

Original Article

Effects of multiple oral dosing of cyclosporine on the pharmacokinetics of quercetin in rats

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Abstract: The aim of this study was to investigate effect of cyclosporine on the pharmacokinetics of quercetin and to explore the underlying mechanisms in rats. The interaction study in male Sprague-Dawley (SD) rats evaluated the effect of cyclosporine (10 mg/kg) following orally administration for 7 consecutive days on the pharmacokinetics of quercetin (50 mg/kg), as well as effect of co-administration of cyclosporine and quercetin on the drug-metabolizing enzymes (DMEs) and drug transporters (DTs) in rat small intestine and liver. The C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ of quercetin in cyclosporine-treated rats increased by 1.6-, 2.3- and 3.8-fold when compared to control values, respectively. Meanwhile, cyclosporine-treated rats displayed significantly higher MRT_{0-t} and VRT_{0-t} value of quercetin than the control, showing 36% and 91.9% rise, respectively. Cyclosporine decreased CL/F of quercetin by 77.8% reduction and consequently increased $t_{1/2}$ value by 2.8-fold, respectively. Importantly, co-administration of cyclosporine and quercetin could significantly decrease the mRNA level of *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Slco1b2*, *Mdr1*, *Bcrp*, and *Mrp2* in the small intestine and liver. In contrast to the mRNA results, the co-administration showed weaker inhibitory effect on protein expression levels. Cyclosporine markedly increased the exposure (AUC) of quercetin in rats, which might be involved in the combined inhibitory effects by the co-administration of cyclosporine and quercetin on small intestinal and hepatic DMEs and DTs. Further research is needed to determine the scope, magnitude and clinical importance of cyclosporine effects on the metabolism and transport of quercetin.

Keywords: Cyclosporine, quercetin, pharmacokinetics, drug-metabolizing enzymes, drug transporters, interplay

Introduction

Flavonoids are the most commonly distributed group of plant polyphenolic compounds. Due to their multiple health benefits flavonoids have been the subject of increasing research interest. Quercetin (3,3',4',5,7-pentahydroxyflavone), a prominent dietary antioxidant ubiquitously present in herbs, food and beverages, is one of the most potent bioflavonoids and possesses diverse pharmacological activities, including anti-inflammatory, anti-neoplastic, cardioprotective, and anti-cancer activities [1-3]. Based on epidemiological studies in the US, Europe, and Asia [4-7], the daily dietary intake of quercetin ranges from 4 to 68 mg, but can be as high as several 100 mg in the dietary supplement and several grams in anticancer therapy [8]. Due to its beneficial health effects, at present, quercetin supplements are widely available through commercial sources in doses ranging from 250 to 1500 mg of quercetin [9].

Upon ingestion, quercetin glycosides are rapidly hydrolyzed during passage across the small intestine or by bacterial activity in the colon to generate quercetin aglycone, which is further metabolized by phase II enzymes, such as UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), into the glucuronidated and/or sulfated derivatives, respectively [10, 11]. These glucuronides and sulfates are subsequently excreted via ABC transporters, e.g, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins 2 (MRP2). Therefore, the interplay between phase II enzymes and efflux transporters might affect the disposition of quercetin and lead to its low bioavailability [10, 12]. Additionally, quercetin, like other flavonoids or drugs, can be metabolized by cytochrome P450 (CYP) enzymes [10, 13]. Furthermore, quercetin can also serve as a chemical regulator that affects the expression or activity levels of drug-metabolizing enzymes (DMEs)

including UGTs, SULTs and glutathione transferases (GSTs), and drug transporters (DTs) including P-gp, BCRP, MRP2, organic anion transporting polypeptide (OATP), and organic anion transporters (OAT) [10, 13]. Since quercetin is the substrate of some DMEs and DTs and can modulate the metabolism and transport pathways shared by many important clinical drugs, drug-quercetin interaction is becoming an increasingly important concern. It was hypothesized that the concomitant administration of DMEs and DTs modulator might alter the pharmacokinetics and pharmacodynamics of quercetin.

As an immunosuppressant, cyclosporine has been widely used in transplant recipients to prevent rejection and in the treatment of autoimmune diseases with successful results [14] and its inhibitory effects on DMEs (e.g. CYP3A4) and DTs (e.g. P-gp, OATP1B, OATP2B, MRP2, and BCRP) activity have been well documented [15]. Interestingly, quercetin was known to have direct or indirect effects on the immune system [16, 17]. Therefore, the co-administration of cyclosporine and quercetin may enhance the immunosuppressive effects on T lymphocyte proliferation, which might be beneficial to the solid organ transplant recipients by their combined immunosuppressive effects.

Since cyclosporine and quercetin may frequently be administered simultaneously, their pharmacokinetic interactions are becoming an increasingly important concern. Although the precise pathways of metabolism and excretion of cyclosporine and quercetin had been identified, only limited data from animal and human studies reported that quercetin had relevant interactions with cyclosporine causing a reduction or increase in cyclosporine blood concentration [18-23]. However, to date, there has been no report on how cyclosporine modulates the pharmacokinetics of quercetin, which in turn would alter the pharmacokinetics of the former.

The major purpose of this study was to investigate the effect of cyclosporine on pharmacokinetics of quercetin after oral administration in rats. Furthermore, the present study measured the mRNA and protein expression levels of DMEs and DTs in the rat small intestine and liver tissue following co-administration of cyclosporine and quercetin over 7 consecutive days.

