Assessment of the inhibition potential of Licochalcone A against human UDP-glucuronosyltransferases

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Abstract

Licochalcone A (LCA) is a major bioactive compound in Licorice, a widely used herbal medicine. In this study, the inhibitory effects of LCA against human UDP-glucuronosyltransferases (UGTs) and LCA associated herb-drug interactions were systematically investigated. Our results demonstrated that LCA displayed broad-spectrum inhibition against human UGTs. LCA exhibited strong inhibitory effects against UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9, and 2B7 (both IC_{50} and K_i values lower than 5 μ M), while showing moderate inhibitory effects against UGT1A8, 1A10, 2B4, 2B15, and 2B17. The inhibitory effects of LCA against two major UGTs, including UGT1A1 and 1A9, were further investigated in human liver microsomes (HLMs), where the potential risks of LCA *via* inhibition of UGT1A1 and 1A9 were predicted by combining the *in vitro* inhibitory data and physiological data. The results from this study also showed that several LCA-containing products were able to increase the area under the curve (AUC) of the substrates that were predominantly metabolized by UGT1A1 or 1A9. These findings together demonstrate that LCA has a potent and broad-spectrum inhibitory effect against most human UGTs and thus suggest that much caution should be exercised when high-dose LCA is co-administered with UGT substrates.

Keywords: Licochalcone A (LCA), UDP-glucuronosyltransferases (UGT), Broad-spectrum inhibitor, Herb-drug interactions (HDIs)

1. Introduction

Licorice is roots of *Glycyrrhiza inflata* (also named Gan-Cao in China), which has been widely used in herbal medicine and as food traditionally in Asia. Licorice is one of the major ingredients used for preparing several famous Traditional Chinese Medicine (TCM) products, such as as *Mistura Glycyrrhizae Composita*, *HuoxiangZhengqi Tincture*, and *Naolejing Oral Liquid* (*Pharmacopoeia of the People`s Republic of China*, 2005). Licochalcone A (**Fig. 1**) is a major bioactive flavonoid isolated from the roots of licorice (Hatano et al., 1988). In the past thirty years, a wide variety of pharmacological studies have shown that LCA possesses many potential biological activities, including anti-parasitic (Ziegler et al., 2004), anti-inflammatory (Kolbe et al., 2006), anti-oxidative (Haraguchi et al., 1998), anti-bacterial (Friis-Møller et al., 2002), and anti-tumor activities (Yo et al., 2009; Xiao et al., 2011; Park et al., 2015). In China, licorice is listed in *Chinese Pharmacopoeia* and the *List of Herbal Materials that Can Be Used for Health Foods* under the *Law on Food Hygiene* by State Food and Drug Administration, and its daily dosage can be as high as up to 60 g/day (Liu et al., 2014). As the most abundant constituent in commercially available licorice in China, the concentration of LCA ranges in 4-10 mg/g of dried licorice (0.4-1% w/w), which translates into upwards of 240-600 mg of LCA as the daily dose (Zhang and Ye, 2009; Shibata, 2000). In such cases of high daily intake of licorice, much attention should be paid to monitor the potential herb-drug interactions (HDIs) since LCA-containing herbal preparations are commonly used with many therapeutic drugs in China.

In recent years, many studies have demonstrated that crude extracts of licorice and LCA can inhibit the activities of human cytochrome P450 enzymes (CYPs) and P-glycoprotein

transporters (P-gps) (Choi et al., 2014; Qiao et al., 2014; Choi et al., 2013). More recently, we have also found that LCA exhibits broad-spectrum inhibitory effects toward human CYP enzymes including CYP1A2, 2C8, 2C9, 2C19, and 3A4 (He et al., 2015). These studies suggest that LCA can reduce the elimination of many therapeutic drugs that are substrates of CYPs and P-gp, which may cause subsequent HDIs. It is also possible that HDIs can result from herbal-associated inhibition against major phase II metabolizing enzymes (Na et al., 2011; Choi et al., 2011; Li et al., 2012; Zheng et al., 2014; Wang et al., 2015). Unlike the extensive investigations on LCA interactions with CYPs, there is currently no reported evidence on the inhibitory effects of LCA and other major constituents of licorice against common phase II metabolizing enzymes. Thus, it is important to evaluate the potential inhibitory effects of LCA or other major constituents isolate from licorice against human phase II metabolizing enzymes, such as UDP-glucuronosyltransferases (UGTs).

