 μM of verapamil and 20 μM of rifampin all exerted no toxic influences on the viability of LS 180 cells (data not shown).

3.4. Acute effects of CR, berberine, coptisine and palmatine on the function of P-gp

Fig. 3 shows the effects of CR, berberine, coptisine and palmatine on the intracellular accumulation of rhodamine 123 in LS 180 cells. The results indicated that berberine and coptisine at 2.5, 5.0 and 10 μM significantly decreased the intracellular accumulation of rhodamine 123 by 24–39%. Palmatine at 5.0 and 10 μM significantly decreased that by 16–18%. CR at 100 and 200 μg/mL significantly decreased that by 32.8% and 32.4%, respectively. Conversely, verapamil (a positive control of P-gp inhibitor) at 100 μM significantly enhanced the intracellular accumulation of rhodamine 123 by 32.5%.

3.5. Chronic effect of CR on the function of P-gp

Fig. 4 shows the chronic effect of CR on the intracellular accumulation of rhodamine 123 in LS 180 cells after incubation for 72 h. The results indicated that CR at 100 and 200 μg/mL significantly decreased the intracellular accumulation of rhodamine 123 by 40.3% and 42.3%, respectively. As a positive control of inducer, rifampin at 20 μM significantly decreased the intracellular accumulation of rhodamine 123 by 22.7%.

3.6. Effect of CRM on the activity of CYP3A4

Fig. 5 shows the effect of CRM on CYP3A4 activity. The results revealed that CRM at 1/4-, 1/2- and 1- fold serum concentrations significantly increased the activity of CYP3A4 by 34.4%, 48.1% and 59.4%, respectively, when compared to those of

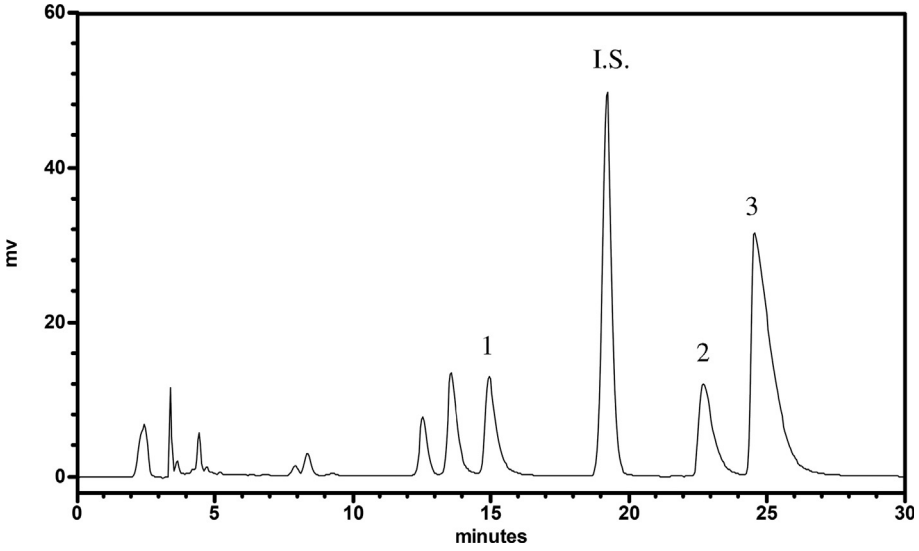

Fig. 1 – HPLC chromatogram of CR decoction. 1. coptisine; 2. palmatine; 3. berberine; I.S.: daidzein.

