Inhibitory effects of quercetin and its major metabolite quercetin-3-O-β-D-glucoside on human UDP-glucuronosyltransferase 1A isoforms by liquid chromatography-tandem mass spectrometry

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Abstract. Quercetin is a flavonoid that is widely present in plant-derived food. Quercetin-3-O-β-D-glucoside (Q3GA) is a predominant metabolite of quercetin in animal and human plasma. The inhibitory effects of the UDP-glucuronosyl transferases (UGTs) caused by herbal components may be a key factor for the clinical assessment of herb-drug interactions (HDIs). The present study aimed to investigate the inhibitory profile of quercetin and Q3GA on recombinant UGT1A isoforms *in vitro*. The metabolism of the nonspecific substrate 4-methylumbelliferone (4-MU) by the UGT1A isoforms was assessed by liquid chromatography-tandem mass spectrometry. Preliminary screening experiments indicated that quercetin exhibited stronger inhibitory effects on UGT1A1, UGT1A3, UGT1A6 and UGT1A9 enzymes than Q3GA. Kinetic experiments were performed to characterize the type of inhibition caused by quercetin and Q3GA towards these UGT isoforms. Quercetin exerted non-competitive inhibition on UGT1A1 and UGT1A6, with half maximal inhibitory concentration (IC_{50}) values of 7.47 and 7.07 μM and inhibition kinetic parameter (K_i) values of 2.18 and 28.87 μM, respectively. Quercetin also exhibited competitive inhibition on UGT1A3 and UGT1A9, with IC_{50} values of 10.58 and 2.81 μM and K_i values of 1.60 and 0.51 μM, respectively. However, Q3GA displayed weak inhibition on UGT1A1, UGT1A3 and UGT1A6 enzymes with IC_{50} values of 45.21, 106.5 and 51.37 μM, respectively. In the present study, quercetin was a moderate inhibitor of UGT1A1 and UGT1A3, a weak inhibitor of UGT1A6, and a strong inhibitor on UGT1A9. The results of the present study suggested potential HDIs that may occur following quercetin co-administration with drugs that are mainly metabolized by UGT1A1, UGT1A3 and UGT1A9 enzymes.

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Abbreviations: Q3GA, quercetin-3-O-β-D-glucoside; 4-MU, 4-methylumbelliferone; UGTs, UDP-glucuronosyltransferase enzymes; NSCs, neural stem cells; HDIs, herb-drug interactions; 4-MU-G, 4-methylumbelliferyl-β-D-glucuronides; UDPGA, uridine-5'-diphosphoglucuronic acid; IC_{50}, half maximal inhibitory concentration; K_i, inhibition kinetic parameter

Key words: quercetin, Q3GA, UGT1As, LC-MS/MS

Introduction

The flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone; Fig. 1A) is one of the most abundant dietary polyphenols. It is mainly present in fruits and vegetables and ~3-40 mg quercetin is consumed in daily diets (1,2). Quercetin is widespread in the flowers, leaves and fruits of various plants and exhibits a multitude of pharmacological activities, including anti-neoplastic (3-5), anti-oxidative (6,7), anti-inflammatory (8,9), anti-thrombotic (10,11), antiviral (12,13), cardiovascular-protective (14,15) and immune-regulatory (16,17) effects. Isolated quercetin is a dietary supplement and its recommended maximum daily dosage is 1,000 mg (18). Following oral ingestion, quercetin is extensively conjugated with glucuronic acid and/or sulfate in the small intestine and liver. Quercetin-3-O-β-D-glucoside (Q3GA; Fig. 1B) is one of the primary metabolites found in the blood circulation (19). Q3GA exerts various pharmacological properties. Quercetin inhibits the viability of neural stem cells via the Akt signaling pathway. However, Q3GA provides a novel therapeutic potential in neurodegenerative diseases (20). In addition, the anti-inflammatory activity of Q3GA was evaluated by assessing the inhibition of LPS-induced NO release *in vitro* (21).

The biological activity of quercetin is notably affected by phase II, and not phase I, metabolism enzymes. As a plant-derived polyphenol, quercetin contains more than one free hydroxyl group, which makes it easy to be metabolized by different types of UGT enzyme isoforms, including UGT1As (22,23). UGTs are considered the indispensable enzymes of phase II metabolism and catalyze the conjugation of several endobiotics or xenobiotics with UDP-glucuronic acid in order to produce more hydrophilic metabolites that are easily excreted via the kidneys or the bile and the gut (24,25). CYP450-mediated herb-drug interactions (HDIs) have been previously investigated by *in vitro* assays using cocktails of probe substrates (26-29). Subsequent studies involving UGT enzymes have demonstrated that drug interactions based on the inhibition of UGTs may lead to clinically important side effects (30,31). Therefore, from a clinical point of view, the study of the inhibition of herbal compounds on UGT-mediated metabolism may aid the understanding of HDIs.