UDP-glucuronosyltransferases are superfamily of membrane-bound enzymes that are responsible for glucuronidation of many endogenous molecules (*e.g.*, bilirubin, steroid hormones, thyroid hormones, bile acids, and fat-soluble vitamins) and exogenous compounds (*e.g.*, drugs, chemical carcinogens, and environmental toxins) (Ritter, 2000). The formation of glucuronides is one of the most important detoxification pathways among vertebrates (Jancova et al., 2010). Moreover, a large number of different UGTs that exhibit a wide range of substrates are distributed throughout human body, which makes them a particularly important group of phase II metabolizing enzymes (Kiang et al., 2005). To date, at least twenty-two human UGTs have been identified, and they are classified into three subfamilies, namely UGT1A, UGT2A, and UGT2B, based on their amino acid sequence (Rowland et al.,

2013). Most UGTs are expressed in the liver, but some isoforms, such as UGT1A7, UGT1A8, and UGT1A10, are expressed predominantly in the gastrointestinal tract (Gregory et al., 2004). Human intestinal UGTs are considered as important determinants in intestinal metabolism, especially for those oral phenolic compounds, such as flavonoids and coumarins (Zhang et al., 2007; Wu et al., 2011; Xia et al., 2015). Therefore, the inhibition against human UGTs in the gastrointestinal tract may make a great impact on the oral bioavailability of many phenolic drugs or natural products.

This study aimed to investigate the inhibitory effects of LCA against human UGTs. To this end, twelve commercially available human UGTs were used to screen the inhibitory potential of LCA *in vitro*, while trifluoperazine (TFP) N-glucuronidation (for UGT1A4) and 4-methylumbelliferone (4-MU) O-glucuronidation (for all other UGT isoforms) were used as probe reactions. The dose-dependent inhibition curve and the half maximal inhibitory concentration (IC_{50}) of LCA against each UGT isoform were determined firstly. For those UGT isoforms which could be potently inhibited by LCA ($IC_{50} < 10 \mu M$), the inhibition mechanism and the inhibition constant (K_i) values of LCA against human UGTs were carefully characterized. Moreover, the inhibitory effects of LCA against UGT1A1 and UGT1A9 were further investigated in human liver microsomes considering their key roles in glucuronidation of both endogenous compounds and therapeutic drugs, as well as the potent inhibitory effects of LCA against these two enzymes. Finally, the changes in the AUCs of UGT1A1 and UGT1A9 substrates following co-administration of LCA-containing herbal products were predicted based on a series of available data on literature.

2. Materials and Methods

2.1 Chemicals and reagents

Licochalcone A (>98%) was purchased from Sichuan Victory Biotechnology Co., Ltd (Sichuan, China). 4-Methylumbelliferone (4-MU), 4-methylumbelliferone glucuronide (4-MUG), trifluoperazine, propofol, propofol O-glucuronide and uridine-5-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NCHN (N-3-carboxypropyl-4-hydroxy-1,8-naphthalimide) and NCHN O-glucuronide were synthesized by the authors, the structures of NCHN and O-glucuronide were fully characterized as described previously (Lv et al., 2015). The purities of NCHN and O-glucuronide were higher than 98% by LC-UV. Pooled human liver microsomes from twenty-five donors were purchased from Research Institute for Liver Diseases (Shanghai, China). Twelve recombinant human UGT SupersomesTM (UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, A10, 2B4, 2B7, 2B15, and 2B17) expressed in baculovirus-infected insect cells were purchased from BD Gentest (Woburn, MA, USA) and stored at -80 °C until use. Tris-HCl buffer (pH 7.4) was prepared by using Millipore filtered water (Millipore, Bedford, USA) and stored at 4 °C until use. All other reagents were of the highest purity commercially available or HPLC grade.

2.2 Assessment of inhibitory effects of LCA against human UGTs

Unlike CYP inhibition assays, in which many specific probe substrates can be used and human liver microsomes are generally utilized as enzyme source, only a limited number of specific substrates can be employed in the evaluation of UGT inhibition (Liu et al., 2012). Therefore, recombinant UGT isoforms are commonly used as enzyme source when

evaluating the inhibitory potential of LCA against UGTs. 4-Methylumbelliferone (4-MU), a non-selective substrate for UGTs, is typically used as a probe substrate for all UGTs isoforms except for UGT1A4 (Uchaipichat et al., 2004).

In this study, 4-MU was incubated with recombinant UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 in both absence and presence of varying LCA concentrations (1 μ M, 10 μ M, and 100 μ M). The positive controls used were nilotinib (0–20 μ M) for UGT1A1, magnolol (0–10 μ M) for UGT1A9, and androsterone (1–100 μ M) for UGT2B7 (Ai et al., 2014; Zhu et al., 2012; Uchaipichat et al., 2006). Each positive control compound was dissolved in dimethyl sulfoxide (DMSO) and its final concentration was 1% (v/v). Corresponding to the reported values of K_m or S_{50} for each UGT isoform, the concentrations of 4-MU were as follows: 110 μ M for UGT1A1, 1200 μ M for UGT1A3, 110 μ M for UGT1A6, 30 μ M for UGT1A7, 750 μ M for UGT1A8, 30 μ M for UGT1A9, 30 μ M for UGT1A10, 1000 μ M for UGT2B4, 350 μ M for UGT2B7, 250 μ M for UGT2B15, and 2000 μ M for UGT2B17 (Uchaipichat et al., 2004). The incubation time and protein concentrations were carried out according to the previous studies with slight modifications (Zhu et al., 2012; Dong et al., 2012). In brief, a typical incubation mixture (a total volume of 200 μ L) contained recombinant UGT isoforms (final concentration: 0.125 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.05 mg/ml, 0.25 mg/ml, 0.05 mg/ml, 0.2 mg/ml, and 0.5 mg/ml for UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, respectively), 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, and 4 mM UDPGA. After pre-incubation at 37°C for 5 min, the reaction was initiated by the addition of UDPGA. The incubation time was 60 min for UGT1A1, 1A3, 1A10, 2B4, 2B7, 2B15, and 2B17, and

30 min for 1A6, 1A7, 1A8, and 1A9. The reactions were terminated by adding 200 μ L ice-cold acetonitrile (equal volume to incubation mixture) followed by centrifugation at 20,000 $\times$ g for 10 min by using Allegra 64R centrifuge (Beckman, USA). The aliquots of supernatant were then taken for ultra-fast liquid chromatography-ultraviolet spectrometry (UFLC-UV) analysis.