Materials and methods

Materials

Quercetin, cyclosporine, internal standard (IS) ferulic acid, β -glucuronidase (type H-3, from *Helix pomatia*) and sulfatase (type H-1, from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cyclosporine formulation was Sandimmune® injection (50 mg/mL) (Novartis PharmaAG, Basel, Switzerland) containing Cremophor® EL. Cremophor® EL (polyethoxylated castor oil) was obtained from BASF (Ludwigshafen, Germany). Trizol reagent was purchased from Invitrogen Life Technologies (San Diego, CA, USA). A first-strand cDNA synthesis kit was from Pharmacia LKB Biotechnology (Tokyo, Japan). SYBR Green mix enzyme was from Toyobo Corporation (Shiga, Japan). All other reagents and kits were provided by commercial companies. Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all preparations.

Animals

Male Sprague-Dawley (SD) rats (weighing 180-220 g) 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 (low quercetin) and tap water. The animals were housed, two per cage, and maintained at $22\pm 2^\circ\text{C}$ and 50-60% relative humidity, under a 12 h light-dark cycle. The experiments were initiated after acclimation under these conditions for at least 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 experiments were performed with approval from the Animal Research Ethics Committee of Union Hospital of Huazhong University of Science and Technology (Permit Number: 2015-015, Wuhan, China).

Animal experiments and drug administration

Eight rats were fasted for 12 h before dosing, and food was withheld for another 3 h and water was supplied *ad libitum*. Quercetin (50 mg/kg) was dissolved in vehicle (Cremophor® EL/de-ionized water). The dosages were

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designed on the basis of clinical doses in human. Cremophor® EL was added to the vehicle to ensure quercetin dissolution and accurate dosing. The cyclosporine solution was prepared by diluting Sandimmune® injection with de-ionized water to afford a concentration of 10 mg/kg. On day 1, the rats received a single oral dose of quercetin alone. From day 3 to day 8, all rats were gavaged (16-gauge gavage needle, Kent Scientific) with 10 mg/kg/day of cyclosporine for 6 consecutive days. After the last administration, the rats were fasted overnight with free access to water. The next morning, cyclosporine was orally administered to the rats and 0.5 h later quercetin orally. On the experimental day (day 1 and day 9), under anesthetization with isoflurane, blood samples (0.3 mL) were collected before quercetin dosing and at 0.167, 0.333, 0.667, 1, 2, 3, 4, 6, 8, 12 and 24 h after dosing by retro-orbital bleeding via heparinized capillary tubes. Blood samples were centrifuged for 10 min at 10,000 g at 4°C to obtain plasma samples, which were frozen at -80°C until analysis.

In addition, twelve rats were randomly divided into two groups (six rats in each group): the control and co-administration of cyclosporine and quercetin group. Rats in the co-administration group were gavaged once daily with cyclosporine (10 mg/kg) and quercetin (50 mg/kg) for 7 consecutive days. Rats in the control group were similarly gavaged with the equivalent volume (5 mL/kg) of vehicle (Cremophor® EL/de-ionized water). Animals were allowed free access to food and water but were fasted overnight before scarification to reduce the small intestinal content. On day 7, after the last ingestion at 0.5 h, rats were sacrificed by cervical dislocation. Organ tissues, including the small intestine and liver, were isolated, rinsed with saline, blotted dried, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Assay of quercetin in plasma by HPLC

Quantitative analysis of quercetin was performed by HPLC method following liquid-liquid extraction with minor modifications as previously described [24, 25]. In brief, for the determination of total quercetin in plasma, an aliquot of 100 µL plasma sample was treated with a mixture of β-glucuronidase and sulfatase 20 µL (final activities: 800 and 200 U/mL glucuronidase and sulfatase, respectively) for cleavage of the conjugates' ester-bonds. After

incubation at 37°C for 60 min, 20 µL of HCl solution (2.5 M) and 1 mL of ethyl acetate were added to plasma sample, followed by the addition of 10 µL of IS (ferulic acid, 200 µg/mL solution in acetonitrile). The supernatant was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 150 µL of mobile phase, and 20 µL was injected into HPLC. The samples were eluted using a reversed-phase column (Inertsil ODS-SP, C-18, 150×4.6 mm, 5 µm) complemented by pre-column (Inertsil ODS-2, C-18, 10×4.6 mm, 5 µm) on a Shimadzu LC-20AT Prominence HPLC system (Shimadzu Corporation, Tokyo, Japan) at 30°C and a flow rate of 1.0 mL/min under isocratic conditions [phosphate buffer (25 mM sodium dihydrogen phosphate, pH 2.4) and acetonitrile, 70:30 v/v]. Quercetin and IS were detected via UV absorbance at 366 nm.

Method validation

Validation was performed with regard to lower limit of quantification (LLOQ), linearity, intra- and inter-day precisions and accuracies, and stability. Calibration curve samples with final concentrations of 0.100, 0.315, 0.629, 1.259, 2.518, 5.035, and 10.070 µg/mL quercetin were prepared. Calibration curves were constructed by plotting the peak area ratio of quercetin to IS against the quercetin concentrations used with least-squares linear regression analysis. Accuracy and precision were evaluated by assessing QC samples at the following concentrations (n=6): low (0.157 µg/mL), medium (1.259 µg/mL), and high (8.050 µg/mL) for quercetin, respectively. The LLOQ was determined as the lowest concentration on the calibration curve which should be reproducible with a precision of 20% and accuracy of 80 to 120%. Extraction efficacy of quercetin was measured by comparing the peak areas of quercetin from plasma samples to those from samples prepared without plasma.