The *in vitro* UGT enzyme assay utilizes the nonspecific substrate 4-MU as a substrate and has various advantages over the use of human liver microsomes that include several specific probe-substrates (32). In the present study, the inhibition type and inhibitory effects of quercetin and its major metabolite Q3GA were assessed on various UGT isoforms (UGT1A1, UGT1A3, UGT1A6 and UGT1A9) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This method was used to detect the changes in the concentration levels of 4-methylumbelliferyl-β-D-glucuronides (4-MU-G). This may provide insight into the potential HDIs regarding quercetin and Q3GA, providing the basis for further drug research and safe drug use.

Materials and methods

Chemicals and reagents. 4-MU and 4-MU-G were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Q3GA was purchased from Chengdu Sino Standards Bio-Tech Co., Ltd. 7-Hydroxycoumarin was obtained from Dalian Meilun Biotechnology Co., Ltd. Quercetin and uridine-5'-diphosphoglucuronic acid (UDPGA; trisodium salt) were purchased from Sigma-Aldrich (Merck KGaA). Recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A6 and UGT1A9) were expressed in baculovirus-infected insect cells that, where were obtained from Corning, Inc. All other reagents were of the highest analytical grade commercially available. The specific reagents were sourced from companies mentioned previously (33).

Inhibition of recombinant UGTs-catalyzed 4-MU glucuronidation by quercetin and Q3GA. The experimental protocol and incubation has been accurately presented in previous studies (34-36). Typical incubations were performed in 200 μl reaction mixture containing 5 mM UDPGA, 5 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 4-MU and recombinant UGTs. In addition, a series of quercetin concentrations and Q3GA were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the total mixture was <0.5% (v/v). Table I indicates the final concentration of UGT1A isoforms and 4-Mu, and the incubation time used for each UGT enzyme. Following pre-incubation at 37˚C for 5 min, the UDPGA was added in the incubation system to initiate the reaction. The samples were incubated for the aforementioned incubation time periods and the reaction was terminated by the addition of 200 μl ice-cold methanol, containing 500 $ng \cdot ml^{-1}$ 7-hydroxycoumarin as an internal standard. Subsequently, the samples were centrifuged at 20,000 x g for 10 min at 4˚C, and 100 μl supernatant was obtained and injected into the LC-MS/MS system for analysis.

Detection of 4-MU-G by LC-MS/MS. 4-MU-G and 7-hydroxycoumarin (internal standard) were analyzed on an API-4000 triple quadruple mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc.) coupled with a Waters ACQUITY Ultra Performance Liquid Chromatograph (Waters Corporation). The separation was performed on an Inertsil ODS-SP column (100x2.1 mm; 3 μm; GL Sciences) with a column temperature of 40˚C. The mobile phase consisted of ultrapure water, containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The following gradient conditions were used: 0-4.00 min, 5-80% B; 4.00-4.10 min, 80-5% B; 4.10-7.00 min, 5% B. The flow rate used was 0.2 ml/min, and the LC retention times of 4-MU-G and 7-hydroxycoumarin were 3.7 and 3.53 min, respectively. The turbo ion spray interface was operated at -4,500 V and the ion source temperature was set at 500˚C in the negative electrospray ionization mode. The multiple reaction monitoring (MRM) mode was employed for quantification using specific precursor/product ion transition. The precursor/product ion transitions were monitored at m/z 351.1→175.1 and 161.0→89.0 for 4-MU-G and 7-hydroxycoumarin, respectively. The optimized working parameters for mass detection of 4-MU-G and 7-hydroxycoumarin were as follows: i) Declustering potential, -50 and -80 V; ii) collision energy, -19 and -40 V; iii) curtain gas, 30 psi; iv) collision activated dissociation gas, 8 psi; v) Gas1, 55 psi and Gas2, 55 psi. The peak areas for all analytes were automatically integrated using the Analyst software (version 1.5.1; Applied Biosystems).