Trifluoperazine (TFP) is a specific probe substrate for UGT1A4 that is used in this study. TFP (50 μ M, near its K_m value) was incubated with recombinant UGT1A4 (0.1 mg/ml) at 37°C for 30 min in both absence and presence of varying LCA concentrations (1 μ M, 10 μ M, and 100 μ M) (Guo et al., 2013; Song et al., 2015). Then the samples were treated and determined as described above.

2.3 Kinetic analyses for LCA-associated inhibition on recombinant human UGTs

Half maximal inhibitory concentration (IC_{50}) was determined using various concentrations of LCA. IC_{50} values were calculated by non-linear regression analysis using Graphpad Prism 6.0 (San Diego, CA, USA). Inhibition kinetic parameters (K_i) were determined by using various concentrations of 4-MU/TFP in the presence or absence of varying concentrations of LCA. The reversible inhibitory type can be categorized into competitive inhibition, non-competitive inhibition, or mixed inhibition (Lu et al., 2013). The corresponding reaction rates were determined by utilizing various concentrations of 4-MU/TFP and LCA. The inhibition kinetic type was evaluated through determining the intersection point in the Dixon and Lineweaver-Burk plots. The second plot of slopes from Lineweaver-Burk plot versus LCA concentrations was utilized to calculate the corresponding inhibition parameter (K_i) value.

2.4 Kinetic analyses for LCA against NCHN O-glucuronidation in both HLM and UGT1A1

N-(3-carboxy propyl)-4-hydroxy-1,8-naphthalimide (NCHN) was a newly developed fluorescent probe for human UGT1A1 (Lv et al., 2015). In this study, NCHN is used as a selective probe substrate for UGT1A1 to determine the inhibitory effects of LCA against NCHN O-glucuronidation in both HLM and UGT1A1 (Wang et al., 2015). The IC_{50} values were determined in two different enzyme sources by using various LCA concentrations, while K_i values were determined by using various concentrations of NCHN in the presence or absence of different concentrations of LCA. In brief, NCHN (40 μ M, final concentration) was incubated with HLM (0.2 mg/ml) at 37°C for 30 min, or incubated with UGT1A1 (0.06 mg/ml) at 37°C for 90 min, in the presence of different LCA concentrations (0-40 μ M). The reactions were terminated and treated as described above, and the aliquots of supernatant were taken for liquid chromatography-fluorescence detector analysis.

2.5 Kinetic analyses for LCA against propofol O-glucuronidation in both HLM and UGT1A9

Propofol O-glucuronidation has been widely used as a probe reaction to phenotype UGT1A9 activity in human liver (Zhu et al., 2012; Miners et al., 2011). To explore whether LCA displayed substrate-dependent inhibition behavior against UGT1A9, as well as to determine the inhibitory effects of LCA against UGT1A9 in HLM, propofol was used as a selective probe substrate for UGT1A9 in this study (Rowland et al., 2008). Briefly, to get the IC_{50} and the K_i values of LCA against propofol O-glucuronidation, propofol was incubated with HLM or UGT1A9 in presence of varying LCA concentrations (0-20 μ M), while all incubations were performed at 37°C for 20 min, with the final protein concentrations were 0.05 mg/ml for HLM and UGT1A9. The reactions were quenched by adding 200 μ L

acetonitrile with 4-MUG (1.5 μM) as internal standard. The samples were treated as described above, and the aliquots of supernatant were taken for LC-MS/MS analysis.

2.6. Prediction of the DDI potential in vivo

The magnitudes of inhibitory interactions of LCA against UGT1A1 and UGT1A9 were estimated as the ratio of the area under the plasma concentration-time curve (AUC) in the presence and absence of the inhibitor. The ratio was predicted using the following equation (Kirby and Unadkat., 2010).

$$\text{AUC ratio} = \frac{1}{f_{\text{hep}} \cdot \left(\frac{1/E_h}{(1/E_h - 1) \cdot (1 + I/K_i) + 1} \right) + (1 - f_{\text{hep}})} \quad (1)$$

Where f_{hep} is the percentage of hepatic clearance mediated by UGT1A1 or UGT1A9; E_h is the hepatic extraction ratio; I is the maximum concentration of inhibitor in human plasma, and K_i (μM) is the inhibitory constant.