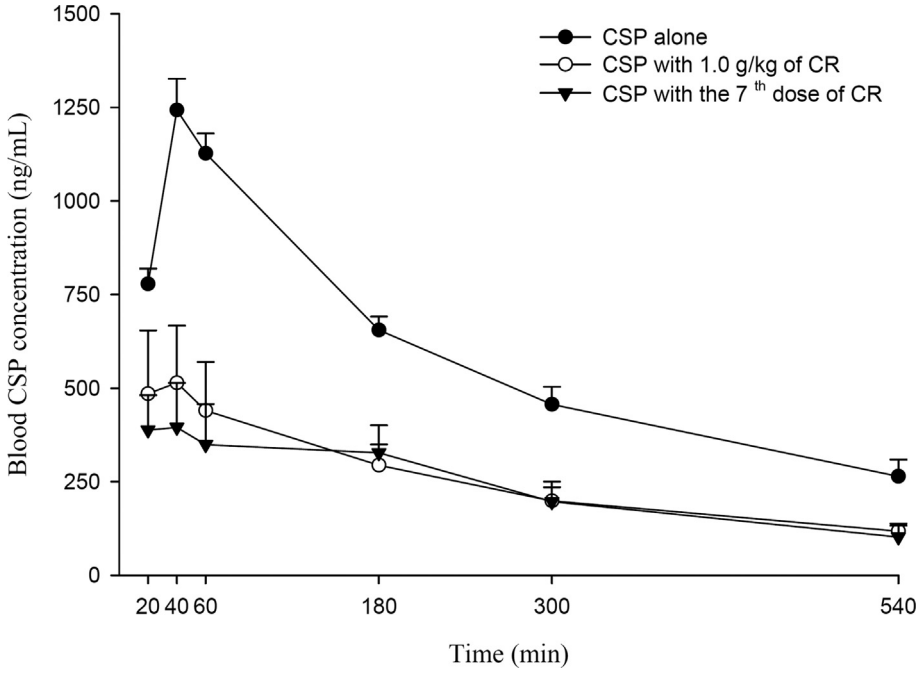

Fig. 2 – Mean (±S.E.) CSP blood concentration–time profiles after oral administration of CSP alone (●) and coadministration with single dose (○) and the 7th dose (▼) of 1.0 g/kg of CR in rats.

correspondent concentration of blank serum specimen. As a positive control, 10 μM of ketoconazole spiked in blank serum significantly decreased the activity of CYP3A4 by 14.3%.

4. Discussion

Quantitation of isoquinoline alkaloids in CR decoction showed that berberine was the major one, while palmatine and coptisine were about a quarter of berberine. This result was in good agreement with previous reports [22–24].

Table 1 – Pharmacokinetic parameters of CSP after administration of CSP alone (2.5 mg/kg) and coadministration of CR (1.0 g/kg) and the 7th dose of CR (1.0 g/kg) in rats.

Parameters	CSP alone (n = 6)	CSP + CR (n = 5)	CSP + 7th dose of CR (n = 5)
T_{max}	46.7 ± 4.2	64.0 ± 29.3	56.0 ± 31.2
C_{max}	1262.3 ± 72.1[a]	544.3 ± 176.0 [b] (-56.9%)	374.2 ± 62.8 [b] (-70.4%)
AUC_{0-t}	311.8 ± 18.6[a]	136.0 ± 32.4 [b] (-56.4%)	97.5 ± 15.6 [b] (-68.7%)
MRT	197.0 ± 8.2	210.2 ± 16.7	204.7 ± 12.2

Data expressed as mean ± S.E.
Means in a row without a common superscript differ. $p < 0.05$.
A mean with a symbol of "a" was significantly different from a mean with a symbol of "b".
T_{max} (min): time to reach peak blood concentration.
C_{max} (ng/mL): peak blood concentration.
AUC_{0-t} (μg•min/mL): area under the blood concentration–time curve.
MRT (min): mean residence time.

The results of CR–CSP pharmacokinetic interaction study showed that single dose of CR significantly decreased both the peak blood concentration and systemic exposure of CSP, indicating that the oral bioavailability of CSP was reduced by CR. Likewise, when rats were repeatedly dosed with CR before CSP dosing, the oral bioavailability of CSP was also significantly decreased. Observing the blood profiles revealed that the curves of CSP during the absorption stage were markedly lowered by both dosage regimens of CR.

It is well known that both P-gp and CYP3A4 play important roles in the oral bioavailability of CSP [25,26]. Therefore, we speculated that the efflux function of P-gp in the intestine and/or the catalytic activity of CYP3A in the intestine and/or liver were probably activated by CR, which resulted in decreased bioavailability of CSP in rats. In order to clarify the possible involvement of P-gp in this interaction, the transport study of rhodamine 123, a typical substrate of P-gp, in LS 180 cells was conducted. The results showing that the intracellular accumulation of rhodamine 123 was significantly decreased by CR revealed that the efflux transport of P-gp was activated. It indicated that the decreased bioavailability of CSP caused by CR might be in part arisen from the increased efflux of CSP into gut lumen mediated by P-gp. We further assumed that berberine, coptisine and palmatine were the probable causative components activating P-gp, and the transport study of rhodamine 123 showed that berberine, coptisine and palmatine all significantly activated P-gp at the concentration ranges tested. Among those three alkaloids, berberine and coptisine showed comparable activation effects on P-gp, and palmatine was a weaker activator. This finding that 2.5–10 μM of berberine exhibited activation modulation on P-gp was in good agreement with a previous study which used primary cultured rat brain microvascular