The specificity of this method was optimal. The linear range was estimated to be 50-5,000 ng/ml with the lower limit of quantification at 50 ng/ml. The RSD% of the intra-assay and inter-assay precisions were both <10%. The extraction recovery ranged between 100.99 and 106.34%. The internal standard normalized matrix factors for the low-, moderate- and high-quality control samples were 1.02, 1.07 and 0.99, respectively. The residues were negligible, and the samples were placed in a sampler at 4˚C for 9 h and left at room temperature for 2 h.

Determination of inhibition kinetic parameters of quercetin and Q3GA on recombinant UGTs. The glucuronidation velocity was determined at various 4-MU, quercetin or Q3GA concentrations. A preliminary screening experiment was performed to assess the inhibitory effects of quercetin and Q3GA. A total of 50 μM was selected for quercetin and Q3GA as the experimental group concentration. The remaining activity of UGTs=average concentration of $4\text{-}MUG_{UGT1A\ enzyme\text{-}quercetin}$/average concentration of $4\text{-}MUG_{UGT1A\ enzyme\text{-}blank}$ x100%. The remaining activity of the blank group without quercetin and Q3GA was 100%

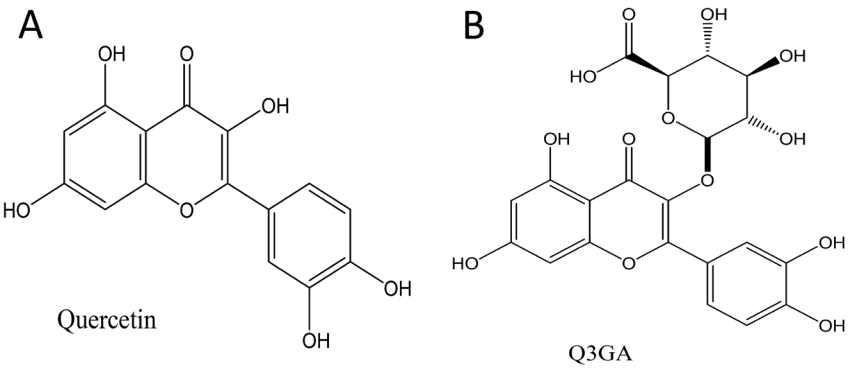

Figure 1. Chemical structures of (A) Quercetin and (B) Q3GA. Q3GA, quercetin-3-O-β-D-glucoside.

in the incubation system. Five or six concentration ranges of quercetin and Q3GA were selected to assess the IC_{50} values for the UGT1A enzymes. The concentration ranges of quercetin used were as follows: 0-10 μM for UGT1A1, 0-20 μM for UGT1A3 and 0-50 μM for UGT1A6 and UGT1A9. In addition, 0-50 μM Q3GA was selected for UGT1A1, UGT1A3 and UGT1A6. The IC_{50} values of quercetin and Q3GA towards UGT1A enzyme activities were calculated by nonlinear regression analysis using the GraphPad Prism 5 (GraphPad Software, Inc.). When the remaining activities of these enzymes in the experimental groups were <50% of the control group, the K_i was calculated. The K_i resulted from fitting data into competitive inhibition, non-competitive inhibition, or mixed inhibition models. The type of inhibition was assessed graphically from the Lineweaver-Burk and Dixon plots (37).

Results

Inhibitory activities of quercetin and Q3GA on recombinant UGT1A isoforms. As shown in Table II, in the presence of 50 μM quercetin, the remaining activity of UGT1A1, UGT1A3, UGT1A6 and UGT1A9 enzymes were <8.4, <30.8, 23.1 and 11.7%, respectively, while the remaining activity of Q3GA on UGT1A1, UGT1A3, UGT1A6 and UGT1A9 was 36.1, 33.9, 28.4 and 74.2%, respectively. As the remaining activity of UGT1A isoforms was <50%, except for UGTIA9, quercetin and Q3GA exhibited inhibitory effects on UGT1A1, UGT1A3, UGT1A6 and UGT1A9 enzymes.

In the presence of 50 μM quercetin, the final concentrations of the metabolite 4-MUG were too low to detect in UGT1A1 and UGT1A3 incubation systems. However, the lowest limit of quantitation for 4-MUG was 50 ng/ml in the present study. The remaining activity of UGT1A1 enzymes <50 ng/ml/593 ng/mlx100%=8.4%, and the remaining activity of UGT1A1 enzymes <50 ng/ml/162.3 ng/mlx100%=30.8%. Therefore, the remaining activities of UGT1A1 and UGT1A3 were less than 8.4 and 30.8%, respectively.