2.7 Instruments and analytical conditions

The LC system (Shimadzu, Kyoto, Japan) contained a SCL-30A system controller, two LC-30AD pumps, a SIL-30AC auto sampler, a DGU-20AC vacuum degasser, a CTO-30AC column oven, a SPD-20AVP UV detector and a RF-20A fluorescence detector. An ODS (5 μm , 150.0 mm $\times$ 2.1 mm, Acchrom Technology Corp., Beijing, China) analytical column was used and the column temperature was kept at 40 $^{\circ}\text{C}$. Acetonitrile (A) and Millipore filtered water with 0.2% formic acid (B) were used as the mobile phase at a flow rate of 0.4 ml/min. For samples containing 4-MU and its glucuronide, the following gradient condition was used: 0–2.0 min, 90% B; 2.01–9.0 min 90% B–25% B; 9.01–10.0 min 25% B–5% B; 10.01–13.0 min, 5% B; 13.01–16.0 min, balance to 90% B. The detection wavelength was set at 317 nm.

For the detection of TFP and its glucuronide, the following gradient condition was used: 0–9.0 min, 70%–50% B; 9.01–10.0 min 25%–5% B; 10.01–13.0 min 5% B; 13.01–16.0 min, balance to 70% B. The detection wavelength was set at 254 nm. A Shim-pack XR-ODS (2.2 μ , 75 mm $\times$ 2.0 mm I.D., Shimadzu) was used to analyze NCHN and its glucuronide. Acetonitrile (A) and Millipore filtered water with 0.2% formic acid (B) were used as the mobile phase at a flow rate of 0.4 ml/min. The following gradient condition was used: 0–4.0 min, 90%-25% B; 4.01–6.0 min 5% B; 6.01–8.0 min, balance to 90% B. NCHN-O-glucuronide was quantified by liquid chromatography with fluorescence detection with excitation wavelength of 364 nm and emission wavelength of 450 nm (Lv et al., 2015).

A LC-MS/MS based method was used to quantify propofol O-glucuronide under the following conditions. Chromatographic separation was performed on an ACCHROM C18HC column (5 μ , 150 mm $\times$ 2.1 mm, Acchrom Technology Corp., Beijing, China) using an ACQUITY™ UPLC system equipped with a binary pump, a vacuum degasser and an auto-sampler (Waters Corp., Milford, MA, USA). Methanol (A) and Millipore water containing 0.1% ammonium hydroxide (B) were used as the mobile phase at a flow rate of 0.2 ml/min. The following gradient condition was used: 0–2.0 min, 100% B; 2.0–6.0 min, 100%-10% B, 6.0 -9.0 min, balance to 100% B. The mass spectrometry detection was performed on an AB Sciex QTrap® 5500 system with a Turbo VTM source (Framingham, MA, USA). Ionization was operated using an electrospray ionization (ESI) source in the negative ion mode. Optimized instrument settings specific for propofol O-glucuronide and IS (4-methyklumbelliferone-D-glucuronide) were as follows: Curtain Gas was 25 psi, Ion Source Gas 1 was 40 psi, Ion Source Gas 2 was 50 psi, IonSpray Voltage was -4500 V, Turbo

Heater Temperature was 600 °C. Quantification and confirmation transitions and their optimized values for Entrance Potential (EP), Collision Cell Exit Potential (CXP) were -10 V and -13 V, respectively. Declustering Potential (DP) for propofol O-glucuronide and IS were -70 psi and -80 psi, respectively. Quantification was performed in multiple reaction monitoring (MRM) mode at m/z 353.2→177.2 for propofol O-glucuronide, and m/z 351.2→175.0 for IS, respectively. The optimized collision energy (CE) was -35 eV for propofol O-glucuronide, and -25 eV for IS, respectively. All data acquired were processed using Analyst Ver. 1.5 software (Applied Biosystems, Toronto, Canada).

The calibration curves and linear ranges for quantification of 4-MU and 4-MUG, TFP and its glucuronide, NCHN and its glucuronide, as well as propofol and its glucuronide were constructed by using serial dilutions of the standard solution with appropriate concentration. The calibration curve for each analyte was linear over the tested concentration ranges (from 0.1 to 10 µM) with good linear regression ($R^2 \geq 0.9998$). The intra-day and inter-day variations in peak area were also evaluated with acceptable relative standard deviation (RSD) values (<3.8%). It is also found that all analytes in the sample solutions are stable within 24 h with acceptable RSD values (<5.0 %).

3. Results

3.1 Inhibition screening of LCA on the activities of recombinant human UGTs

To investigate the inhibitory effects of LCA against the catalytic activities of human UGTs, trifluoperazine (TFP) N-glucuronidation (for UGT1A4) and 4-methylumbelliferone (4-MU) O-glucuronidation (for all other UGT isoforms) were used to measure the activities

of twelve human UGTs with or without LCA. As seen in **Fig. 2**, upon addition of LCA (10 μ M, final concentration), the catalytic activities of most human UGTs were strongly inhibited. Specifically, the residual activities of UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 were 16.7%, 31.8%, 12.3%, 0%, 75.6%, 3.2%, 54.1%, 61.9%, 28.2%, 29.1%, and 67.8%, respectively, of their null controls. At the same dosage of LCA, the residual activity of UGT1A4-mediated TFP N-glucuronidation was 29.1%. Notably, the catalytic activities of all tested human UGTs could be inhibited by LCA near completely at high concentration. As shown in **Fig. 2** upon addition of LCA (100 μ M, final concentration), the residual activities of UGT1A3 and UGT2B17 were 4.5%, and 6.6%, respectively, while the activities of the other ten human UGTs were totally inhibited.

