Impact of quercetin-induced changes in drug-metabolizing enzyme and transporter expression on the pharmacokinetics of cyclosporine in rats

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Abstract. The aim of the present study was to evaluate whether quercetin (Que) modulates the mRNA and protein expression levels of drug-metabolizing enzymes (DMEs) and drug transporters (DTs) in the small intestine and liver, and thus modifies the pharmacokinetic profile of cyclosporine (CsA) in rats. This two-part study evaluated the pharmacokinetic profiles of CsA in the presence or absence of Que (experiment I) and the involvement of DMEs and DTs (experiment II). In experiment I, 24 rats received single-dose CsA (10 mg/kg) on day 1, single-dose Que (25, 50 and 100 mg/kg/day; eight rats in each group) on days 3-8, and concomitant CsA/Que on day 9. In experiment II, the mRNA and protein expression levels of cytochrome P (CYP)3A1, CYP3A2, UDP glucuronosyltransferase family 1 member A complex locus, organic anion-transporting polypeptide (OATP)2B1, OATP1B2, P-glycoprotein, breast cancer resistance protein, and multidrug resistance-associated protein 2 in the small intestine and liver of rats were analyzed following oral administration of Que at 25, 50 and 100 mg/kg in the presence or absence of CsA (10 mg/kg) for seven consecutive days. Co-administration of Que (25, 50 and 100 mg/kg) decreased the maximum serum concentration of CsA by 46, 50 and 47% in a dose-independent manner. In addition, the area under the curve to the last measurable concentration and area under the curve to infinite time were decreased, by 21 and 16%, 30 and 33%, and 33 and 34% ($P < 0.01$), respectively. However, the mRNA and protein expression levels of the above-mentioned DMEs

and DTs were inhibited by Que in a dose-dependent manner ($P < 0.01$) to a similar extent in the small intestine and liver. It was demonstrated that Que was able to reduce the bioavailability of CsA following multiple concomitant doses in rats. Overlapping modulation of intestinal and hepatic DMEs and DTs, as well as the DME-DT interplay are potential explanations for these observations.

Introduction

Flavonoids are important phytochemicals, prevalent in the human diet, which are claimed to exert a variety of biological effects (1). Herbal preparations containing high doses of flavonoids have become widespread as interest in healthy living and alternative medicine increases. Therefore, potential herb-drug interactions (HDIs) may be a major concern in the co-administration of flavonoids and other medicines (2,3).

Quercetin (3,3',4',5,7-pentahydroxyflavone; Que), the predominant flavonoid, is ubiquitously present in edible plants, herbs, beverages and dietary supplements, including onions, grapes, berries, apples, red wine, tea, St. John's wort and ginkgo (3). Due to its beneficial effects on health, it has also been marketed as a dietary supplement (3), with a recommended daily dose of 200-1200 mg (4). It exhibits antioxidant, anti-inflammatory, anticancer, neuroprotective, anti-anaphylaxis and anti-aging effects (5-7). In addition, Que is well-known to protect tissue against damage induced by chemicals. For example, Que protects against cyclosporine (CsA)-induced nephrotoxicity and hepatotoxicity (8,9), acetic acid- and trinitrobenzene sulphonic acid-induced inflammatory bowel disease-like symptoms (10,11), atrazine-induced cytotoxicity in cultured Sertoli-germ cells and Leydig cells (12,13), ethanol-induced dyslipidemia and mitochondrial oxidative damage (14), and high glucose-induced Schwann cell damage (15).

It has been reported that Que modulates the phase I and phase II drug-metabolizing enzymes (DMEs), including cytochrome P (CYP) 1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2D6, CYP3A4, UDP glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) (16-20), and drug transporters (DTs), including P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), breast cancer resistance

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protein (BCRP), organic anion transporter (OAT) and organic cation transporter (16,21-23). However, the effect of Que on DMEs and DTs *in vitro* and *in vivo* (16,24-26) remains to be elucidated.

As a calcineurin (CN) inhibitor, CsA is widely used to prevent rejection of transplanted organs (27). It is a substrate for CYP3A and P-gp (27), UGT1A and 2B (28), and MRP2 (29). However, it is also a potent inhibitor of CYP3A4, P-gp, OATP1B and 2B, MRP2, and BCRP (30,31). Consequently, foods or dietary supplements that influence DMEs, DTs and/or their interplay may alter the pharmacokinetics of CsA, resulting in increased toxicity and/or diminished efficacy. Previous studies have reported that Que interacts with CsA resulting in a reduction or increase in the serum concentration of CsA (31-37). These conflicting results may be due to differences in the subjects used, the method of administration and the dose. Furthermore, the underlying mechanisms by which the effects of Que are mediated are poorly understood.

Increased knowledge of the interactions between DMEs and DTs in drug absorption and disposition, as well as complex HDIs, facilitates prediction of the pharmacokinetic properties of drugs and potential HDIs (38,39). There may be serious risks, particularly for drugs such as CsA with a narrow therapeutic index, if other drugs or food constituents that interfere with DMEs and DTs are deliberately or unintentionally co-administered (37).

As a novel CN inhibitor, Que demonstrates noncompetitive inhibition of CN (40), suggesting that it may have immunosuppressant properties and may potentially enhance the effect of CsA. As Que is widely distributed in foods and is available as a dietary supplement, Que and CsA may frequently be administered simultaneously. It is therefore crucial to understand the effect of Que on DMEs and DTs, and their interactions. Thus far, to the best of our knowledge, there have been no studies investigating the simultaneous modulation of DME and DT expression levels by Que in the small intestine and liver.

The aim of the present study was to evaluate whether multiple-dose oral administration of Que influenced the pharmacokinetics of CsA in rats. Furthermore, to investigate the underlying mechanisms, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting were performed to measure the mRNA and protein expression levels of DMEs and DTs in the small intestine and liver of rats, following Que consumption in the presence or absence of CsA for seven consecutive days.

Materials and methods

Materials. Que, CsA and Cyclosporine D were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CsA formulation was Sandimmune® injection (50 mg/ml; Novartis International AG, Basel, Switzerland) containing Cremophor® EL (polyethoxylated castor oil), and each ml of infusion concentrate was diluted in de-ionized water prior to use. TRIzol® reagent was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Cremophor® EL was obtained from BASF SE (Ludwigshafen, Germany). The first-strand complementary (c) DNA synthesis kit and THUNDERBIRD SYBR qPCR Mix were purchased from Toyobo Co., Ltd. (Osaka, Japan). Milli-Q plus water (EMD Millipore, Billerica, 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 free access to a commercial rat chow diet (low Que) and tap water. The animals were housed two per cage, and maintained at 22±2˚C and 50-60% relative humidity, under a 12-h light/dark cycle. The experiments were initiated following acclimation to these conditions for at least one week. All experiments were performed with the approval of the Animal Research Ethics Committee of Union Hospital of Huazhong University of Science and Technology (permit no. 2015-015; Wuhan, China).

Experiment I. A total of 24 rats were randomly divided into three groups, and received either solely CsA or an identical dose of CsA together with low-, moderate- or high-dose Que in a before-and-after design. The rats were fasted for 12-h prior to dosing, and food was also withheld for 3 h subsequent to dosing. Water was supplied *ad libitum*. The CsA solution was prepared by diluting Sandimmune® injection with de-ionized water to a concentration of 2 mg/ml. Que was dissolved in vehicle (Cremophor® EL/de-ionized water). Drugs were administered by oral gavage using a 16-gauge gavage needle (Kent Scientific Corporation, Torrington, CT, USA). On day 1, 24 rats assigned to the low-dose, moderate-dose and high-dose Que treatment groups received a single oral dose of 10 mg/kg CsA alone. From days 3 to 8, rats received 25, 50 or 100 mg/kg/day of Que for six consecutive days. Following the final dose, rats were fasted overnight with free access to water. The following morning, Que was again administered, followed 0.5 h later by CsA (10 mg/kg dose). The dosages were selected based on clinical doses administered to humans. Cremophor® EL was added to the vehicle to ensure Que dissolution and accurate dosing. On experimental days 1 and 9, blood samples (0.2 ml) were collected via the tail vein prior to, and at 0.5, 1, 3, 5, 8, 12, 24, 36 and 48 h following, CsA administration and deposited into heparinized tubes (BD Biosciences, Franklin Lakes, NJ, USA). Blood samples were stored at -80˚C until analysis.