Inhibition type and kinetics of quercetin towards UGT1A1, UGT1A3, UGT1A6 and UGT1A9. The inhibitory parameters of quercetin on these UGT isoforms were characterized/calculated by further kinetic experiments. Quercetin inhibited UGT1A1, UGT1A3, UGT1A6 and UGT1A9 activity in a dose-dependent manner and the IC_{50} values were 7.47, 10.58, 7.07 and 2.81 μM, respectively (Figs. 2A, 3A, 4A and 5A). Furthermore, the inhibition types were determined from Dixon and Lineweaver-Burk plots. As shown in Fig. 2B and D and in Fig. 4B and D, the inhibitory effect of quercetin on UGT1A1 and UGT1A6-catalyzed 4-MU glucuronidation was characterized as non-competitive inhibition, while the inhibitory activity of quercetin on UGT1A3 and UGT1A6 was characterized as competitive inhibition (Figs. 3B and D; 5B and D). The calculated K_i were 2.18, 1.60, 28.87 and 0.51 μM for the inhibitory effect of quercetin on UGT1A1, UGT1A3, UGT1A6 and UGT1A9, respectively (Figs. 2C, 3C, 4C and 5C).

Inhibition type and kinetics of Q3GA towards UGT1A1, UGT1A3 and UGT1A6. Preliminary screening experiments indicated that Q3GA exhibited inhibitory effects on UGT1A1, UGT1A3 and UGT1A6. The parameters of the kinetic experiments are presented in Fig. 6. The inhibitory activity of Q3GA on UGT1A1, UGT1A3 and UGT1A6 enzymes was dose-dependent, with IC_{50} values of 45.21, 106.5 and 51.37 μM, respectively. Therefore, Q3GA may display weakly inhibit UGT1A1, UGT1A3 and UGT1A6.

Discussion

In recent years, dietary supplements and alternative medicine therapies have become increasingly accepted. A previous study (38) has demonstrated that the absorption and disposition of the active ingredients of dietary supplements are significant in determining their biological activity. In addition, accumulated evidence with regard to HDIs between drug-metabolizing enzymes and drug transporters may be used to predict the pharmacokinetic profile of drugs and the underlying HDIs (39,40). These potential interactions may cause significant risks to the patients, particularly for drugs with narrow therapeutic indices.

It has been demonstrated that quercetin may prevent cyclosporine A-induced nephrotoxicity and hepatotoxicity (41,42) as it exerts various effects on the pharmacokinetics of cyclosporine A. However, the underlying mechanisms for these interactions remain unclear. Our previous studies have demonstrated that Q3GA increases the C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of cyclosporine A (19,43). In order to further elucidate the underlying mechanisms of quercetin and Q3GA on the pharmacokinetics of cyclosporine A, the inhibitory effects of quercetin and Q3GA on the enzyme activity of recombinant UGT1A isoforms were investigated *in vitro*.

Table I. Substrate concentration, enzyme concentration and incubation time of each UGT enzyme.

UGT enzyme	Enzyme concentration, mg/ml	Incubation time, min	Substrate concentration, μM
UGT1A1	0.125	120	30
UGT1A3	0.05	120	1,200
UGT1A6	0.025	30	110
UGT1A9	0.05	30	30

UGT, UDP-glucuronosyltransferase.

Table II. Preliminary inhibition screening of quercetin and Q3GA toward activities of recombinant UGT1A isoforms.

	Remaining enzyme activity	
UGT1A enzyme	Quercetin, (%)	Q3GA, (%)
UGT1A1	<8.4	36.1
UGT1A3	<30.8	33.9
UGT1A6	23.1	28.4
UGT1A9	11.7	74.2

Q3GA, quercetin-3-O-β-D-glucoside; UGT, UDP-glucuronosyltransferase.

The inhibitory potential of the compounds was determined based on their Ki values and they were classified as potent inhibitors (Ki<1 μM), moderate inhibitors (1 μM < Ki<10 μM) and weak inhibitors (Ki>10 μM). Based on the IC_{50} values of Q3GA on UGT1A1, UGT1A3 and UGT1A6 enzymes that were much higher than 10 μM, and the fact that the remaining activity of UGT1A9 was >50% following Q3GA incubation, these data suggested that Q3GA displayed limited inhibitory effects on these UGT isoforms. Therefore, the subsequent enzymatic kinetics experiments were not performed, so the inhibition constant Ki of Q3GA on UGT1A enzymes could not be calculated. However, quercetin was more active in suppressing the activity of UGT isoforms compared with Q3GA. Its inhibitory effect on UGT1A1 and UGT1A6 was noncompetitive, with IC_{50} values of 7.47 and 7.07 μM, respectively, and Ki values of 2.18 and 28.87 μM, respectively. By contrast to these two isoforms, quercetin inhibited UGT1A3 and UGT1A9 competitively with IC_{50} values of 10.58 and 2.81 μM, respectively, and Ki values of 1.60 and 0.51 μM, respectively. These results indicated that quercetin was a moderate inhibitor of UGT1A1 and UGT1A3, and a strong inhibitor of UGT1A9, while Q3GA exhibited weak inhibitory effects on the activity of UGT1A1, UGT1A3, UGT1A6 and UGT1A9 isoforms. There was a small slope in Fig. 4C, which may be due to the fact that quercetin only exerted a weak inhibitory effect on UGT1A6. Furthermore, in the UGT1A3 incubation system, the inhibitory effect of 5 μM quercetin did not have a linear relationship with the inhibitory effect of other concentrations of quercetin, which may be the reason for the small slope in Fig. 3C.