The stability of stock solution of quercetin and IS standards storing at room temperature for 24 hours and 4°C for 1 week were assessed. In addition, the stability of quercetin in plasma after three freeze-thaw cycles was investigated as well as the stability at room temperature (25°C) for 6 hours. The stability of quercetin in plasma at -80°C was investigated by analysis of the study samples before and after storing at -80°C for 7, 14, and 28 days. Furthermore, we investigated the stability of extracted samples

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in the auto-sampler tray at ambient temperature (25°C) for 3 days. All analyses were done in sextuplicate.

Measurement of small intestinal and hepatic mRNA expression

In the small intestine and liver tissue the amounts of mRNA encoding CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP, and MRP2 were quantified by real-time PCR method. The tissues (100 mg) were homogenized in 1 mL of Trizol reagents (Invitrogen, Carlsbad, CA, USA). RNA was extracted using chloroform and precipitated using isopropanol. The quantity of total RNA extracted was assessed using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE). RNA quality was verified by ensuring that all RNA samples had an absorbance ratio (OD_{260}/OD_{280}) between 1.8 and 2.0. Subsequently, RNA was converted to cDNA using high-capacity cDNA reverse transcription kit (TOYOBO First Strand cDNA Synthesis Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR assay was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan) and an Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies, Japan).

Specific primers for *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Slco1b2*, *Mdr1*, *Bcrp*, *Mrp2*, and the housekeeping gene β -actin were synthesized from Invitrogen Biotechnology Co, Ltd (Shanghai, China), which were shown as follows: *Cyp3a1* (170-bp PCR product): 5'-ACTGCATTGGCATGAGGTTTG-3' (sense) and 5'-ATCCCGTGGCACAACCTTT-3' (antisense), *Cyp3a2* (158-bp): 5'-ATTCTAAGCATAAGCACCGAGTG-3' (sense) and 5'-TGTGCTGCTGGTGGTTTCAT-3' (antisense), *Ugt1a1* (231-bp): 5'-ACTATTCTTGTCATATGGCTACCC-3' (sense) and 5'-GTTTTCCAAATCATCGGCAGT-3' (antisense), *Slco2b1* (162-bp): 5'-TCGCTGTTGTGCTGCTACTCAG-3' (sense) and 5'-AACAGGGTTAAAGTCATCTGATTGG-3' (antisense), *Slco1b2* (162-bp): 5'-TTCGTGGTGATAAGAAGCCG-3' (sense) and 5'-CAATTCAGGTTGACGCTCTT-3' (antisense), *Mdr1* (179-bp): 5'-TCCTATGCTGCTTGTTCCTG-3' (sense) and 5'-ATCCTGATGATGTGGGATGCT-3' (antisense), *Bcrp* (236-bp): 5'-ATTGGTGCCCTTACTTTGGTC-3' (sense) and 5'-ACACTTGGCAAGAACCTCATAAGG-3' (antisense), *Mrp2* (246-bp): 5'-TGTGGCAGTTGAGCGAATAAGT-3' (sense) and 5'-AAGAGGCAGTTTGTGAGGGATG-3' (antisense), and β -actin (110-bp): 5'-CGTTGACATCCGTAAAGACC-

TC-3' (sense) and 5'-TAGGAGCCAGGGCAGTAATCT-3' (antisense). The following cycling parameters were used: one cycle of 1 min at 95°C, followed by 40 cycles of de-naturation for 15 sec at 95°C, annealing for 20 sec at 58°C, and extension for 20 sec at 72°C. For the final cycle only, the duration of the elongation step was 5 min. The relative mRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Measurement of small intestinal and hepatic protein expression

In the small intestine and liver tissue the protein levels of CYP3A1, CYP3A2, UGT1A1, OATP2B1, OATP1B2, P-gp, BCRP, and MRP2 were analyzed using Western blotting method. Protein concentrations were determined using the BioRad Protein Assay (Bio-Rad, Hercules, CA, USA). Protein samples (40 μ g each) were separated by electrophoresis on 8% to 20% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Eschborn, Germany). Membrane was blocked for 1 h with Tris-buffered saline (TBS)=0.1% Tween-20 (TBST) containing 5% skim milk and incubated with primary antibody overnight at 4°C, washed three times with TBST, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 30 min at room temperature.