Experiment II. Rats were randomly divided into eight groups with three rats in each group. Rats in the Que-treated groups were gavaged once daily with Que at 25, 50 or 100 mg/kg without (Que-WOC) or with (Que-WC) CsA (10 mg/kg) for 7 consecutive days. Rats in the CsA-treated group were gavaged once daily with CsA (10 mg/kg) for 7 consecutive days. Rats in the CsA-treated group were only gavaged with CsA (10 mg/kg); however, the Que-WC group were treated with Que and CsA by two separate gavages within 1 min. 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 throughout the experiment; however, they were fasted overnight prior to sacrifice to reduce the intestinal content. On day 7, 0.5 h following the final dose, rats were sacrificed by cervical dislocation. Tissues, including the small intestine

Table I. Summary of the gene-specific polymerase chain reaction primer sequences, product size and annealing temperature.

Description	Genebank	Sense primer, 5'-3'	Antisense primer, 5'-3'	Product size, bp	Tm, °C
β-actin	NM_031144	CGTTGACATCCGTAAAGACCTC	TAGGAGCCAGGGCAGTAATCT	110	58
Cyp3a1	NM_013105.2	ACTGCATTGGCATGAGGTTTG	ATCCCGTGGCACAACCTTT	170	58
Cyp3a2	NM_153312.2	ATTCTAAGCATAAGCACCGAGTG	TGTGCTGCTGGTGGTTTCAT	158	58
Ugt1a1	NM_012683.2	ACTATTCTTGTCAAATGGCTACCC	GTTTTCCAAATCATCGGCAGT	231	58
Slco2b1	NM_080786.1	TCGCTGTTGTGTCTGCTACTCAG	AACAGGGTTAAAGTCATCTGATTGG	162	58
Slco1b2	NM_031650.3	TTCGTGGTGATAAGAAGCCG	CAATTCAGGTTGGACGCTCTT	162	58
Mdr1	NM_012623.2	TCCTATGCTGCTTGTTTCCG	ATCCTGATGATGTGGGATGCT	179	58
Bcrp1	NM_181381.2	ATTGGTGCCCTTTACTTTGGTC	ACACTTGGCAAGAACCTCATAGG	236	58
Mrp2	NM_012833.2	TGTGGCAGTTGAGCGAATAAGT	AAGAGGCAGTTTGTGAGGGATG	246	58

Tm, annealing temperature; Cyp, cytochrome P; Ugt1a1, UDP glucuronosyltransferase family 1 member A complex locus; Slco, solute carrier organic anion transporter family member; Mdr1, multi-drug resistance 1; Bcrp, breast cancer resistance protein; Mrp2, multidrug resistance-associated protein 2.

and liver, were isolated, rinsed with saline, blotted dry, snap-frozen in liquid nitrogen and stored at -80°C until use.

Detection of the cyclosporine blood concentration in rats by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Blood concentrations of CsA were measured using validated LC-MS/MS using an internal standard of Cyclosporine D. The standards in the rat samples were analyzed on an API-4000 triple quadruple mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.) under an electrospray ionization negative mode (standard curves ranged from 1.00 to 4,000 ng/ml; $r^2 > 0.99$). The lower limit of quantitation for CsA was 1.00 ng/ml. The assay accuracy (% bias), and precision (% relative standard deviations) of the quality control samples were within ± 15%.

Measurement of intestinal and hepatic mRNA expression levels. The expression levels of mRNA encoding CYP3A1, CYP3A2, UGT1A, OATP2B1 (small intestine only), OATP1B2 (liver only), P-gp, BCRP and MRP2 in the small intestine and liver were quantified by RT-qPCR. The tissues (100 mg) were homogenized in 1 ml TRIzol® reagent. Total RNA was extracted according to the manufacturer's instructions. The RNA was quantified by the standard optical density (OD)$_{260}$ method (41). The OD_{260}/OD_{280} ratio for each RNA sample ranged from 1.8 to 2.2. Subsequently, RNA was converted to cDNA using the high-capacity First Strand cDNA Synthesis kit (Toyobo, Co., Ltd.), according to the manufacturer's instructions. qPCR was performed using THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Co., Ltd.) and a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers for *Cyp3a1, Cyp3a2, Ugt1a,* solute carrier organic anion transporter family member *(Slco) 2b1, Slco1b2,* multi-drug resistance 1 *(Mdr1), Bcrp, Mrp2* and the housekeeping gene β-actin were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. and are listed in Table I. The PCR cycling protocol consisted of one cycle of 1 min at 95°C, followed by 40 cycles of denaturation 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 expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (42).

Measurement of intestinal and hepatic protein expression levels. The protein expression levels of CYP3A1, CYP3A2, UGT1A, OATP2B1 (small intestine only), OATP1B2 (liver only), P-gp, BCRP and MRP2 in the small intestine and liver were analyzed by western blotting. The small intestine and liver samples were homogenized in 10X ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich), and centrifuged at 12,000 x g for 5 min at 4°C. The supernatants were stored at -80°C until analysis. Protein concentrations were determined using the BioRad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples (40 µg) were loaded onto 8-20% SDS-PAGE gels and subjected to electrophoresis at 120 V for 90 min. The proteins were transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 h with Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) containing 5% skim milk and incubated with primary antibody overnight at 4°C. Membranes were subsequently washed three times with TBST, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 30 min at room temperature. The following primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): mouse anti-CYP3A1 monoclonal antibody (1:200; sc-53246), mouse anti-P-gp monoclonal antibody (1:500; sc-71557), rabbit anti-BCRP polyclonal antibody (1:500; sc-25822), rabbit anti-MRP2 polyclonal antibody (1:500; sc-20766) and mouse anti-β-actin monoclonal antibody (1:5,000; TDY051). Additional antibodies purchased from Abcam (Cambridge, MA, USA) were: rabbit anti-UGT1A polyclonal antibody (1:1,000; ab194697) and rabbit anti-OATP2B1 polyclonal antibody (1:1,000; sc-376904). Rabbit anti-CYP3A2 polyclonal antibody (1:500; AB1276) was supplied by Merck Millipore. Secondary antibodies were goat

anti-mouse IgG-HRP (1:10,000; 074-1806) and goat anti-rabbit IgG-HRP (1:10,000; 074-1506), which were purchased from Santa Cruz Biotechnology, Inc. Protein bands were visualized using Enhanced chemiluminescence plus western blotting detection system (GE Healthcare Life Sciences, Chalfont, UK) followed by exposure to Kodak films (Kodak, Rochester, NY, USA) and densitometry analyses (Kodak 1D3 image analysis software version 3.6.1; Kodak). Results were normalized relative to β-actin expression.

Pharmacokinetic analysis. The plasma concentration data were analyzed by a non-compartmental method using Drug and Statistics software version 3.0 (Mathematical Pharmacology Professional Committee of China, Shanghai, China). The elimination rate constant (K_{el}) was calculated by log-linear regression of CsA data during the elimination phase. The terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}) of CsA in plasma were derived directly from the concentration-time curve. 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 from zero to infinity ($AUC_{0-\infty}$) 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 vs. time curve, calculated in a similar fashion to the AUC.