Quercetin and Q3GA exhibited similar inhibitory effects on the protein and mRNA expression levels of UGT1A1 in the small intestine and the liver. This is consistent with our preliminary data and previously published studies (19,43). It has also been reported that quercetin inhibits glucuronidation of ethanol in human liver microsomes and in recombinant UGT1A1, UGT1A3, UGT 1A4, UGT1A6 and UGT1A9 enzymes (44). However, quercetin has been reported to induce UGT1A6 mRNA expression in human intestinal tissues *in vitro* (45). Notably, quercetin induces UGT1A expression in Caco-2 cells (46). It has also been reported that this flavonoid increases UGT enzyme activities in hepatic, and small and large intestinal tissues of male Wistar rats (47). A previous study has reported that the age-associated differences in the UGT-catalyzed glucuronidation of quercetin depend on the intestinal segment, particularly the proximal and distal segments (48). In brief, humans and animals express different protein and activity levels of UGT1A isoforms.

There are limitations to the present study, including the fact that quercetin or Q3GA were added separately to the UGT1A incubation systems evaluate their inhibitory effects. However, to the best of our knowledge, there are no effective methods to distinguish the role of the quercetin/Q3GA vs. quercetin plus Q3GA, as quercetin may be metabolized to Q3GA by UGT1A in the incubation (49-51), and Q3GA may be hydrolyzed to quercetin in the incubation in activated mouse macrophages (49). The results of the present study demonstrated that the inhibitory effects of quercetin and Q3GA on UGT1A enzymes were significantly different. Additionally, compared with quercetin, the inhibitory effect of Q3GA on UGT1A enzymes was very weak. Therefore, we hypothesized that Q3GA had a separate inhibitory effect on the UGT1A enzymes, rather than inhibiting the UGT1A enzymes through hydrolysis to quercetin.

A previous study (52) has reported that 3-hydroxyflavone has a higher catalytic rate than 7-hydroxyflavone, due to differences in the hydroxyl positions. Therefore, following conjugation of the C-3 hydroxyl group with the glucuronic acid to yield Q3GA, a decrease in the catalytic rate of Q3GA may be noted. In addition, Boersma *et al* (53) reported that quercetin inhibited UGT1A6 less efficiently than luteolin, which was possibly due to the lack of the C-3 hydroxyl group. This result suggested that flavonoids without the C-3 hydroxyl group exhibit a significant inhibitory effect on the UGT1A6-catalyzed reaction. This blocking effect is more pronounced for UGT1A6 when a larger group is conjugated with the C-3 hydroxyl group. In general, when Q3GA and quercetin