Then, the following primary antibodies (Santa Cruz Biotechnology, CA, USA) and working dilutions were used: mouse anti-CYP3A1 monoclonal antibody (1:200), mouse anti-P-gp monoclonal antibody (1:500), rabbit anti-BCRP polyclonal antibody (1:500), rabbit anti-MRP2 polyclonal antibody (1:500), mouse anti- β -actin monoclonal antibody (1:5,000). Additional antibodies purchased from Abcam (Cambridge, MA, USA) were: rabbit anti-UGT1A1 polyclonal antibody (1:1,000), rabbit anti-OATP2B1 polyclonal antibody (1:1,000). Rabbit anti-CYP3A2 monoclonal antibody (1:500, Millipore, Germany) was also used. Secondary antibodies (Santa Cruz Biotechnology, CA, USA) were goat anti-mouse IgG-HRP, goat anti-rat IgG-HRP, goat anti-rabbit IgG-HRP, and rabbit anti-goat IgG-HRP. Results were normalized relative to β -actin expression. The relative levels of each protein were visualized using electrochemiluminescence (ECL) Plus Western Blotting detection system (GE Healthcare/Amersham) followed by exposure to Kodak films (Kodak) and

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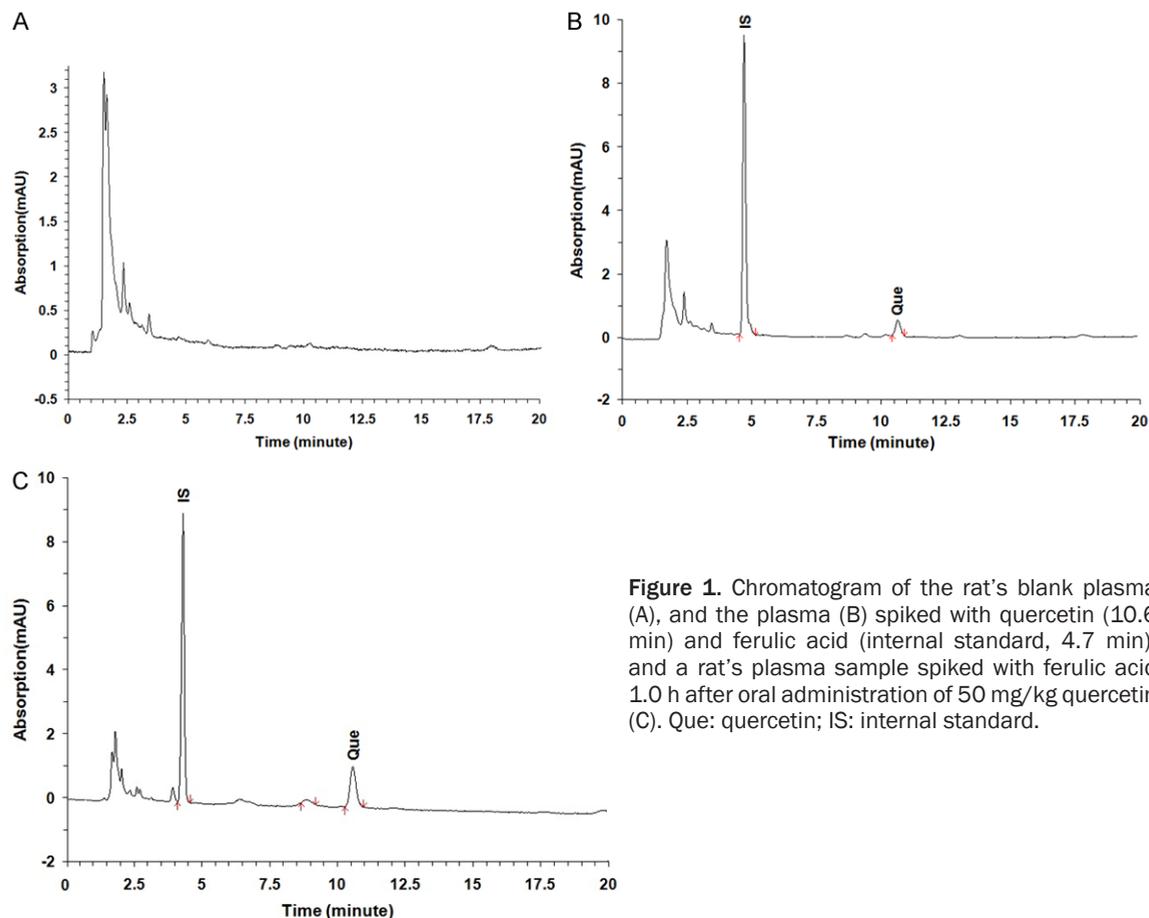


Figure 1. Chromatogram of the rat's blank plasma (A), and the plasma (B) spiked with quercetin (10.6 min) and ferulic acid (internal standard, 4.7 min), and a rat's plasma sample spiked with ferulic acid 1.0 h after oral administration of 50 mg/kg quercetin (C). Que: quercetin; IS: internal standard.

densitometry analyses (Kodak 1D3 image analysis software, Kodak).

Pharmacokinetic analysis

The plasma concentration data were analyzed by the non-compartmental method using Drug and Statistics software (DAS, version 3.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China). The peak plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) of plasma quercetin were derived directly from the concentration-time curve. The elimination rate constant (K_{el}) was calculated by log-linear regression of quercetin data during the elimination phase and the terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The area under the plasma concentration-time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el} . The mean residence time (MRT) was calculated as $MRT=AUMC/AUC$,

where AUMC represented the area under the first moment versus time curve.

Statistical analysis

Results are expressed as mean values \pm SD in the text, tables and figures. Statistical differences were performed with GraphPad Prism 6.0 (GraphPad Software Inc, San Diego, CA, USA). Statistical comparisons were performed using unpaired or paired Student's *t* test. The differences were considered statistically significant at a *P*-value of less than 0.05.