Statistical analysis. Statistical analyses were performed with GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired Student's *t* tests were performed for comparison between independent groups. The influences of Que on changes in mRNA and protein expression levels, as well as pharmacokinetic parameters of CsA were evaluated by paired Student's *t* tests. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's post hoc test was performed for each group. All tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation.

Results

Effect of Que on CsA pharmacokinetic. Mean plasma concentration-time profiles of CsA in rats following oral administration of 10 mg/kg CsA in the presence or absence of Que (25,50 or 100 mg/kg) are presented in Fig. 1; the corresponding pharmacokinetic parameters are presented in Table II. The presence of Que significantly altered the pharmacokinetic parameters of CsA. C_{max} of CsA in the absence of Que on day 1 was 2412.66±544.85 ng/ml (n=24), while that of CsA in the Que-treated rats decreased by 46 (P=0.0094), 50 (P=0.0175) and 47% (P=0.0015) in the low-, moderate- and high-dose groups, respectively (day 9, n=8 each group). In addition, AUC_{0-t} and $AUC_{0-\infty}$ of CsA in Que-treated rats decreased, by 21 (P=0.3392) and 16% (P=0.5694), 30 (P=0.2567) and 33% (P=0.4101), 33 (P=0.0028) and 34% (P=0.0036), respectively. Furthermore, Que-treated rats exhibited significantly increased MRT_{0-t}
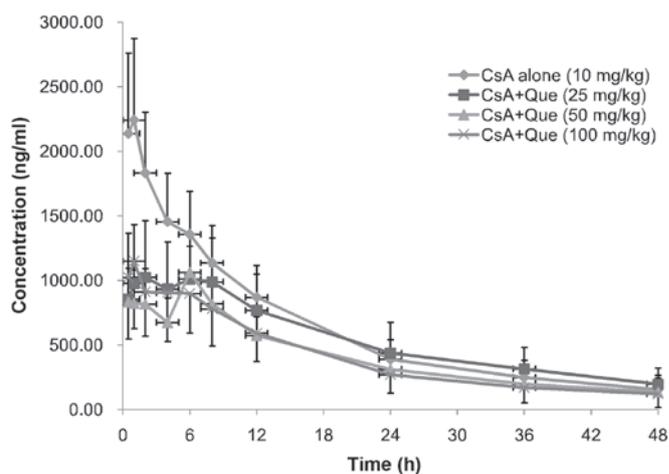

Figure 1. Mean plasma concentration-time profiles of CsA. On day 1, the rats received a single oral dose of CsA (10 mg/kg) alone. From days 3 to 8, all rats assigned to the Que-treatment groups were gavaged with 25, 50 or 100 mg/kg/day of Que for 6 consecutive days. On day 9, animals were pretreated with the relevant dosage of Que 0.5 h prior to the final dose of CsA (10 mg/kg), and blood samples were collected at various time points for pharmacokinetic analysis. Data are presented as the mean ± standard deviation for each data point (Que-treated groups, n=8; CsA alone group, n=24). CsA, cyclosporine; Que, quercetin.

values compared with the control rats, increasing by 16 (P=0.0426), 19 (P=0.0458), and 9% (P=0.0180) in the low-, moderate- and high-dose groups, respectively. However, there were no significant differences in C_{max} (P=0.6562), AUC_{0-t} (P=0.3087), $AUC_{0-\infty}$ (P=0.2197) and MRT_{0-t} (P=0.1498) between the three Que treatment groups, or in the T_{max} (P=0.2359), CL/F (P=0.3325) and $t_{1/2}$ (P=0.0540) of CsA between Que-treated and non-treated groups.

mRNA expression levels of DMEs and DTs in the small intestine and liver. The intestinal and hepatic mRNA encoding CYP3A1, CYP3A2, UGT1A, OATP2B1 (small intestine only), OATP1B2 (liver only), P-gp, BCRP and MRP2 were measured by RT-PCR analysis using intestinal and hepatic RNA prepared from rats (Fig. 2).

mRNA expression levels of Cyp3a1 and Cyp3a2. As presented in Fig. 2A and B, a dose-dependent decrease (P=0.0099 and P<0.0001, respectively) was observed in the intestinal mRNA expression levels of *Cyp3a1* and *Cyp3a2* in Que-WOC rats when compared to the control (vehicle) group, by 45 and 61% (P=0.0033 and P=0.0006, respectively), 83 and 70% (P<0.0001 and P=0.0004, respectively), and 88 and 90% (P<0.0001 and P=0.0001, respectively) in the low-, moderate- and high-dose groups, respectively. By contrast, CsA treatment increased the intestinal mRNA expression levels of *Cyp3a1* and *Cyp3a2* by 90 (P=0.0008) and 63% (P=0.0022). When compared with CsA treatment alone, a dose-dependent decrease (P=0.0324 and P<0.0001, respectively) was observed in the intestinal mRNA expression levels of *Cyp3a1* and *Cyp3a2* in Que-WC rats, by 46 and 58% (P=0.0006 and P=0.0002, respectively), 83 and 71% (P<0.0001 and P<0.0001, respectively), and 81 and 88% (P<0.0001 and P<0.0001, respectively) in the low-, moderate- and high-dose Que co-administration groups, respectively.

Table II. Predominant pharmacokinetic parameters of CsA following the oral administration of CsA (10 mg/kg) to rats in the absence or presence of Que (25, 50 or 100 mg/kg).

Parameters	Que, 25 mg/kg		Que, 50 mg/kg		Que, 100 mg/kg	
	CsA alone	CsA+Que	CsA alone	CsA+Que	CsA alone	CsA+Que
AUC_{0-t}(ng·h/ml)	32116.48±9821.92	25274.44±9623.67	28280.09±9226.11	19682.86±6525.58	29301.87±8789.25	19398.18±7754.19[b]
$AUC_{0-\infty}$(ng·h/ml)	37368.60±14650.46	31479.18±13392.53	34135.53±17290.60	23042.65±10567.90	32643.59±12685.94	21665.49±10194.81[b]
$AUMC_{0-t}$(ng·h²/ml)	456519±181803	415848±180689	369183±155544	310041±144983	393299±166468	282844±149577[a]
MRT_{0-t}(h)	13.95±1.64	16.13±1.52[a]	12.80±1.62	15.27±1.86[a]	13.06±1.68	14.27±2.05[a]
VRT_{0-t}(h²)	149.03±22.23	156.35±26.29	135.55±28.71	150.97±20.45	136.57±23.31	145.26±23.98
$t_{1/2}$	16.64±6.49	23.27±10.47	19.29±9.32	15.71±5.49	14.07±4.77	13.60±4.24
V/F	6.68±1.90	10.65±4.00[a]	7.98±2.92	10.29±1.89	6.44±1.40	10.83±7.04
CL/F	0.30±0.11	0.38±0.24	0.35±0.14	0.50±0.17	0.34±0.12	0.57±0.30
T_{max}	0.93±0.19	3.56±3.22	0.90±0.22	3.58±2.69	0.81±0.26	1.56±1.52
C_{max}(ng/ml)	2405.07±447.95	1288.11±430.75[c]	2307.00±705.51	1146.85±111.26[b]	2485.35±579.03	1326.37±421.57[b]

On day 1, rats received a single oral dose of CsA (10 mg/kg) alone. From day 3 to day 8, all rats assigned into three different treatment groups were gavaged with 25, 50 or 100 mg/kg/day of Que for 6 consecutive days. On day 9, animals were pretreated with the relevant dose (25,50 or 100 mg/kg) of Que 0.5 h prior to the final dose of CsA (10 mg/kg) given to all rats for pharmacokinetic study. Data are presented as the mean ± standard deviation; n = 8. [a]P<0.05, [b]P<0.01, [c]P<0.001, compared with CsA alone. AUC_{0-t}, area under the plasma concentration-time curve from time zero to the time of last measured concentration; $AUC_{0-\infty}$, area under the plasma concentration-time curve from 0 h to infinity; AUMC, area under the moment curve; MRT, mean residence time; VRT, variance of the mean residence time; $t_{1/2}$; terminal half-life; V/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.