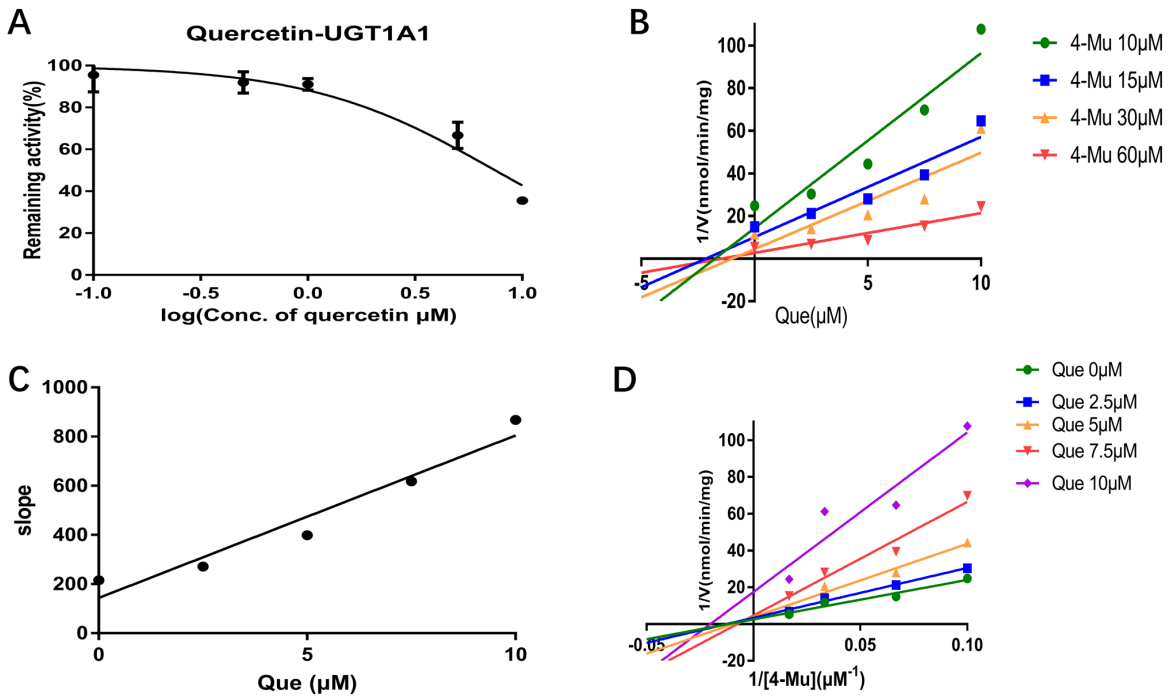

Figure 2. Determination of the inhibitory effect of quercetin on UGT1A1, and the kinetic parameters. (A) Dose-dependent inhibition of quercetin on recombinant UGT1A1-catalyzed 4-MU glucuronidation. (B) Dixon plot of the inhibition of quercetin towards recombinant UGT1A1-catalyzed 4-MU glucuronidation. (C) Second plot comparing the slopes from the Lineweaver-Burk plot with the concentrations of quercetin. (D) Lineweaver-Burk plot of the inhibition of quercetin towards recombinant UGT1A1-catalyzed 4-MU glucuronidation. UGT, UDP-glucuronosyltransferase; 4-MU, 4-methylumbelliferone; Que, quercetin.

