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In vitro and *in vivo* evaluation of cucurbitacin E on rat hepatic CYP2C11 expression and activity using LC-MS/MS

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This study explored the effects of cucurbitacin E (CuE), a bioactive compound from *Cucurbitaceae*, on the metabolism/pharmacokinetic of tolbutamide, a model CYP2C9/11 probe substrate, and hepatic CYP2C11 expression in rats. Liquid chromatography-(tandem) mass spectrometry (LC-MS/MS) assay was used to detect tolbutamide as well as 4-hydroxytolbutamide, and then successfully applied to the pharmacokinetic study of tolbutamide in rats. The effect of CuE on CYP2C11 expression was determined by western blot. CuE (1.25–100 μmol L^{-1}) competitively inhibited tolbutamide 4-hydroxylation (CYP2C11) activity only in concentration-dependent manner with a K_i value of 55.5 μmol L^{-1} *in vitro*. In whole animal studies, no significant difference in metabolism/pharmacokinetic of tolbutamide was found for the single pretreatment groups. In contrast, multiple pretreatments of CuE (200 μg kg^{-1} d^{-1}, 3 d, i.p.) significantly decreased tolbutamide clearance (CL) by 25% and prolonged plasma half-time ($T_{1/2}$) by 37%. Moreover, CuE treatment (50–200 μg kg^{-1} d^{-1}, i.p.) for 3 d did not affect CYP2C11 expression. These findings demonstrated that CuE competitively inhibited the metabolism of CYP2C11 substrates but had no effect on rat CYP2C11 expression. This study may provide a useful reference for the reasonable and safe use of herbal or natural products containing CuE to avoid unnecessary drug-drug interactions.

cucurbitacin E, CYP2C11, LC-MS/MS, pharmacokinetic, tolbutamide

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Cytochrome P450 (CYP) enzymes belong to a super family of haem-proteins that play an important role in catalyzing the oxidation of many endogenous and exogenous substances. CYP enzymes are primarily located in the liver, as well as widely distributed in other tissues, such as intestine, lung, kidney, and brain [1]. Drug-drug interactions (DDI) usually occur as CYP have broad substrate specificity and overlapping substrate selectivity [2]. Up to now, DDI have become more common as a result of polypharmacy and the increasing use of alternative medicine, including herbs and natural products [3]. In particular, CYP inhibition after exposure to different drugs and chemicals is directly linked to a number of drug-induced toxicity [2]. Therefore, much attention should be paid to assess the CYP inhibitory properties of drugs and chemicals especially in the early stage of the discovery.

The *Cucurbitaceae* family, mainly in tropical and subtropical regions, ranks among the highest of plant families for number and percentage of species used as human food [4]. Advances in separation techniques have enabled the isolation and chemical characterization of the active components of *Cucurbitaceae*, with extensive research currently undertaken to investigate the pharmacology of the individual components of the plant [4,5]. Cucurbitacin E (CuE, Figure 1), one of the major triterpenoid of *Cucurbi-*

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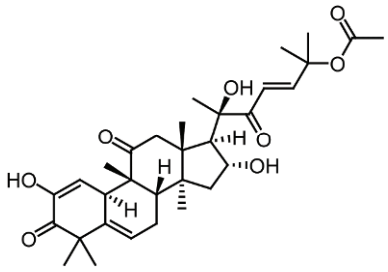

Figure 1 Chemical structure of cucurbitacin E.

taceae, has been reported to possess a number of pharmacological properties such as anti-inflammatory [6,7], anti-tumor [8–12], and immunomodulatory activities [13]. Previous studies in our laboratory have also shown that CuE inhibits tumor angiogenesis through VEGFR2-mediated Jak2-STAT3 signaling pathway [9], and inhibits breast tumor metastasis by suppressing cell migration and invasion [11]. In addition, some herbal products containing CuE such as cucurbitacin tablets/capsules have been widely used in China to treat liver diseases [14]. However, the interactions between CuE and CYP enzymes remain unclear.