Results

HPLC method validation

Chromatograms of rat's blank plasma and the plasma spiked with quercetin and ferulic acid are shown in **Figure 1**. The peaks of quercetin and IS separated clearly. The retention times of IS and quercetin were 4.7 and 10.6 min, respectively. The calibration curves of quercetin were linear over the concentration range of 0.100-

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Table 1. Precision and accuracy for the determination of quercetin in rat plasma

Added concentration µg/mL	Day	Intra-day (n=6)			Inter-day (n=18)		
		Mean ± SD µg/mL	Accuracy %	CV %	Mean ± SD µg/mL	Accuracy %	CV %
0.157	1	0.144±0.007	93.89	4.58	0.144±0.005	94.05	3.64
	2	0.147±0.004	95.85	2.79			
	3	0.141±0.004	90.99	3.06			
1.259	1	1.138±0.021	90.41	1.80	1.145±0.030	90.98	2.60
	2	1.168±0.022	92.78	1.91			
	3	1.130±0.034	92.41	3.04			
8.056	1	7.694±0.081	95.51	1.05	8.021±0.271	99.57	3.38
	2	8.226±0.084	102.12	1.02			
	3	8.144±0.198	90.74	2.43			

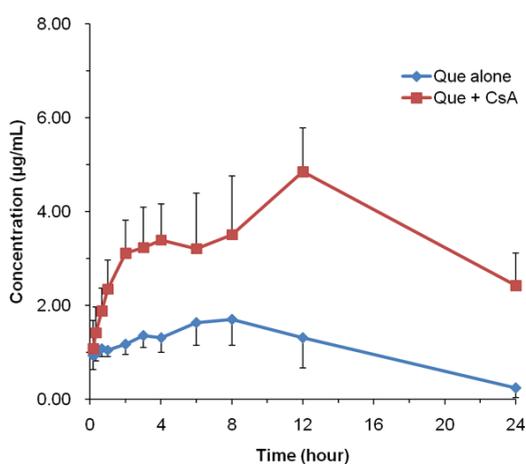


Figure 2. Mean plasma concentration-time profiles of quercetin. On day 1, the rats received a single oral dose of quercetin (50 mg/kg) alone. From day 3 to day 8, all rats were gavaged with 10 mg/kg/day of cyclosporine for 6 consecutive days. On day 9, animals were pretreated with cyclosporine (10 mg/kg) at 0.5 h prior to the last dose of quercetin (50 mg/kg) given to all rats for pharmacokinetic study. Values are expressed as mean ± SD for each data point (n=8). CsA: cyclosporine; Que: quercetin.

10.070 µg/mL (*r* values were typically greater than 0.9998) and the LLOQ was 0.100 µg/mL (CV<20%). For precision, % CV values for quercetin were less than 5% for both intra- and inter-day analysis. The intra- and inter-day percentage accuracies for quercetin were 90.41-105.12% (Table 1). The mean extraction recoveries of quercetin at low, medium, and high QC concentrations were (96.60±4.63)%, (92.42±2.38)%, and (99.46±1.92)%, respectively.

The stock solution of quercetin and IS standards was stable at room temperature for at least 24 hours and 4°C for 1 week. After stor-

age for 28 days at -80°C and at room temperature (25°C) for 5 hours, the stability of quercetin and IS in plasma was found within the acceptance range of 90%-110%, based on peak areas in comparison with freshly prepared solution. Repeated freezing and thawing (three cycles) of plasma samples spiked with quercetin did not affect the stability of quercetin with a bias range being between -5.10% and 6.39%. Finally, the stability in the extracted samples was stable in auto-sampler tray at ambient temperature (25°C) for 12 hours. Therefore, the results showed good selectivity and sensitivity, a wide linear range, precision and accuracy, which is suitable for routine analysis of quercetin formulations.

Effect of cyclosporine on the pharmacokinetics of quercetin

Mean plasma concentration-time profiles of quercetin following an oral administration of quercetin (50 mg/kg) to rats in the presence or absence of cyclosporine (10 mg/kg) for 7 consecutive days are shown in Figure 2; the corresponding pharmacokinetic parameters are shown in Table 2. Cyclosporine significantly altered the pharmacokinetic parameters of quercetin. The C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ in quercetin without cyclosporine-treated rats (n=8) on day 1 was 1.97±0.41 µg/mL, 25.93±6.47 µg·h/mL and 29.07±8.24 µg·h/mL, respectively, while those in the quercetin with cyclosporine-treated rats (day 9) increased by 156.2% (*P*<0.001), 232.9% (*P*<0.001) and 378.2% (*P*<0.01) rise, respectively. Meanwhile, cyclosporine-treated rats displayed significantly higher MRT_{0-t} and VRT_{0-t} value than the control, showing 36.0% and 91.9% enhancement (*P*<0.001), respectively. Cyclosporine treatment also increased the $t_{1/2}$ value by 2.8-fold

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Table 2. The main pharmacokinetic parameters of quercetin after oral administration of quercetin (50 mg/kg) to rats in the absence or presence of cyclosporine (10 mg/kg) for 7 consecutive days (Mean \pm SD, n=8)