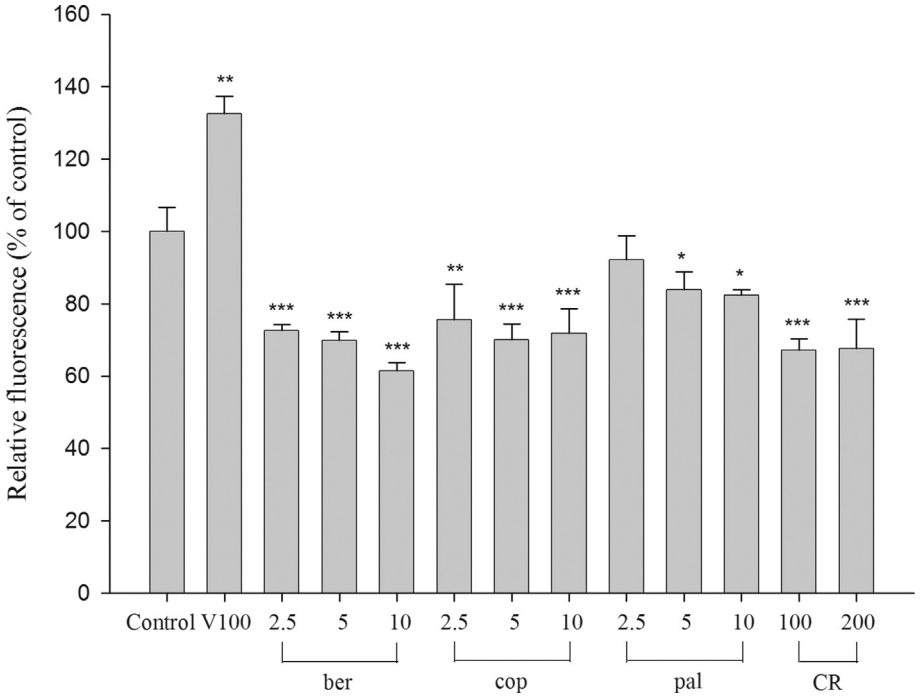

Fig. 3 – Acute effects of CR (μg/mL), berberine (ber, μM), coptisine (cop, μM), palmatine (pal, μM) and verapamil (V, μM) on the intracellular accumulation of rhodamine 123 in LS 180 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

endothelial cells and reported that berberine activated P-gp at lower concentrations [9].

With regard to the effects of CR on CYPs, we have to consider the metabolic fates of isoquinoline alkaloids. It has been reported that the major molecules after berberine intake included phase I and phase II metabolites, such as berberrubine, thalifendine, demethyleneberberine and their correspondent glucuronides or sulfates, whereas only a trace of berberine parent form was detected [22,27]. The other two isoquinoline alkaloids should have similar metabolic fates like berberine, i.e. via extensive phase I and phase II metabolisms. Therefore, we had prepared CRM for mimicking the metabolites of CR interacting with CYPs in the enterocytes and/or hepatocytes in order to investigate the *in vivo* modulation effect of CR on CYP3A4. Since the authentic standards of the phase I and phase II metabolites of isoquinolines were not

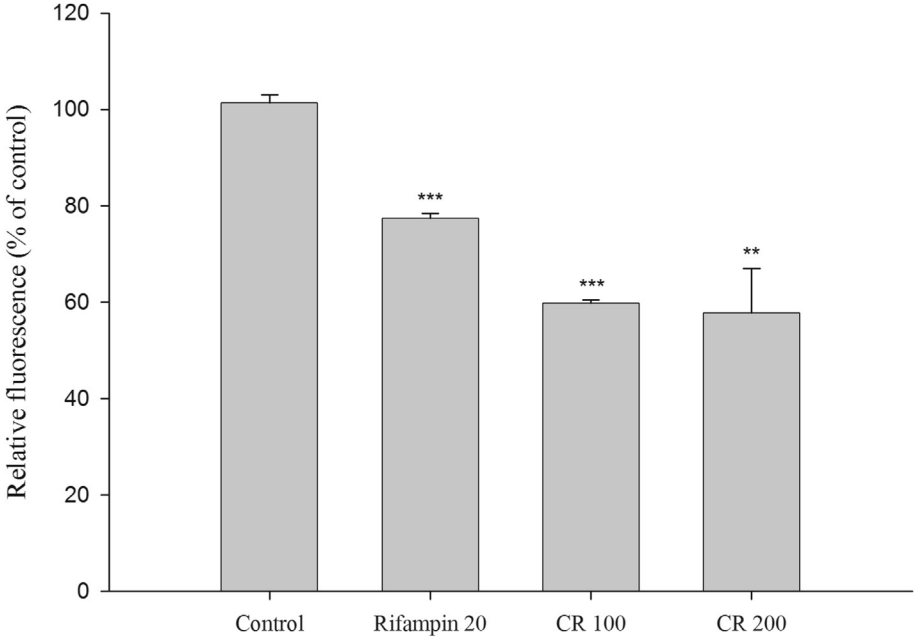

Fig. 4 – Chronic effects of CR (μg/mL) and rifampin (μM) on the intracellular accumulation of rhodamine 123 after 72-h incubation in LS 180 cells. ** $p < 0.01$, ***$p < 0.001$.