Recently, we reported CuE-warfarin interactions involved possible underlying pharmacodynamic and pharmacokinetic mechanisms [15]. CuE not only exaggerated the pharmacological effects of warfarin by prolonging the prothrombin time, an indicator of anticoagulation action, but also decreased the elimination of warfarin in rats [15]. Warfarin is metabolized through different CYP metabolic pathways, such as via CYP1A2 to form 6-hydroxy- and 8-hydroxy-warfarin, CYP2C9 to form 6-hydroxy- and 7-hydroxy-warfarin, and CYP3A4 to 4-hydroxy- and 10-hydroxy-warfarin [16–18]. Hence warfarin is not an ideal model substrate to investigate the potential inhibition of CuE for the individual CYP isoforms. In this study, the effects of CuE on CYP2C11 activity were investigated *in vitro* and *in vivo* in the rat, using the probe substrate tolbutamide and LC-MS/MS method. Moreover, the western blot analysis was also used to determine the effect of CuE on hepatic CYP2C11 expression.

1 Materials and methods

1.1 Chemicals and reagents

Tolbutamide, chlorpropamide, sulfaphenazole, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), β-nicotinamide adenine dinucleotide phosphate (NADP), and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Chemical Co. (USA). 4-hydroxytolbutamide was obtained from Toronto Research Chemical (CA). Cucurbitacin E (purity>98%) was purchased from Shanghai Zhanshu Chemical Technology Co. (Shanghai). Fluconazole was supplied by Energy Chemical (Shanghai). Carbamazepine was obtained from J&K Scientific Co. (Beijing). Acetonitrile (ACN) and methanol (all high performance liquid chromatography (HPLC) grade) were purchased from Merck (Germany). Acetic acid glacial (HPLC grade) was purchased from TEDIA (USA). Ethyl acetate (HPLC grade) was purchased from Fisher chemicals (UK). Distilled water was purified in a Millipore system Milli Q.

1.2 Animals

Male Sprague-Dawley (SD) rats (200–250 g) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch of China. The animals were kept in animal holding room under standard conditions with 12 h light-dark cycles, with free access to rodent cubes and tap water. Animals were maintained according to the National Institutes of Health standards established in the "Guidelines for the Care and Use of Experimental Animals", and all the experimental protocols were approved by the Ethical Committee on Animal Experimentation of the East China Normal University (Shanghai).

1.3 Preparation of rat liver microsomes

In this study, the rats (200–250 g) were fasted overnight and killed by cervical dislocation before removal of the liver. The liver was excised, rinsed with ice-cold saline (0.9% NaCl w/v), weighed and homogenised in a 0.05 mmol L^{-1} Tris/KCl buffer (pH 7.4). The homogenate was centrifuged at 10,000×*g* at 4°C for 30 min. The supernatant was then centrifuged at 105,000×*g* at 4°C for 60 min. The pellet was reconstituted with 0.05 mmol L^{-1} Tris/KCl buffer (pH 7.4) and stored at −150°C until used. The protein concentration was determined by a protein quantitative assay using bicinchoninic acid [19]. Cytochrome P450 content of the rat liver microsomes was quantified as previously described [20].

1.4 Analysis of rat CYP2C11 enzyme activity

The effects of CuE on CYP2C11 activity in rat liver microsomes *in vitro* were evaluated by adding CuE at various concentrations (1.25–100 μmol L^{-1}) to tolbutamide (50 μmol L^{-1}). Control incubations for tolbutamide 4-hydroxylation did not contain CuE. Liver microsomes (1 mg mL^{-1}) were incubated with 0.05 mol L^{-1} Tris/HCl buffer (pH 7.4) with NADPH regenerating system (1 mmol L^{-1} NADP, 5 mmol L^{-1} G6P, 1 unit mL^{-1} G6PDH, and 5 mmol L^{-1} magnesium chloride). The incubation mixtures were incubated in an Eppendorf Thermomixer at 800 rev min^{-1} at 37°C. The reaction was initiated by adding the NADP into incubation mixture. Termination of the enzyme reaction was by addition of 500 μL of ice-cold acetonitrile. The HPLC analysis of 4-hydroxytolbutamide was as previ-

ously described [21]. The inhibitory effect of CuE on CYP2C11 was evaluated by the percentage of the formation of 4-hydroxytolbutamide compared with the control.