To further characterize the inhibitory effects of LCA against UGTs activities, dose-dependent inhibition curves were plotted using different LCA concentrations. It can be seen from **Fig.3** and **Fig.4** that LCA inhibited all the tested UGTs in a concentration-dependent manner. The calculated IC_{50} values are 0.97 μ M, 4.19 μ M, 4.26 μ M, 2.52 μ M, 0.09 μ M, 17.64 μ M, 0.37 μ M, 15.65 μ M, 24.20 μ M, 6.37 μ M, 14.15 μ M, and 32.05 μ M for UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, respectively (**Table 1**). For comparison, three well-known UGT inhibitors were used as positive controls for inhibition assays. Nilotinib, magnolol, and androsterone are commonly used inhibitors against UGT1A1, UGT1A9, and UGT2B7, respectively (Ai et al., 2014; Zhu et al., 2012; Uchaipichat et al., 2006). The IC_{50} value of nilotinib against UGT1A1 was determined as 0.10 μ M, while magnolol showed a strong inhibitory effect against UGT1A9 with the IC_{50} value of 0.08 μ M, and androsterone inhibited UGT2B7 activity with the IC_{50}

value of 12.58 μM . It is evident from these results that LCA is found to be a strong inhibitor against UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9, and 2B7 ($\text{IC}_{50} < 10 \mu\text{M}$) while serving as a moderate inhibitor against UGT1A8, 1A10, 2B4, 2B15, and 2B17 (IC_{50} range from 14 to 32 μM). These findings prompted us to further investigate the inhibition kinetic types and the corresponding inhibition parameters of LCA, specifically for UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9, and 2B7.

3.2 Kinetic analysis for inhibition of LCA on recombinant UGTs

To assess the possible mechanisms of LCA inhibition against human UGT isoforms, inhibition kinetic analysis were carried out. As shown by **Fig.3**, both Lineweaver-Burk and Dixon plots demonstrates that LCA is a competitive inhibitor for UGT1A1-, UGT1A7-, and UGT2B7-mediated 4-MU O-glucuronidation. Second kinetic plots deduced from the Lineweaver-Burk plot slopes were then used to determine the corresponding inhibition parameters. Here, the K_i values were determined as 0.78 μM , 0.04 μM , and 2.12 μM for UGT1A1, UGT1A7, and UGT2B7, respectively.

Likewise, **Fig.4** shows the noncompetitive inhibition mechanism of LCA against UGT1A3-, UGT1A6-, and UGT1A9-mediated 4-MU O-glucuronidation and against UGT1A4-mediated TFP N-glucuronidation. The K_i values of LCA against these four enzymes in **Fig.4** were determined as 3.17 μM , 2.32 μM , 2.60 μM , and 0.43 μM for UGT1A3, UGT1A4, UGT1A6, and UGT1A9, respectively. The inhibition types and K_i values for these UGTs are listed in **Table 1**. The inhibition types for UGT1A8, UGT1A10, UGT2B4, UGT2B15, and UGT2B17 were not determined because inhibitory activities of LCA against these UGT isoforms were relatively low. These findings suggest that LCA is a strong and

non-selective inhibitor of most members from the UGT1A subfamily and of UGT2B7 from the UGT2B subfamily.

3.3 Kinetic analysis for LCA against NCHN O-glucuronidation in both HLM and UGT1A1

To explore whether the inhibitory effect of LCA against UGT1A1 is substrate-dependent or enzyme source-dependent, a highly specific UGT1A1 probe substrate, NCHN was used to investigate the inhibitory effect of LCA in both recombinant UGT1A1 and HLM. As shown in **Fig.5** and **Table 2**, LCA displayed potent inhibition against NCHN O-glucuronidation in both UGT1A1 and HLM. The IC_{50} values of LCA against NCHN O-glucuronidation in both UGT1A1 and HLM were 0.13 μ M and 0.84 μ M, respectively. The K_i values for LCA against NCHN O-glucuronidation in both HLM and UGT1A1 were determined as 0.54 μ M and 0.23 μ M, respectively. These results together indicated that LCA could potentially inhibit UGT1A1 even in complex biological samples such as liver preparations.