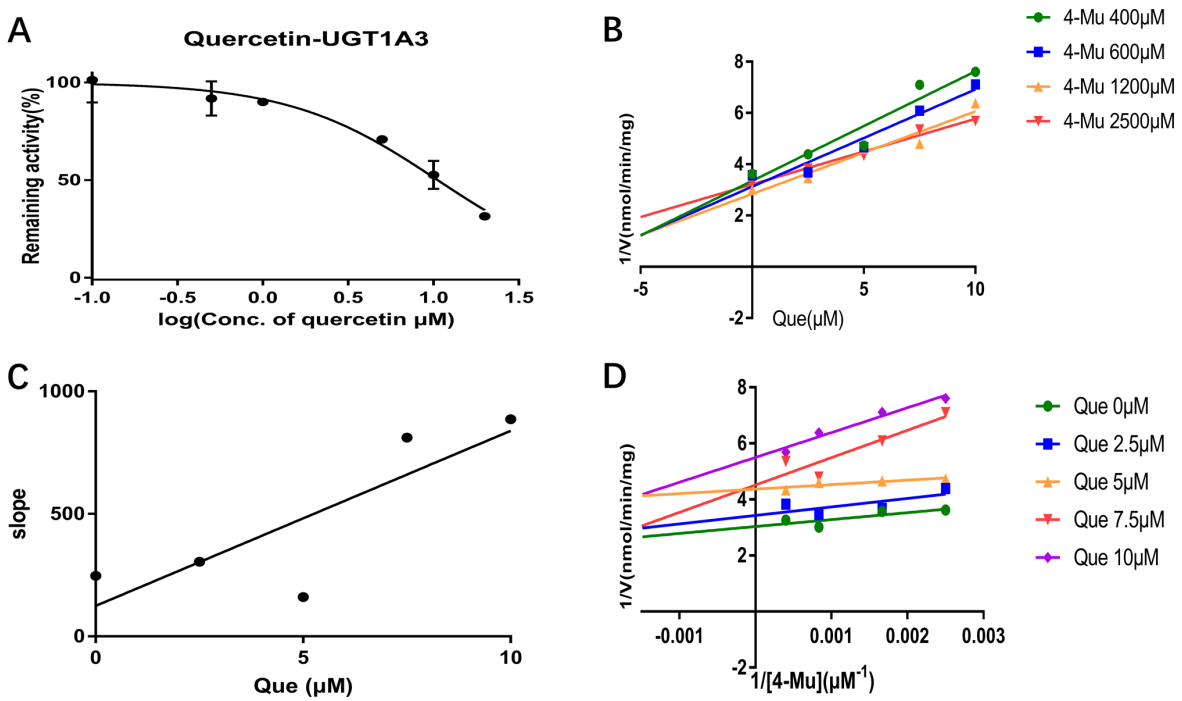

Figure 3. Determination of the inhibitory effect of quercetin on UGT1A3, and the kinetic parameters. (A) Dose-dependent inhibitory effects of quercetin on recombinant UGT1A3-catalyzed 4-MU glucuronidation. (B) Dixon plot of the inhibitory effect of quercetin on recombinant UGT1A3-catalyzed 4-MU glucuronidation. (C) Second plot comparing the slopes of the Lineweaver-Burk plot with the concentrations of quercetin. (D) Lineweaver-Burk plot of the inhibitory effect of quercetin on recombinant UGT1A3-catalyzed 4-MU glucuronidation. UGT, UDP-glucuronosyltransferase; 4-MU, 4-methylumbelliferone; Que, quercetin.

are combined with UGT1As, the steric hindrance effect of Q3GA greatly decreases the rate and extent of this catalytic reaction.

In conclusion, the results of the present study indicated that the inhibitory effect of quercetin on UGT1A1, UGT1A3, UGT1A6 and UGT1A9 was more potent than that of Q3GA. Furthermore, the results demonstrated that quercetin was a moderate inhibitor of UGT1A1 and UGT1A3, and a strong inhibitor of UGT1A9, and therefore HDIs may occur when

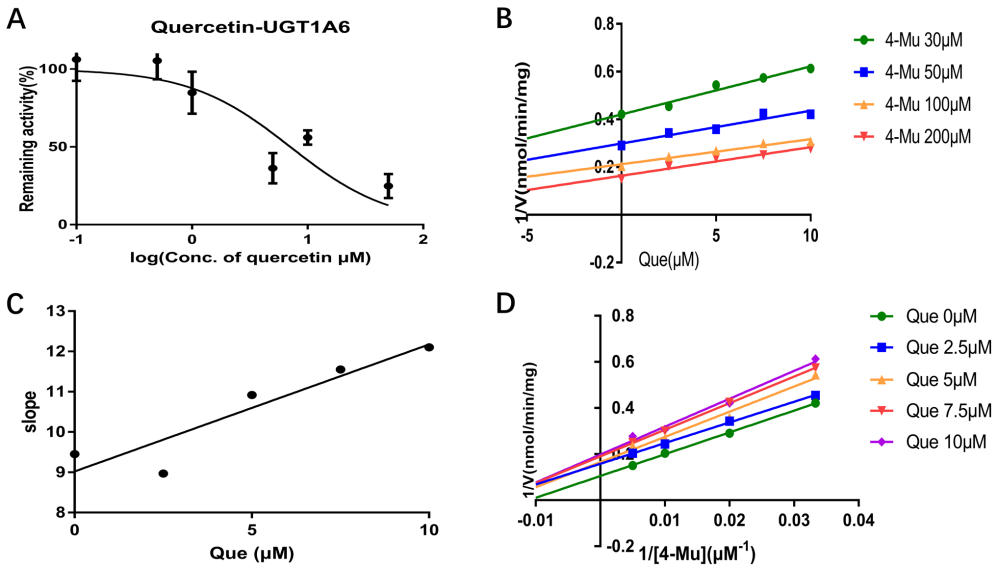

Figure 4. Determination of the inhibitory effect of quercetin on UGT1A6, and the kinetic parameters. (A) Dose-dependent inhibitory effect of quercetin on recombinant UGT1A6-catalyzed 4-MU glucuronidation. (B) Dixon plot of the inhibitory effect of quercetin on recombinant UGT1A6-catalyzed 4-MU glucuronidation. (C) Second plot comparing the slopes from the Lineweaver-Burk plot with the concentrations of quercetin. (D) Lineweaver-Burk plot of the inhibitory effect of quercetin on recombinant UGT1A6-catalyzed 4-MU glucuronidation. UGT, UDP-glucuronosyltransferase; 4-MU, 4-methylumbelliferone; Que, quercetin.