Similarly, in the liver, a dose-dependent decrease (P=0.0048 and P=0.0002, respectively) was observed in the mRNA expression levels of *Cyp3a1* and *Cyp3a2* in Que-WOC rats when compared to the control group, by 37 and 21% (P=0.0092 and P=0.0036, respectively), 86 and 37% (P<0.0001), and 98 and 49% (P<0.0001) in the respective treatment groups. Contrasting with the small intestine, the hepatic mRNA expression level of *Cyp3a1* was not significantly influenced by CsA treatment (P=0.0563); however, there was a significant rise (23%; P=0.0083) in *Cyp3a2*. When compared to CsA treatment alone, Que-WC treatment led to a decrease in the hepatic mRNA expression levels of *Cyp3a1* and *Cyp3a2*, by 31 (P=0.0206) and 16% (P=0.0398), 77 (P<0.0001) and 48% (P=0.0002), and 65 (P<0.0001) and 57% (P<0.0001) in the respective treatment groups. These results revealed that the mRNA expression levels of *Cyp3a1* and *Cyp3a2* were inhibited by Que to a similar extent in the small intestine and liver.

mRNA expression levels of Ugt1a1. The mRNA expression of *Ugt1a1* in the Que-WOC rats was significantly decreased in a dose-dependent manner (P=0.0070) by 20 (P=0.0045), 76 (P<0.0001) and 84% (P<0.0001) in the small intestine, and by 41, 81 and 92% in the liver (P<0.0001), in the respective treatment groups (Fig. 2C). CsA treatment led to a significant increase (109%; P=0.0002) in the intestinal mRNA expression level of *Ugt1a1* compared with the control group, while the hepatic mRNA expression level of *Ugt1a1* was not significantly influenced by CsA treatment (P=0.0970). Similarly, when compared to CsA treatment alone, Que-WC treatment led to a dose-dependent decrease (P=0.0061 and P=0.0005, respectively) in the intestinal and hepatic mRNA expression levels of *Ugt1a1*, by 32 (P=0.0062) and 28% (P=0.0088), 76 (P<0.0001) and 71% (P<0.0001), and 84 (P<0.0001) and 92% (P<0.0001) in the respective treatment groups.

mRNA expression levels of Slco2b1/Slco1b2. As presented in Fig. 2D, a dose-dependent decrease (P=0.0010 and P=0.0052, respectively) was observed in the mRNA expression levels of *Slco2b1* in the small intestine and *Slco1b2* in the liver of Que-WOC rats when compared to the control (vehicle) group, by 84 (P<0.0001) and 79% (P=0.0005), 92 (P<0.0001) and 92% (P=0.0002), and 97 (P<0.0001) and 95% (P=0.0002) in the low-, moderate- and high-dose groups, respectively. By contrast, CsA treatment led to a significant increase in the mRNA expression levels of *Slco2b1* in the small intestine (54%; P=0.0057) and *Slco1b2* in the liver (29%; P=0.0292) compared with the control. Similarly, when compared with CsA treatment, Que-WC treatment led to a marked decrease in the intestinal *Slco2b1* and hepatic *Slco1b2* mRNA expression levels, by 66 (P=0.0087) and 72% (P<0.0001), 92 (P=0.0001) and 90% (P<0.0001), and 92 (P=0.0001) and 94% (P<0.0001) in the respective treatment groups. These results revealed that Que had a potent inhibitory effect on the intestinal *Slco2b1* and hepatic *Slco1b2* mRNA expression levels.

mRNA expression levels of Mdr1. In the small intestine, as presented in Fig. 2E, moderate- and high-dose Que-WOC treatment led to an increase in the mRNA expression levels of *Mdr1* by 75 (P=0.0008) and 80% (P=0.0004), respectively, compared with the control group, while low-dose treatment did not produce a significant effect (P=0.2312). Similarly, in the Que-WC rats, moderate- and high-dose Que co-treatment led to a decrease in the mRNA expression level of *Mdr1* by 78% when compared with CsA treatment alone (P=0.0033 and P=0.0034, respectively), while low-dose treatment did not produce a significant effect (P=0.2661). By contrast, CsA treatment increased the mRNA expression levels of *Mdr1* in the small intestine and liver, by 74 (P=0.0305) and 19% (P=0.0239), respectively.

In the liver, a dose-dependent decrease (P=0.0066) was observed in the mRNA expression levels of *Mdr1* in Que-WOC rats when compared with the control (vehicle) group, by 64, 87 and 91% in the respective treatment groups (P<0.0001). Similarly, when compared with CsA treatment alone, Que-WC treatment led to a decrease in the hepatic mRNA expression levels of *Mdr1*, by 68, 68 and 90% in the respective treatment groups (P<0.0001).

mRNA expression levels of Bcrp. A dose-dependent decrease (P<0.0001 and P=0.0002, respectively) was observed in the mRNA expression levels of *Bcrp* in the small intestine and liver of Que-WOC rats when compared to the control (vehicle) group, by 46 and 55%, 70 and 77%, and 92 and 88% in the respective treatment groups (P<0.0001, Fig. 2F). CsA treatment led to a significant increase in the intestinal mRNA expression levels of *Bcrp* (75%; P=0.0007), while in the liver *Bcrp* was not significantly influenced by CsA treatment (P=0.1736). When compared with CsA treatment alone, Que-WC treatment led to a dose-dependent decrease (P<0.0001) in the *Bcrp* mRNA expression levels, by 50 (P=0.0002) and 68% (P=0.0008), 68 (P<0.0001) and 77% (P=0.0004), and 91 (P<0.0001) and 89% (P=0.0002<0.001) in the respective treatment groups. These results revealed that the mRNA expression levels of *Bcrp* were inhibited by Que to a similar extent in the small intestine and liver.

mRNA expression levels of Mrp2. As presented in Fig. 2G, a dose-dependent decrease (P<0.0001) was observed in the mRNA expression levels of *Mrp2* in the small intestine and liver of Que-WOC rats compared with the control (vehicle) group, by 45 and 45%, 69 and 52%, and 96 and 98% in the respective treatment groups (P<0.0001). CsA treatment led to a significant increase in the intestinal mRNA expression levels of *Mrp2* (65%; P=0.0025), while in the liver *Mrp2* was not significantly influenced by CsA treatment (P=0.2855). When compared with CsA treatment alone, Que-WC treatment led to a dose-dependent decrease (P<0.0010.05) in the *Mrp2* mRNA expression levels in the small intestine and liver, by 48 (P=0.0015) and 38% (P=0.0006), 68 (P=0.0004) and 36% (P<0.001), and 85 (P=0.0002) and 99% (P<0.0001) in the respective treatment groups.

Taken together, these results revealed that the mRNA expression levels of the investigated DMEs and DTs were inhibited by Que in a dose-dependent manner, and to a similar extent in the small intestine and liver. In addition, when compared with CsA treatment alone, Que-WC treatment demonstrated a dose-dependent inhibitory effect in the small intestine and liver of the respective treatment groups.