3.4 Kinetic analysis for LCA against propofol O-glucuronidation in both HLM and UGT1A9

The inhibitory effect of LCA against UGT1A9 was also further investigated in both recombinant human UGT1A9 and HLM by incorporating propofol O-glucuronidation as the probe reaction. As shown in **Fig.6** and **Table 3**, LCA displayed potent inhibition against propofol O-glucuronidation in both UGT1A9 and HLM, with its IC_{50} values of 0.56 μ M and 0.26 μ M, respectively. Dixon and Lineweaver-Burk plots revealed that the inhibition type of LCA against UGT1A9 in both recombinant human UGT1A9 and HLM was best fitted as noncompetitive inhibition. The K_i values for LCA against propofol O-glucuronidation in both HLM and UGT1A9 were also calculated to be 0.61 μ M and 0.37 μ M, respectively (**Table 3**). These findings demonstrate that LCA is a potent inhibitor against UGT1A9 in both

recombinant UGT1A9 enzyme and human liver preparations.

3.5. Quantitative prediction of DDI Risks (AUC ratios)

The magnitudes of this inhibitory interactions of LCA with UGT1A1 and 1A9 were also evaluated by estimating the changes in the AUCs of the substrates that are predominantly metabolized by UGT1A1 or 1A9 after co-administration of two LCA-containing marketed TCM products, HuoxiangZhengqi Tincture and Naolejing Oral Liquid. The AUC ratios were calculated based on several available data, including the hepatic extraction ratio (E_h), the percentage of hepatic clearance mediated by target UGTs relative to the total clearance of the substrates(f_{hep}), the K_i values for LCA against UGT enzymes and the predicted maximum concentration of LCA in human plasma (C_{max}). As shown in **Table 4**, LCA increased the AUCs of the UGT1A1 substrates by 71-341% when they were co-administrated with LCA-containing herbal products. Likewise, LCA also increased the AUCs of the UGT1A9 substrates by 64-334% when they were co-administrated with LCA-containing herbal products.

4. Discussion

As an important class of drug-metabolizing enzymes, UGTs participate in metabolic elimination of many endogenous compounds, such as bilirubin and steroids, and account for more than 35% of phase II drug metabolism (King et al., 2000). However, there is no published report to date regarding the inhibitory effects of LCA against human UGTs. In our study, the inhibitory effects of LCA against human UGTs were investigated for the first time, and the results demonstrate that LCA is a potent and broad-spectrum inhibitor of human

UGTs. Notably, LCA displayed potent inhibitory effects against several UGTs including UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9 and 2B7, with low K_i values ranging from 0.04 to 3.17 μM . Although many non-selective UGT inhibitors have been reported (Fang et al., 2013; Jiang et al., 2013; Choi et al., 2014), none of them can inhibit all human UGTs as LCA is able to. It should be noted that LCA (100 μM , final concentration) can inhibit the catalytic activities of all tested human UGTs near completely (**Fig.2**). To the best of our knowledge, LCA is the first reported broad-spectrum inhibitor of human UGTs, and it can be used as a broad-spectrum inhibitor of human UGTs in UGT-related drug metabolism or toxicological studies.

In clinical settings, adverse effects caused by UGT inhibitions have been widely reported in literature. For instance, a strong inhibitory effect of UGT1A1 activity can lead to elevated levels of unconjugated bilirubin in serum following administration of HIV drug, indinavir, and as well as the anticancer agent, sorafenib (Zucker et al., 2001; Meza-Junco et al., 2009). In China, several TCM preparations containing licorice have also been used as adjuvant therapy for the treatment of cancer (Yang et al., 2015). It is also well-established that licorice can be used to reduce mucositis including mouth ulcers (Aly et al., 2005; Agarwal et al., 2009). Moreover, it is well known that UGT1A1 and UGT1A9 are two major metabolizing enzymes responsible for the metabolic elimination of SN-38, the active metabolite of anti-cancer agent irinotecan, which has well-documented adverse events of gastrointestinal perforations (Nagar and Blanchard, 2006; Hecht, 1998). Thus, due to the potent inhibitory effects of LCA against UGT1A1 and UGT1A9 found in this study, the likelihood of the interactions between LCA-containing herbals or TCM preparations and irinotecan is very

high when they are administered concomitantly. Likewise, LCA has the potential to block the glucuronidation process in human gastrointestinal tract for many UGT substrates, and thus improve the oral bioavailability of many phenolic drugs or natural products. Particularly, for drugs with relatively high therapeutic index that are predominantly metabolized by UGTs, such as the anti-inflammatory agent, daphnetin (Liang et al., 2010), the cholagogue drug, armillarisin A (Sun et al., 2014), the anesthetic agent, propofol (Liang et al., 2011), and the antiretroviral agent, zidovudine (Sun et al., 2015), the co-administration of LCA may extend their half-life and subsequently increase their efficacy *in vivo* and also potentially reduce the treatment cost.