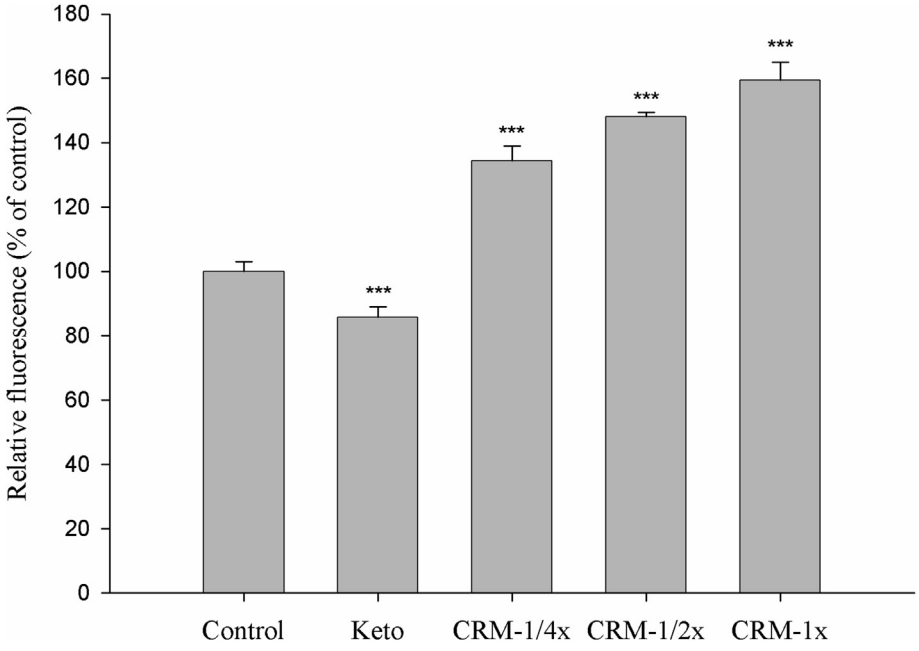

Fig. 5 – Effects of CRM (1/4-, 1/2- and 1-fold serum concentrations) and ketoconazole (keto, 10 μM spiked in deprotenized blank serum) on CYP3A4 activity. ***$p < 0.001$.

available, the characterization of CRM was not performed, which was the limitation of this study. The activity assay of CYP 3A4 showing CRM at 1-, 1/2- and 1/4-fold serum concentrations significantly increased CYP3A4 activity indicated that the decreased bioavailability of CSP caused by CR could be in part explained by the increased metabolism of CSP catalyzed by CYP3A4. As a positive control of CYP3A4 inhibitor, ketoconazole was spiked into blank serum and compared with blank serum control. The results showed that 10 μM of ketoconazole exhibited weak inhibition effect, which might be due to the masking effect of blank serum matrix. Compared to a previous study reporting that CR and isoquinoline alkaloids inhibited CYP3A4 [10], our result conversely showed that the metabolites of CR activated CYP 3A4 rather than inhibition. Apparently, our result was more satisfactory to account for the decreased bioavailability of CSP in rats. This finding strongly suggested that the metabolic fates of isoquinoline alkaloids should be taken into consideration when using *in vitro* assay to evaluate their effects on CYPs. Taken together, oral intake of CR activated both P-gp and CYP3A4, which interplayed to additively decrease the oral bioavailability of CSP.

In regard to the relative effects caused by two dosage regimens of CR, the effect sizes were comparable, implying that the mechanisms underlying two treatments were essentially the same, which made us to speculate that the protein levels of P-gp and CYP 3A4 had not been changed after repeated dosing. In order to verify the null effects on the protein levels of P-gp and CYP 3A4, western blotting analysis was performed after 72-h incubation of CR with LS 180 cells. The results showed that the protein levels of P-gp and CYP 3A4 were indeed not changed by CR (data not shown). Moreover, the efflux transport of P-gp after 72-h incubation of CR showed comparable activation with that after 4-h incubation of CR. Taken together, we can infer that the chronic effect of CR merely activated the functions of P-gp and CYP 3A4, rather than changed their protein levels.

It is noteworthy that P-gp and CYP3A4 have a broad overlap in their substrate spectrum, such as everolimus, sirolimus, verapamil, nicardipine, lovastatin, indinavir and amitriptyline [28–31]. We speculate that beyond CSP, CR may also markedly decrease the oral bioavailabilities of above-mentioned medicines, which might result in treatment failure. Therefore, due to the broad specificity of substrates of P-gp and CYP 3A4, it is suggested that CR is better avoided to be taken concurrently with western medicines. In conclusion, through using CSP as an *in vivo* probe substrate, we have verified that oral intake of CR activated the functions of P-gp and CYP3A based on *in vivo* and *in vitro* studies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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