Protein expression levels of DMEs and DTs in the small intestine and liver. The intestinal and hepatic proteins
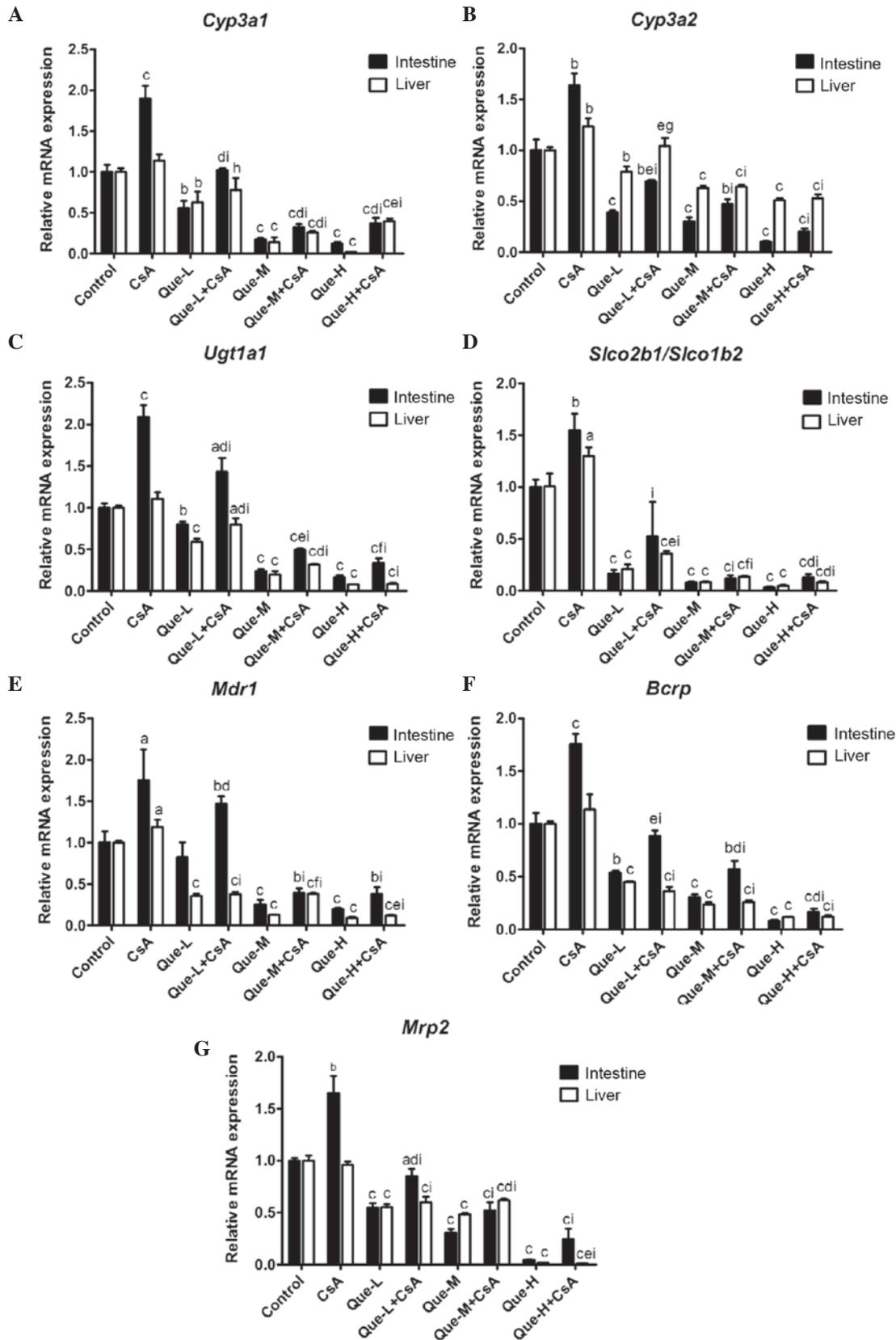
Figure 2. Effect of Que on the intestinal and hepatic mRNA expression levels of CYP3A1, CYP3A2, UGT1A, SLCO2B1, SLCO1B2, MDR1, BCRP and MRP2. In rats of the control, CsA treatment, and Que treatment without (Que-WOC) and with (Que-WC) CsA for 7 consecutive days groups, the mRNA expression levels were measured by reverse transcription-quantitative polymerase chain reaction and calculated as expression levels relative to the control using the $2^{-\Delta\Delta Cq}$ method. mRNA expression levels of (A) *Cyp3a1*, (B) *Cyp3a2*, (C) *Ugt1a1*, (D) *Slco2b1/Slco1b2*, (E) *Mdr1*, (F) *Bcrp* and (G) *Mrp2* were measured in the small intestine and liver of rats. β-actin was used as a loading control. Data are presented as the mean ± standard deviation (n=3). [a]P<0.05, [b]P<0.01 and [c]P<0.001, compared with the control; [d]P<0.05, [e]P<0.01, and [f]P<0.001, compared to the Que-WOC group; [g]P<0.05, [h]P<0.01, and [i]P<0.001, compared to the CsA treatment alone group. Que-L, Que low dose (25 mg/kg); Que-M, Que moderate dose (50 mg/kg); Que-H, Que high dose (100 mg/kg); CsA, cyclosporine; Que, quercetin; Cyp, cytochrome P; Ugt1a1, UDP glucuronosyltransferase family 1 member A complex locus; Slco, solute carrier organic anion transporter family member; Mdr1, multi-drug resistance 1; Bcrp, breast cancer resistance protein; Mrp2, multidrug resistance-associated protein 2.
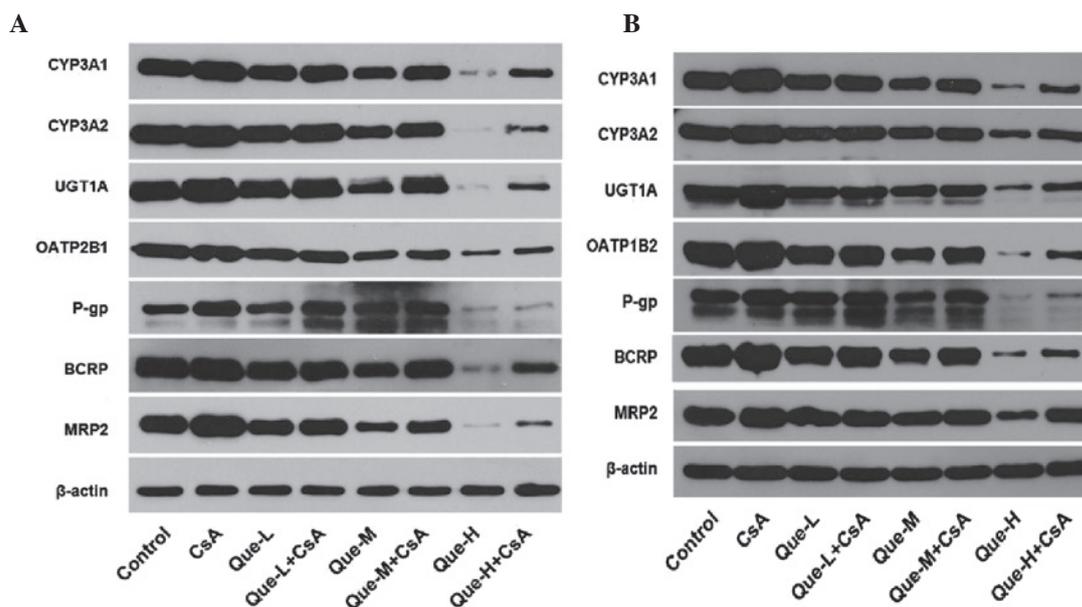
Figure 3. Effect of Que on the intestinal and hepatic protein expression levels of CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP and MRP2 assessed by western blotting. In rats of the control, CsA treatment, and Que treatment without (Que-WOC) or with (Que-WC) CsA for 7 consecutive days groups, western blotting analysis was performed and β-actin was used as a loading control. (A) Image of western blotting results in the small intestine. (B) Image of western blotting results in the liver. Que-L, Que low dose (25 mg/kg); Que-M, Que moderate dose (50 mg/kg); Que-H, Que high dose (100 mg/kg); CsA, cyclosporine; Que, quercetin; CYP, cytochrome P; UGT1A1, UDP glucuronosyltransferase family 1 member A; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP2, multidrug resistance-associated protein 2.

CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP and MRP2 were measured by western blotting analysis in all treated rats (Fig. 3). Images of western blots performed on the small intestine and liver of rats are presented in Fig. 3A and B respectively. Quantification of western blots is presented in Fig. 4.

Protein expression levels of CYP3A1 and CYP3A2. As presented in Fig. 4A and B, a dose-dependent decrease (P<0.0001) was observed in the intestinal protein expression levels of CYP3A1 and CYP3A2 in Que-WOC rats when compared with the control (vehicle) group, by 17 (P=0.1181) and 14% (P=0.3328), 45 (P=0.0078) and 40% (P=0.0270), and 91 (P=0.0003) and 95% (P=0.0007) in the low-, moderate- and high-dose groups, respectively. Contrasting with the mRNA expression level results, CsA treatment did not alter the protein expression levels of CYP3A1 or CYP3A2 in the small intestine or liver. This may be due to the high basal level of CYP3A, meaning an additional increase in mRNA expression levels would not result in a significant change in protein expression levels. When compared with CsA treatment alone, a dose-dependent decrease (P<0.001) was observed in the intestinal protein expression levels of CYP3A1 and CYP3A2 in Que-WC rats, by 22 (P=0.0121) and 18% (P=0.1467), 38 (P=0.0055) and 35% (P=0.0241), and 67 (P<0.0001) and 71% (P=0.0006) in the low-, moderate- and high-dose Que co-administration groups, respectively.

High-dose Que treatment led to an decrease in the hepatic protein expression levels of CYP3A1 and CYP3A2 by 70 (P=0.0022) and 73% (P=0.0075), respectively, compared with the control, while moderate- (P=0.0501 and P=0.0867, respectively) and low-dose (P=0.1219 and P=0.2661, respectively) treatment did not produce significant effects. However, a dose-dependent decrease (P=0.0006) was observed in Que-WOC rats. Similarly,

when compared with CsA treatment alone, only in the high-dose co-administration groups was a significant decrease observed in the hepatic protein expression levels of CYP3A1 and CYP3A2 in Que-WC rats, by 56 (P=0.0071) and 54% (P=0.0163), respectively.

Protein expression levels of UGT1A1. When compared with the control, the protein expression levels of UGT1A1 in the Que-WOC rats was significantly decreased in a dose-dependent manner by 12 (P=0.0888), 41 (P=0.0024) and 91% (P=0.0001) in the small intestine, and by 16 (P=0.6453), 42 (P=0.2090) and 87% (P=0.0231) in the liver (Fig. 4C). Similar to the mRNA expression level results, CsA treatment led to a significant increase in the intestinal protein expression level of UGT1A1 (27%; P=0.0380) compared with the control group, while the hepatic protein expression level of UGT1A1 was not significantly influenced by CsA treatment (P=0.6597). Compared with CsA treatment, Que-WC treatment led to a dose-dependent decrease (P<0.0001) in the intestinal protein expression levels of UGT1A1, by 22 (P=0.0490), 39 (P=0.0079) and 74% (P=0.0003) in the respective treatment groups. However, only high-dose Que-WC treatment led to a decrease in the hepatic protein expression levels of UGT1A1 (72%; P=0.0448) compared with CsA treatment alone, while low- and moderate-dose treatment did not produce significant effects (P=0.4835 and P=0.1782, respectively).

Protein expression levels of OATP2B1/OATP1B2. Similar to the mRNA results, a dose-dependent decrease (P<0.001) in the moderate- and high-dose groups was observed in the protein expression levels of OATP2B1 in the small intestine and OATP1B2 in the liver in Que-WOC rats when compared with the control (vehicle) group, by 43 (P=0.0282) and 48%

(P=0.0222), and 76 (P=0.0031) and 86% (P=0.0027), respectively, while low-dose treatment did not produce a significant effect (P=0.1498 and P=0.3482, respectively; Fig. 4D). CsA treatment did not alter the protein expression levels of OATP2B1 and OATP1B2 in the small intestine and liver. Similarly, when compared with CsA treatment, only moderate- and high-dose Que-WC treatment led to a marked decrease, by 42 (P=0.0297) and 38% (P=0.0254), and 63 (P=0.0071) and 72% (P=0.0028), respectively. When compared with the mRNA results, Que demonstrated a weak inhibitory effect on intestinal OATP2B1 and hepatic OATP1B2 protein expression.

Protein expression levels of P-gp. As presented in Fig. 4E, high-dose Que treatment led to a significant decrease in the intestinal and liver protein expression levels of P-gp by 81 (P=0.0059) and 84% (P=0.0153), respectively, compared with the control group, while low- (P=0.7061 and P=0.7554, respectively) and moderate-dose (P=0.2448 and P=0.0758, respectively) treatment did not produce a significant effect. Similarly, only high-dose Que-WC treatment led to a marked decline in the intestinal and liver protein expression levels of P-gp when compared with CsA treatment, by 83 (P=0.0108) and 76% (P=0.0330), respectively.

Protein expression levels of BCRP. A dose-dependent decrease (P<0.0001) was observed in the protein expression levels of BCRP in the small intestine and liver of Que-WOC rats when compared with the control (vehicle) group, by 17 (P=0.2130) and 7% (P=0.6968), 49 (P=0.0109) and 27% (P=0.1563), and 90 (P=0.0009) and 75% (P=0.0072) in the respective treatment groups. When compared with CsA treatment alone, Que-WC treatment led to a dose-dependent decrease (P<0.001) in the BCRP protein expression levels, by 22 (P=0.0262) and 23% (P=0.0841), 36 (P=0.0044) and 31% (P=0.0475), and 69 (P=0.0004) and 67% (P=0.0015) in the respective treatment groups (Fig. 4F). Therefore, in the small intestine and liver the protein expression levels of BCRP were inhibited by Que to a similar extent.

Protein expression levels of MRP2. A dose-dependent decrease (P<0.0001) was observed in the protein expression levels of MRP2 in the small intestine and liver of Que-WOC rats compared with the control (vehicle) group, by 13 (P=0.0874) and 14% (P=0.2608), 45 (P=0.0011) and 27% (P=0.0693), and 93 (P<0.0001) and 58% (P=0.0041) in the respective treatment groups. Similar to the mRNA expression level results, CsA treatment led to a significant increase in the intestinal protein expression level of MRP2 (45%; P=0.0187), while in the liver MRP2 was not significantly influenced by CsA treatment (P=0.6103). When compared with CsA treatment, Que-WC treatment led to a dose-dependent decrease (P<0.0001 and P=0.0260, respectively) in MRP2 protein expression levels, by 32 (P=0.0164) and 22% (P=0.0934), 52 (P=0.0023) and 23% (P=0.0776), and 82 (P=0.0004) and 35% (P=0.0235) in the respective treatment groups (Fig. 4G).

Taken together, these results revealed that the protein expression levels of the investigated DMEs and DTs were dose-dependently inhibited by Que to a similar extent in the small intestine and liver. However, in contrast to the mRNA

expression level results, the low-dose (25 mg/kg) Que treatment did not demonstrate a significant inhibitory effect on the protein expression levels when compared with the control.

Discussion

Currently, flavonoid-drug interactions are gaining the attention of the scientific community, particularly with regard to clinical practice. Increasing evidence suggests that Que may interact with numerous xenobiotics. For example, Que has been demonstrated to increase the bioavailability of various drugs, including fexofenadine (43), rosiglitazone (44) and CsA (33) in humans; paclitaxel (45), valsartan (46), ranolazine (47), tamoxifen (48) and doxorubicin (49) in rats; and digoxin (50) in pigs. By contrast, Que decreased the bioavailability of talinolol (51) in humans, metoprolol (52) in rats, simvastatin (53) in pigs and CsA (32,34-36) in pigs and rats. Therefore, the HDIs of Que co-administration with other drugs, as well as the effect of Que on CYP3A and P-gp, remain to be fully elucidated. To the best of our knowledge, this is the first report to systematically demonstrate the impact of multiple-dose administration of Que on DMEs and DTs in the small intestine and liver, as well as on the pharmacokinetics of CsA.