Unlike the cytochrome P450 enzymes, the UGT enzymes in mammals have significantly varying species differences in both expression and function, while the substrate and inhibitor spectra of the major UGT enzymes are much different from species to species (Higashi et al., 2014; Liang et al., 2011; Guo et al., 2015; Wen et al., 2007). In these cases, we predicted the potential risk of LCA-associated inhibition on human UGTs based on a series of available data (**Table 4**). In these predictions, the contents of licorice in two marketed TCM products, HuoxiangZhengqi Tincture and Naolejing Oral Liquid, which have been reported were used to calculate the AUCs (Pharmacopoeia of the People`s Republic of China, 2005; Shibata, 2000). The bioavailability of LCA in human has been predicted by a system pharmacology based method (Liu et al., 2013). Based on these data, the maximum plasma concentration of LCA in human was estimated up to 20.8-92.0 μM following administration of the two marketed TCM products. It is evident from this estimated *in vivo* concentration that the $[I]/K_i$ values are much greater than 1.0 for human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9, and 2B7,

where [I] is the inhibitor level at steady state with the highest clinical dose (Williams et al., 2004). Furthermore, the potential risks of UGT1A1 and 1A9 inhibitions were also predicted based on the inhibitory constant of LCA in HLM. The prediction results show that co-administration of LCA-containing TCM products has the potential to increase the AUCs of substrates that are predominantly metabolized by UGT1A1 or 1A9. It is easily conceivable then that administration of LCA-containing TCM products at high doses may affect the metabolic elimination of bilirubin and consequently lead to the elevated levels of unconjugated bilirubin in human serum as previously discussed. Therefore, for safety considerations, the high dose of LCA-containing herbal preparations should not be used concomitantly with the drugs which are primarily metabolized by the above mentioned seven UGT enzymes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A9, and 2B7), especially for those patients with liver injury or hyperbilirubinemia.

Additionally, other factors such as the polymorphism of UGTs might complicate these predictions for HDIs (Burchell et al., 2000). For examples, patients possessing UGT1A1*28 genotype or UGT2B7 mutations (UGT2B7*2, UGT2B7*5, and UGT2B7*71S), the expression and the glucuronidation activity of these enzymes will be relatively lower compared to those of their wild-type counterparts (Zhang et al., 2007; Bjornsson et al., 2003; Coffman et al., 1997). In these genetically polymorphic cases, the patients will have impaired glucuronidation capability for the drugs that are predominantly metabolized by UGT1A1 or UGT2B7. And thus, they are going to be more susceptible to adverse HDIs when LCA-containing herbal preparations are co-administered. This will be particularly true for those drugs with narrow therapeutic index that are substrates for the above mentioned

polymorphic UGT isoforms. Thus, the concomitant use of licorice is not advised in these cases.

It should also be noted that LCA has been identified as a potent inhibitor against human CYP and P-gp (He et al., 2015; Choi et al., 2014). As the most abundant CYP enzyme in the liver, CYP3A4 is the most important phase I metabolic enzyme that involves biotransformation of up to 50% of prescribed drugs and numerous xenobiotic (Dorne et al., 2003). P-glycoprotein is an important transporter molecule and is considered to play a key role in absorption, distribution, and elimination of many therapeutic drugs (Balayssac et al., 2005). In this study, our findings demonstrate that LCA is also a potent and broad-spectrum inhibitor of most human UGTs. Therefore, it is very likely that LCA changes the pharmacokinetic profiles of many therapeutic drugs *via* inhibiting one or more drug metabolizing enzymes, or *via* inhibiting P-gps, or *via* inhibiting both drug metabolizing enzymes and P-gps. As such, the strong inhibitory effects of LCA against multiple human metabolizing enzymes and transporters may exacerbate the risk of HDIs in patients following administration of LCA-containing herbal preparations.

In summary, our findings show that LCA is a potent and broad-spectrum inhibitor against human UGT activities. Specifically, LCA displays strong inhibitory effects against UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, and UGT2B7, while shows moderate inhibitory effects against UGT1A8, UGT1A10, UGT2B4, UGT2B15, and UGT2B17. Moreover, our prediction results for *in vivo* HDIs demonstrate that the interactions are much likely to occur between high dose of LCA and the drugs which are substrates for the above mentioned seven UGTs. The findings from this study are not only useful for guiding

reasonable applications of LCA and its related medical preparations, but also important in laying out the foundation for further *in vivo* investigations on the HDI risks caused by LCA-associated inhibition against human UGTs.

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Figure Legends

Figure 1. The chemical structure of LicochalconeA (LCA)

Figure 2. The inhibitory effects of LCA (1 μ M, 10 μ M, and 100 μ M) on the catalytic activities of human UGT isoforms. The data are presented as the means $\pm$ the S.D.s of triplicate determinations.

Figure 3. Inhibition curves and Lineweaver–Burk plots showing competitive inhibitions by LCA against UGT1A1 (A), UGT1A7 (B), and UGT2B7 (C). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing competitive inhibitions. All data represent the means of triplicate determinations.

Figure 4. Inhibition curves and Lineweaver–Burk plots showing noncompetitive inhibitions by LCA against UGT1A3 (A), UGT1A4 (B), UGT1A6 (C) and UGT1A9 (D). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.

Figure 5. Inhibition curves and Lineweaver–Burk plots of LCA against UGT1A1-mediated NCHN-O-glucuronidation in both HLM (A) and recombinant human UGT1A1 (B). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.

Figure 6. Inhibition curves and Lineweaver–Burk plots of LCA against UGT1A9-mediated propofol-O-glucuronidation in both HLM (A) and recombinant human UGT1A9 (B). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.

Table 1. Inhibition constants of LCA towards twelve human UGTs.