Parameters	Que alone	Que+CsA
AUC _{0-t} (μ gh/mL)	25.93 \pm 6.47	86.35 \pm 13.69***
AUC _{0-∞} (μ gh/mL)	29.07 \pm 8.24	139.03 \pm 56.95**
MRT _{0-t} (h)	8.64 \pm 1.64	11.75 \pm 0.69***
VRT _{0-t} (h ²)	24.07 \pm 9.54	46.18 \pm 7.83***
t _{1/2} (h)	6.10 \pm 2.84	17.05 \pm 7.30*
t _{max} (h)	8.63 \pm 3.89	10.75 \pm 2.38
V _d /F (L/kg)	14.96 \pm 4.44	10.29 \pm 4.14**
CL/F (L/h/kg)	1.85 \pm 0.53	0.41 \pm 0.15**
C _{max} (μ g/mL)	1.97 \pm 0.41	5.04 \pm 0.85***

AUC_{0-t}: area under the plasma concentration-time curve from time zero to the time of last measured concentration; AUC_{0- ∞} : area under the plasma concentration-time curve from 0 h to infinity; MRT: mean residence time; VRT: variance of residence times; t_{1/2}: terminal half-life; V_d/F: apparent volume of distribution; CL/F: apparent total clearance; C_{max}: peak plasma concentration; t_{max}: time to reach C_{max}; CsA: cyclosporine; Que: quercetin. For comparison, paired Student's *t* test was used to compare two groups. Significance is indicated as **P*<0.05, ***P*<0.01, ****P*<0.001, compared to the quercetin alone control.

(*P*<0.05). In contrast, in the quercetin with cyclosporine-treated rats (day 9) the V_d/F and CL/F were decreased by 31.2% and 77.8% reduction (*P*<0.01), respectively. When compared to quercetin alone control, in contrast, there was no significant difference (*P*>0.05) in the t_{max} in the presence of cyclosporine.

mRNA expression levels of DMEs and DTs in the small intestine and liver

The small intestinal and hepatic mRNA levels of *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Mdr1*, *Bcrp*, and *Mrp2* were measured by RT-PCR analysis using small intestinal and hepatic RNA prepared from the rats (**Figure 3**).

As shown in **Figure 3A**, it was demonstrated that co-administration of cyclosporine and quercetin (7-day) significantly decreased the small intestinal mRNA level of *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Mdr1*, *Bcrp*, and *Mrp2*, with 67.9% (*P*<0.001), 52.8% (*P*=0.001), 50.4% (*P*<0.001), 88.2% (*P*<0.001), 60.9% (*P*=0.002), 43.2% (*P*=0.004), and 47.9% (*P*<0.001) reduction, respectively when compared to the control group. Likewise, **Figure 3B** showed that the co-administration could significantly decrease the

mRNA level of the respective DMEs and DTs, with 74.3%, 35.9%, 68.3%, 86.7%, 61.7%, 74.2%, and 38.3% reduction (*P*<0.001) in the liver, respectively. Therefore, these results revealed that the mRNA expression levels of DMEs and DTs were significantly inhibited by the co-administration of cyclosporine and quercetin to a similar extent in the small intestine and liver.

Protein expression levels of DMEs and DTs in the small intestine and liver

The small intestinal and hepatic protein levels of CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP, and MRP2 were measured by Western blotting analysis in rats (**Figure 4**).

As shown in **Figure 4A**, when compared to the control, in the small intestine co-administration of cyclosporine and quercetin (7-day) significantly decreased the protein level of CYP3A1, CYP3A2, UGT1A, OATP2B1, P-gp, BCRP, and MRP2, with 30.6% (*P*<0.044), 24.6% (*P*=0.131), 22.8% (*P*=0.031), 37.8% (*P*=0.037), 8.7% (*P*=0.658), 29.0% (*P*=0.056), and 30.5% (*P*=0.007) reduction, respectively. However, **Figure 4B** indicated that the co-administration did not show significant inhibitory effect on the hepatic protein expression levels of DMEs and DTs investigated. Therefore, these results revealed that the expression levels of DMEs and DTs were not inhibited by the co-administration to a similar extent in the small intestine and liver. It should be noteworthy, when compared to the potent inhibitory effect on mRNA results, the co-administration had a relatively weaker inhibitory effect on the protein expression levels.

Discussion

To the best of our knowledge, this is the first report to systematically demonstrate the impact of cyclosporine on pharmacokinetics of quercetin, as well as co-administration of cyclosporine and quercetin on the DMEs and DTs in the small intestine and liver. The results indicated that co-administration of cyclosporine (10 mg/kg) for 7 consecutive days significantly increased the exposure (AUC) of quercetin (50 mg/kg) and reduced its oral clearance.

Because quercetin is rapidly metabolized into glucuronidated and/or sulfated metabolites in