CsA is an immunosuppressant that is routinely used to prevent rejection of kidney, liver, heart and bone marrow transplants and in addition is used to treat various autoimmune diseases (27). Clinically, a supra-therapeutic CsA blood level may result in adverse effects including nephrotoxicity, hepatotoxicity and neurotoxicity (27). Conversely, a sub-therapeutic blood level may result in allograft rejection by transplant recipients (54). As CsA is effective within a narrow therapeutic index (37), a thorough understanding of its propensity for HDIs is required prior to co-administration with novel pharmacologic agents that may affect its efficacy.

CsA is primarily metabolized in the small intestine and liver by isoenzymes CYP3A4 and CYP3A5 (27,55). In addition, UGT1A and 2B, P-gp and MRP2 are involved in CsA bioavailability (27-29). Numerous factors, including food ingestion, changes in gastric motility, diarrhea, diabetes and genetic polymorphism (56), may affect CsA metabolism and bioavailability, and information is required by clinicians and patients to prevent the inadvertent alteration of CsA serum levels. In addition, a leading cause of altered CsA metabolism is the co-administration of herbal medicines that affect the activity of DMEs or DTs, or alter their interactions with CsA, including ginkgo, St John's wort, ginger, ginseng, garlic and berberine (2,37).

Besides the ameliorative effect of Que on CsA-induced nephrotoxicity and hepatotoxicity (8,9), Que is known to affect the immune system (40,57). Que has been reported to inhibit the production of IL-2 by human T cells in a dose-dependent manner (57), which may explain its immunosuppressive effects. Co-administration with CsA may therefore enhance immunosuppression.

In the present study, the pharmacokinetic interaction between Que and CsA was investigated in rats. Based on the ratio of surface area (human/rat), the doses of 25, 50 and 100 mg/kg were tested in rats in the present study, with the corresponding doses in humans being 250, 500 and 1000 mg/day. The findings of the present study demonstrated that concomitant oral admin-
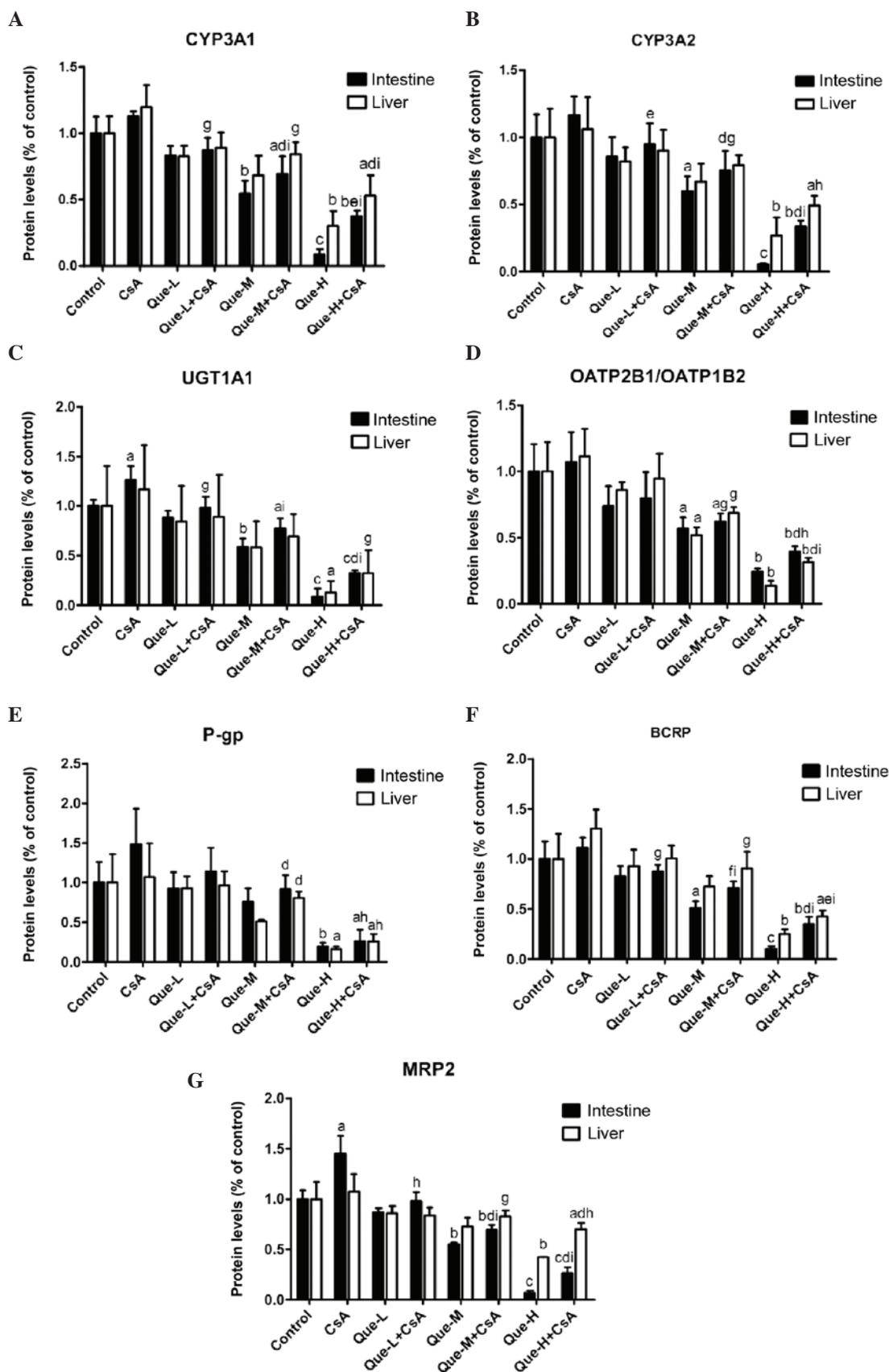
Figure 4. Quantification of the effect of Que on intestinal and hepatic protein expression levels of CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP and MRP2 assessed by western blotting. Quantifiction of western blotting results revealed the protein expression levels of (A) CYP3A1, (B) CYP3A2, (C) UGT1A, (D) OATP2B1/OATP1B2, (E) P-gp, (F) BCRP and (G) MRP2 measured in the small intestine and liver of rats. Data are presented as the mean ± standard deviation (n=3). ^{a}P<0.05, ^{b}P<0.01 and ^{c}P<0.001, compared with the control; ^{d}P<0.05, ^{e}P<0.01 and ^{f}P<0.001, compared to the Que-WOC group; ^{g}P<0.05, ^{h}P<0.01 and ^{i}P<0.001, compared to the CsA treatment group. Que-L, Que low dose (25 mg/kg); Que-M, Que moderate dose (50 mg/kg); Que-H, Que high dose (100 mg/kg); CsA, cyclosporine; Que, quercetin; CYP, cytochrome P; UGT1A, UDP glucuronosyltransferase family 1 member A; OATP, organic anion-transporting polypeptide; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP2, multidrug resistance-associated protein 2.

istration of Que (25,50 and 100 mg/kg) dose-independently decreased C_{max} by 46, 50, and 47% (P<0.01). In addition, the AUC_{0-t} and $AUC_{0-\infty}$ of CsA was decreased, by 21 and 16%, 30 and 33%, and 33 and 34% (P<0.01), respectively. Furthermore, Que-treated rats displayed significantly increased MRT_{0-t} values compared with control rats, with dose-independent increases of 16, 19, and 9% (P<0.05) in the low-, moderate- and high-dose groups, respectively. Notably, there were no significant differences (P>0.05, by ANOVA) in C_{max}, AUC_{0-t}, $AUC_{0-\infty}$ and MRT_{0-t} among three-dose Que co-administration groups in the present study. Furthermore, no significant differences were observed in the T_{max}, CL/F and $t_1/_2$ of CsA in the presence of Que when compared to CsA alone. Therefore, Que co-administration had a significant effect on CsA pharmacokinetics. These results were consistent with previous reports that the bioavailability of CsA in pigs and rats was reduced when Que or Que-derived products were co-administered (32,34-36). However, in a study by Choi *et al* (33), the AUC of CsA was increased by 18% when Que was orally co-administered (CsA 300 mg plus Que 5 mg/kg), by 36% when healthy male subjects received Que 30 min prior to CsA treatment, and by 47% when subjects received Que for three days prior to CsA treatment. This result contrasts with the results of the present study, which may be due to differences in the subjects, the methods of administration and the doses.