Enzyme	Substrate	IC ₅₀ (μM)	K _i (μM)	Type of inhibition	Goodness of Fit (R ²)
UGT1A1	4-MU	0.97 ± 0.08	0.78	Competitive	0.99
UGT1A3	4-MU	4.19 ± 0.35	3.17	Noncompetitive	0.99
UGT1A4	TFP	4.26 ± 0.32	2.32	Noncompetitive	0.99
UGT1A6	4-MU	2.52 ± 0.26	2.6	Noncompetitive	0.99
UGT1A7	4-MU	0.09 ± 0.02	0.04	Competitive	0.99
UGT1A8	4-MU	17.64 ± 2.92	--	--	--
UGT1A9	4-MU	0.37 ± 0.03	0.43	Noncompetitive	1.00
UGT1A10	4-MU	15.65 ± 1.79	--	--	--
UGT2B4	4-MU	24.20 ± 3.73	--	--	--
UGT2B7	4-MU	6.37 ± 0.84	2.12	Competitive	0.99
UGT2B15	4-MU	14.15 ± 2.39	--	--	--
UGT2B17	4-MU	32.05 ± 4.25	--	--	--

Table 2. Inhibition constants of LCA against NCHN-O-glucuronidation in both HLM and recombinant human UGT1A1.

Enzyme source	IC ₅₀ (μM)	K _i (μM)	Type of inhibition	Goodness of Fit (R ²)
HLM	0.84 ± 0.09	0.54	Noncompetitive	0.97
UGT1A1	0.13 ± 0.02	0.23	Noncompetitive	1.00

Table 3. Inhibition constants of LCA against propofol-O-glucuronidation in both HLM and recombinant human UGT1A9.

Enzyme source	IC ₅₀ (μM)	K _i (μM)	Type of inhibition	Goodness of Fit (R ²)
HLM	0.26 ± 0.03	0.61	Noncompetitive	0.99
UGT1A9	0.56 ± 0.07	0.37	Noncompetitive	0.96

Table 4. Prediction of the potential herb-drug interaction risks of LCA *in vivo* based on the AUC ratios.

Substrate	E_h ^a	f_{hep} ^b	K_i (μM)	I (μM) ^c	AUC ratios ^d	AUC increase (%)
UGT1A1 Substrate	0.4-0.9	0.8	0.54	5.82-25.8	1.71-4.41	71-341%
UGT1A9 Substrate			0.61		1.64-4.34	64-334%

a. E_h is the hepatic extraction ratio ranging from 0.4 to 0.9 for UGT substrates.

b. The f_{hep} was set to the mean value of each UGT substrates.

c. The maximum concentration of inhibitor (I) in human plasma was calculated as $f_u \times C_{\text{max}}$. The crude extract of Liquorice is a major material for producing several famous marketed TCM products (e.g., HuoxiangZhengqi Tincture and Naolejing Oral Liquid). The daily dose of these two marketed TCM products contained the crude extract from 20-35.4 g of Liquorice. LCA accounts for approximately 0.4-1% (w/w) of liquorice (Shibata, 2000). An estimated oral bioavailability of LCA in human has been reported as 45.75% (Liu et al., 2013). The average volume of human blood is approximately 5.2 L (Feldschuh and Enson, 1977). On the basis of the above data, the C_{max} of LCA in humans was estimated up to 20.8-92.0 μM. In addition, f_u is the unbound fraction of LCA in plasma, which was predicted as 0.28 by using the following equation (Austin et al., 2002).

$$f_u = \frac{1}{[P] \cdot 10^{0.56 \cdot \log(P/D) - 1.41} + 1}$$

Here $[P]$ is the mean value of human plasma protein concentration (70 mg/ml).

d. Prediction methods as described in the Materials and Methods.

Fig. 1. The chemical structure of Licochalcone A (LCA)

Fig. 2.The inhibitory effects of LCA (1 μ M, 10 μ M, and 100 μ M) on the catalytic activities of human UGT isoforms. The data are presented as mean $\pm$ S.D. of triplicate determinations.

Fig. 3. Inhibition curves and Lineweaver–Burk plots showing competitive inhibitions by LCA against UGT1A1 (A), UGT1A7 (B), and UGT2B7 (C). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing competitive inhibitions. All data represent the means of triplicate determinations.

Fig. 4. Inhibition curves and Lineweaver–Burk plots showing noncompetitive inhibitions by LCA against UGT1A3 (A), UGT1A4 (B), UGT1A6 (C) and UGT1A9 (D). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.

Fig. 5. Inhibition curves and Lineweaver–Burk plots of LCA against UGT1A1-mediated NCHN-O-glucuronidation in both HLM (A) and recombinant human UGT1A1 (B). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.

Fig.6. Inhibition curves and Lineweaver–Burk plots of LCA against UGT1A9-mediated propofol-O-glucuronidation in both HLM (A) and recombinant human UGT1A9 (B). Left: the dose-dependent inhibition curves; Right: the Lineweaver–Burk plots showing noncompetitive inhibitions. All data represent the means of triplicate determinations.