Effects of cyclosporine on pharmacokinetics of quercetin

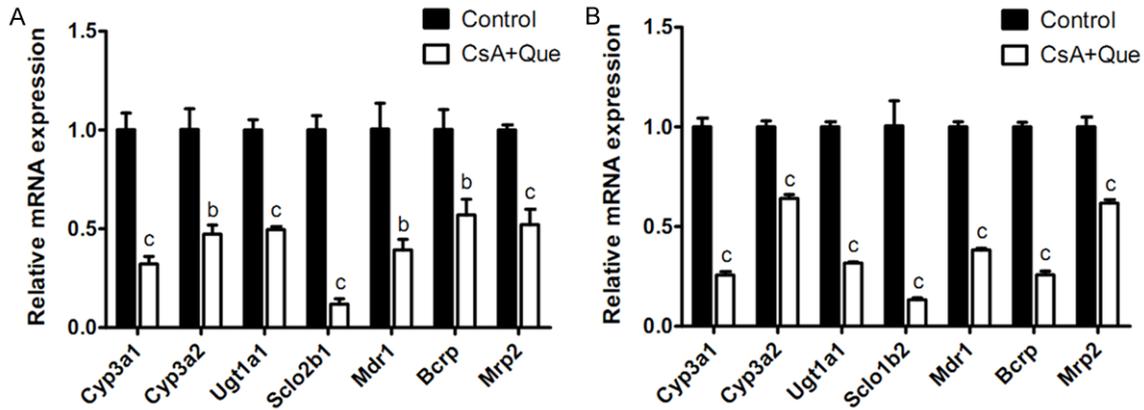


Figure 3. Effect of co-administration of cyclosporine and quercetin on the mRNA expression levels encoding CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP, and MRP2 in the small intestine (A) and liver (B). In rats of the control, co-administration of cyclosporine and quercetin group, the mRNA contents were measured by real-time PCR and calculated as comparative levels over control using $2^{-\Delta\Delta Ct}$ method. β -actin was used as a loading control. Vertical bars represent mean \pm SD (n=6). For comparison, unpaired Student's *t* test was used to compare two independent groups. Statistical significance is indicated as ^a*P*<0.05, ^b*P*<0.01, ^c*P*<0.001, compared to control. CsA: cyclosporine; Que: quercetin.

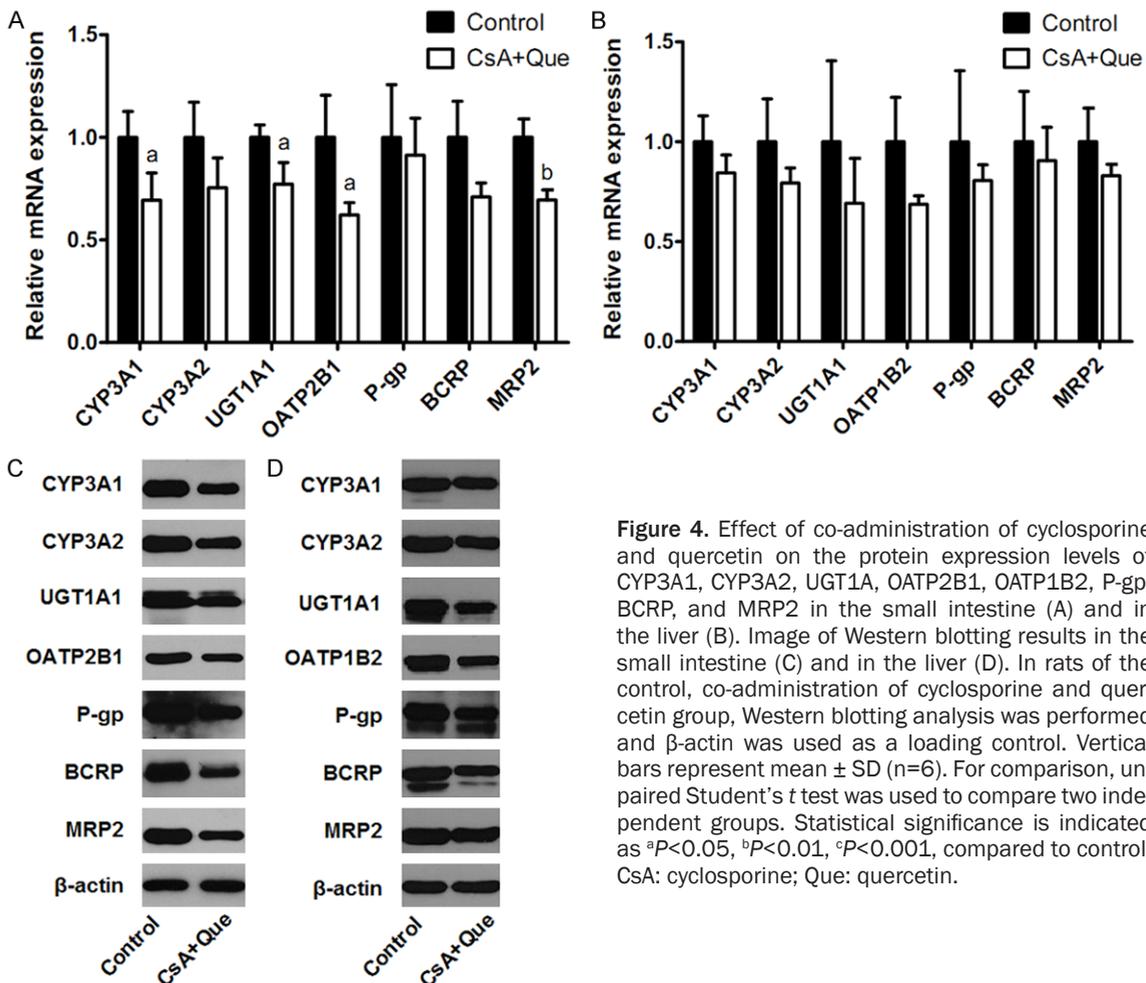


Figure 4. Effect of co-administration of cyclosporine and quercetin on the protein expression levels of CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP, and MRP2 in the small intestine (A) and in the liver (B). Image of Western blotting results in the small intestine (C) and in the liver (D). In rats of the control, co-administration of cyclosporine and quercetin group, Western blotting analysis was performed and β -actin was used as a loading control. Vertical bars represent mean \pm SD (n=6). For comparison, unpaired Student's *t* test was used to compare two independent groups. Statistical significance is indicated as ^a*P*<0.05, ^b*P*<0.01, ^c*P*<0.001, compared to control. CsA: cyclosporine; Que: quercetin.