Furthermore, the results of the present study demonstrated that Que produced a significant inhibitory effect on the mRNA and protein expression of DMEs and DTs in the small intestine and liver of rats. Significantly, Que administered orally was capable of changing the mRNA and protein expression levels in the rat small intestine, and also modified the mRNA and protein expression levels in the rat liver. Notably, it was revealed that the mRNA expression levels of *Cyp3a1*, *Cyp3a2*, *Ugt1a1*, *Slco2b1*, *Slco1b2*, *Mdr1*, *Bcrp* and *Mrp2* were inhibited by Que in a dose-dependent manner (P<0.05) to a similar extent in the small intestine and liver. Additionally, in the small intestine and liver, when compared with CsA treatment alone, Que-WC treatment led to a dose-dependent decrease (P<0.05) in the mRNA expression levels in the low-, moderate- and high-dose co-administration groups. Notably, Que exerted a marked inhibitory effect on intestinal *Slco2b1* and hepatic *Slco1b2* mRNA expression, with reductions of 84 and 79%, 92 and 92%, and 97 and 95% (P<0.001) in the low-, moderate- and high-dose groups, respectively. These effects should be further investigated in future research. Similarly, the protein expression levels of CYP3A1, CYP3A2, UGT1A, OATP2B1, OATP1B2, P-gp, BCRP and MRP2 were inhibited by Que in a dose-dependent manner (P<0.05) to a similar extent in the small intestine and liver. However, in contrast to the mRNA results, the low-dose (25 mg/kg) Que treatment did not produce a significant inhibitory effect on the protein expression levels when compared with the control. Notably, when compared with the potent inhibitory effect observed on mRNA expression levels, Que had a relatively weaker inhibitory effect on the protein expression levels.

The results of the present study were consistent with previous observations made *in vitro* and *in vivo* following co-treatment with Que (18,20-22,26). However, evidence has not always been consistent (16,17). Rats fed a diet containing 1% Que demonstrated significantly increased activity of UGTs in the liver and, to a lesser extent, in the small intestine (58). Que significantly induced CYP3A activity and this induction was somewhat associated with the *CYP3A5* genotype, being more prominent in *CYP3A5*1/*1* and *CYP3A5*1/*3* individuals (17). A previous study in healthy Chinese subjects demonstrated that Que significantly induced the activity of P-gp and this effect was more pronounced in individuals with the *MDR1* 3435 TT polymorphism (25). Thus, the effects of Que on P-gp remain to be fully understood (16). In a previous study of vincristine in MBEC4 cells, Que was observed to decrease the uptake of vincristine at low concentrations (10 μM); however, it increased its uptake at high concentrations (50 μM) (59). These biphasic *in vitro* results were supported by a study on ddY mice *in vivo* with co-administration of vincristine and Que at low (0.1 mg/kg) and high doses (1.0 mg/kg) (59). The biphasic effects were due to an alteration in the function of P-gp. At low concentrations, P-gp activity was increased as a result of enhanced phosphorylation, while at high concentrations P-gp was inhibited (59). A study identified that the major phase II metabolites of Que (including 3'-O-methylquercetin, 4'-O-methylquercetin, quercetin-3-O-β-glucoside, and quercetin-3-O-rhamnosylglucoside) inhibited MRP2 functions to a similar extent as the original compound (60). Que at a concentration of 25 μM markedly induced mRNA and protein expression of BCRP in Caco-2 cells, possibly through regulation of the aryl hydrocarbon receptor (61). The majority of flavonoids have been demonstrated to acutely inhibit the activity of DMEs and DTs; however, chronically, DMEs and DTs had enhanced expression and/or activity (16). Aside from the previously mentioned exceptions (16-17,25,58-61), the majority of the evidence supports an inhibitory role of Que on DMEs and DTs.

Interplay between P-gp and CYP3A has been previously demonstrated (38,62). P-gp increases the availability of a drug for metabolism by intestinal CYP3A by expelling it from enterocytes into the lumen of the intestine, thus promoting its metabolism (38). In addition, a previous study revealed that in CYP3A4-expressing Caco-2 cells, P-gp activity increased CsA metabolism (63). However, in the present study, the decrease in intestinal P-gp did not result in a marked increase in CsA plasma concentration. Therefore, other factors that remain to be identified, such as the interplay between other DMEs and DTs, may be involved in the metabolism of CsA.

As an extension to the idea of interplay between CYPs and efflux transporters, the dependence of phase II metabolic enzymes on efflux transporters was systematically illustrated utilizing various model systems, which may drive the metabolic disposition and clearance processes of flavonoids (16,64). Notably, synergistic interplay between multiple phase II enzyme-efflux transporter combinations has been investigated, including UGTs-MRPs, UGTs-BCRP, SULTs-BCRP and GST-MRPs (64). Transport of metabolites increased the total metabolite formation (64), although the mechanistic basis of the synergistic interplay of DMEs and DTs remains to be elucidated. Therefore, the interplay between DMEs and DTs is hypothesized to explain the pharmacokinetic interactions between Que and CsA in the present study (38).

As CsA is a dual substrate for DMEs and DTs, *ex vivo* investigations may not fully characterize the complex HDIs involved in the interplay between DMEs and DTs. As Que is an inhibitor of various DME and DT proteins, the results of the present study do not reveal whether the decrease in CsA concentration is the result of effects on DME/DT interplay, resulting in a decrease in the absorption of CsA, increased metabolism or a combination of the two. Therefore, the underlying mechanisms of the interaction revealed in the present study remain to be fully elucidated. *In vitro* studies may facilitate the identification of the underlying mechanisms involved in DME/DT interplay, and studies in human cells are required to determine any species-specific variations (16,38).

Notably, Que primarily exists in plants as glycosides. Que glycosides are hydrolyzed and metabolized by DMEs in the intestines of animals and humans (65). Furthermore, it was recently demonstrated that Que 3-O-β-D-glucuronide and Que-3'-O-sulphate are the primary Que conjugates in human and animal plasma, in which Que glycosides or Que aglycone could not be detected (66,67). In the present study, therefore, the Que metabolites rather than Que, may interact with DMEs and DTs *in vivo*. Various *in vitro* studies have revealed that the major phase II metabolites of Que were equal to or more potent than Que in the inhibition of MRP1, MRP2 and OAT1 (60,68). Therefore, it is crucial to understand the metabolism of compounds in order to investigate their effects. Further investigation is required to determine the effects of Que metabolites on DMEs and DTs *in vitro* and *in vivo*.

In conclusion, for the first time, to the best of our knowledge, the present study demonstrated that, following multiple-dose co-administration to rats, Que reduced CsA bioavailability, seemingly in contrast to the individual inhibitory effect on mRNA and protein expression levels of DMEs and DTs in the small intestine and liver. Overlapping modulation of intestinal and hepatic DMEs and DTs, as well as their interplay, may be responsible for this observation. The results of the present study suggest a novel mechanism underlying flavonoid-drug interactions, and may be clinically significant for patients taking CsA and Que or Que-containing dietary/herbal supplements simultaneously.

Acknowledgements

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