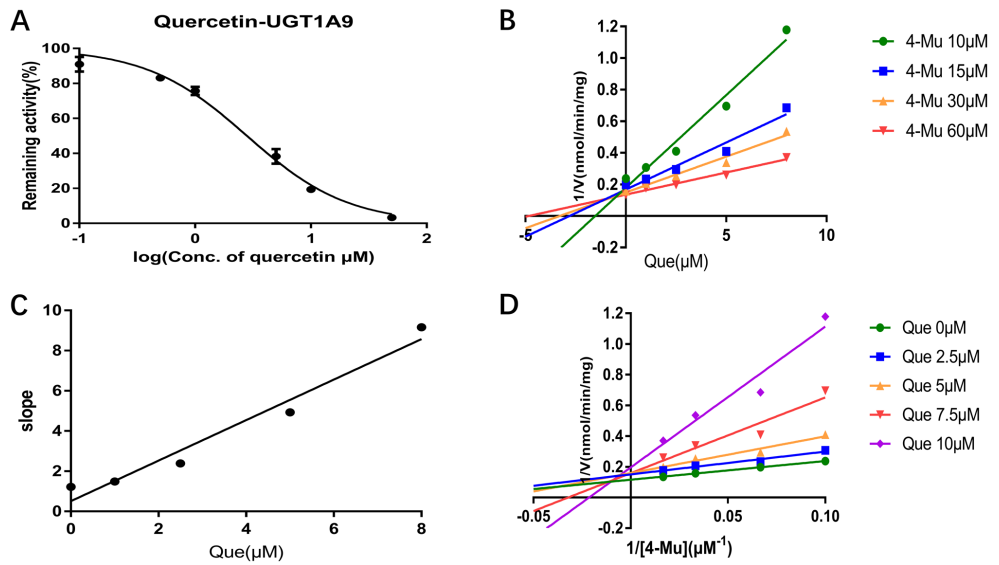

Figure 5. Determination of the inhibitory effect of quercetin on UGT1A9, and the kinetic parameters. (A) Dose-dependent inhibitory effects of quercetin on recombinant UGT1A9-catalyzed 4-MU glucuronidation. (B) Dixon plot of the inhibitory effect of quercetin on recombinant UGT1A9-catalyzed 4-MU glucuronidation. (C) Second plot comparing the slopes from the Lineweaver-Burk plot with the concentrations of quercetin. (D) Lineweaver-Burk plot of the inhibitory effect of quercetin on recombinant UGT1A9-catalyzed 4-MU glucuronidation. UGT, UDP-glucuronosyltransferase; 4-MU, 4-methylumbelliferone; Que, quercetin.

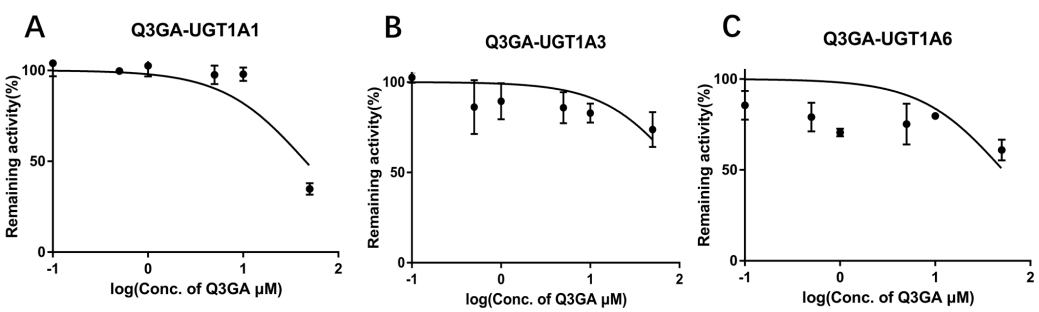

Figure 6. Determination of IC_{50} values of (A) UGT1A1, (B) UGT1A3 and (C) UGT1A6 enzymatic activity.

quercetin is co-administered with drugs that are mainly metabolized by UGT1A1, UGT1A3 and UGT1A9.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

RZ, YL and SS designed all the experiments. YW and TY performed the incubation experiments. Statistical analysis was performed by XH, JZ, JNZ and CY. YL and SS confirm the authenticity of all the raw data. YW, RZ, YL and SS wrote the manuscript. All authors read and approved the final manuscript.RZ, YL and SS designed all the experiments. YW and TY performed the incubation experiments. Statistical analysis was performed by XH, JZ, JNZ and CY. YL and SS confirm the authenticity of all the raw data. YW, RZ, YL and SS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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