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humans and animals and plasma concentrations of free quercetin are very low, a huge amount of scientific data generated along decades with the unconjugated compounds *in vitro* has been questioned [26]. Therefore, the main concern of the present study was to investigate the pharmacokinetics of combined quercetin instead of each separately, following co-administration of cyclosporine. The combined conjugates might represent the dominant metabolites of quercetin metabolism and have been shown to exert a wide range of biological effects, such as anti-oxidative activity, blood pressure lowering effect, and anti-inflammatory properties of the parent aglycone [11, 27-29]. Thus, the combined conjugate concentration can represent a meaningful entity for pharmacokinetic and pharmacodynamic studies. Therefore, in the present study, an enzymatic hydrolytic method was used to convert quercetin sulfate and the glucuronide conjugates to quercetin aglycone using β -glucuronidase/sulfatase.

When compared with control values, the C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ of quercetin with cyclosporine-treated rats increased by 1.6-, 2.3- and 3.8-fold, respectively. Meanwhile, cyclosporine-treated rats showed significantly higher MRT_{0-t} and VRT_{0-t} value than the control, showing 36.0% and 91.9% enhancement, respectively. Cyclosporine-treatment decreased the CL/F by 77.8% reduction and consequently increased the $t_{1/2}$ value by 2.8-fold, respectively. Therefore, co-administration of cyclosporine had a meaningful effect on the pharmacokinetics of quercetin. These results were consistent with the previous reports that the bioavailability of drugs or products, such as, ginkgolic acids [30], rosvastatin [31], atorvastatin [32], repaglinide [33], was increased when they were co-administered with cyclosporine. Additionally, a few studies had investigated the quercetin-cyclosporine pharmacokinetic interactions, showing that quercetin could alter the bioavailability of cyclosporine [18-23]. However, the available results were conflicting and the underlying mechanisms were poorly understood.

In the present paper, the results demonstrated that co-administration of cyclosporine and quercetin could significantly reduce the mRNA expression levels of *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Slco1b2*, *Mdr1*, *Bcrp*, and *Mrp2*. However, when compared to the potent inhibitory effect on mRNA results, the co-administra-

tion had a relatively weaker inhibitory effect on the protein expression levels. For example, in the small intestine the co-administration showed a weak but significant inhibitory effect on the protein expression levels of CYP3A1, UGT1A1, OATP2B1, and MRP2. In contrast, in the liver the co-administration did not exhibit significant inhibitory effects on the protein levels of CYP3A1, CYP3A2, UGT1A, OATP1B2, P-gp, BCRP, and MRP2. Importantly, these results revealed that the co-administration had the most potent inhibitory effect on the small intestinal uptake transporters *Slco2b1* and hepatic *Slco1b2* mRNA expression, which were consistent with the previous report [15]. Cyclosporine had the highest interaction potential against liver uptake transporters, with a maximal reduction of >70% in OATP1B1 activity; the effect on hepatic efflux and metabolism was minimal [15]. Therefore, the results that co-administering cyclosporine (10 mg/kg) could significantly increase the exposure (AUC) of quercetin (50 mg/kg) might due to the inhibition of different DMEs and DTs by the co-administration.

It had been shown that cyclosporine had inhibitory effects on DMEs and DTs activity, such as CYP3A, uptake and efflux transporters (OATP1B1, OATP1B3, NTCP, P-gp, MRP2, BSEP and BCRP) in the liver and small intestine [15, 29, 34]. Additionally, quercetin can also modulate the activity or expression levels of UGTs, SULTs, GSTs, P-gp, MRP2, BCRP, OATP and OAT [10, 13, 35, 36]. Thus, the combined regulation of small intestinal and hepatic phase I and II DMEs, uptake and efflux DTs is mediated by cyclosporine and quercetin. In recent years, the functional interplay between DMEs and DTs in drug absorption and disposition, as well as the complex drug interactions, has becoming an intriguing contention [37, 38]. In the present study, therefore, the interplay between DMEs and DTs would be considered to explain the pharmacokinetic interactions between quercetin and cyclosporine.

As a limitation of the present study, our study did not investigate effect of the co-administration on the activity of DMEs and DTs in the small intestine and liver, which should be very crucial for elucidating the underlying mechanism of this interaction. Additionally, the present study did not compare the effects of quercetin or cyclosporine alone with co-administration of cyclosporine and quercetin on DMEs

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and DTs in rat small intestine and liver. Therefore, further studies are needed to clarify the mechanism.

Collectively, the co-administration of cyclosporine might be an approach to improve the exposure (AUC) of quercetin. Further studies in humans are needed to elucidate the clinical implication of these findings in rats.

Conclusion

We reported for the first time that cyclosporine could significantly increase the exposure (AUC) of quercetin in rats. The combined regulation of small intestinal and hepatic DMEs and DTs by cyclosporine and quercetin, as well as their interplay might be responsible for this phenomenon. This interaction could be of clinical significance. However, further clinical studies are needed to confirm this interaction.

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Disclosure of conflict of interest

None.

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