1.5 Analysis of inhibition kinetics

To determine the mechanism of CuE inhibition of tolbutamide 4-hydroxylase, a range of tolbutamide (25, 50, 75, 100 μmol L^{-1}) and CuE (5, 10, 25, 50 μmol L^{-1}) concentrations were used for inhibition kinetics studies. Sulfaphenazole (5–50 μmol L^{-1}), a selective rat CYP2C11 inhibitor, was used as a positive control [21,22]. The mode of inhibition was evaluated by visual inspection of Lineweaver-Burk plots, which were obtained from Michaelis-Menten non-linear regression equation. The inhibition constant (K_i) was obtained by a secondary plot using the slope of the primary Lineweaver-Burk plot (K_m/V_{max} versus inhibitor concentration) and fitted by GraphPad Prism 5.0 (GraphPad Software Inc., USA).

1.6 Analysis of time-dependent inhibition

To investigate whether inhibition of CYP2C11 by CuE was time-dependent, time-dependent inhibition experiments were carried out [23]. Primary incubations included CuE (final concentration approaching IC_{50}), 0.05 mol L^{-1} Tris/HCl buffer (pH 7.4), 5 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} NADP, 5 mmol L^{-1} G6P, 1 unit mL^{-1} G6PDH and 1 mg mL^{-1} microsomal protein. The mixture was incubated at 37°C for various time points (0, 5, 10, and 20 min). At each pre- incubation time point, 50 μL aliquots of the primary incubation mixtures were transferred to a secondary incubation to a final volume of 500 μL, which included 50 μmol L^{-1} tolbutamide,1 mmol L^{-1} NADP, 5 mmol L^{-1} G6P, 1 unit mL^{-1} G6PDH, and 5 mmol L^{-1} magnesium chloride, and 0.05 mol L^{-1} Tris/HCl (pH 7.4). The tolbutamide mixture was incubated at 37°C for 90 min and stopped by the addition of 500 μL of ice-cold acetonitrile. The samples were treated as described above and then subject to HPLC analysis.

1.7 Effects of CuE treatments on the pharmacokinetics of tolbutamide

Intraperitoneal administration of CuE was selected in this study over the oral route of administration because our previous report suggested possible interaction between CYP3A and CuE [24]. Significant gastrointestinal absorption and first pass metabolism by CYP3A in the small intestine and CYP3A-linked drug transporter proteins such as P-gp may thus influence the interpretation of the CYP2C11 data in this study. For the acute treatments, rats were treated with CuE (50–200 μg kg^{-1}, i.p.), or saline with 0.1% dimethyl sulfoxide (DMSO) (control). A single dose of tolbutamide (10 mg kg^{-1}) was given via caudal vein 60 min after the treatment with CuE. The dosage of tolbutamide used was based on similar herb-drug interaction study carried out previously [21]. Serial blood samples (approximately 0.5 mL) were collected via the orbital venous plexus at 10, 20, 30, 60, 90, 120, 240 and 360 min after tolbutamide administration. Plasma was separated and stored at −20°C prior to analysis by LC-MS/MS. Fluconazole (60 mg kg^{-1}, i.p.) was used as a positive control for enzyme inhibition studies [25].

For the 3 d treatments, rats were pre-treated with CuE (50–200 μg kg^{-1} day^{-1}, i.p.) or saline with 0.1% DMSO (control, i.p.) for 3 d. During the pre-treatment period, the rats were kept in a 12 h light/dark cycle animal room with controlled temperature and humidity. Free access to laboratory rodent diet and tap water was allowed. One day after the final pre-treatment, the rats were injected tolbutamide (10 mg kg^{-1}) via caudal vein and experiments were performed as described in previous section. Carbamazepine (60 mg kg^{-1} day^{-1}, i.p., 3 d) was used as a positive control for enzyme induction [26].

1.8 Plasma sample preparation

Liquid-liquid extraction in an acidic environment was used to extract tolbutamide, 4-hydroxytolbutamide and chlorpropamide (IS) from the rat plasma. IS solution (5 μL, 1 μg mL^{-1} in methanol) and deionized water containing 1% acetic acid (5 μL) were added into the rat plasma (50 μL), and then extracted by ethyl acetate (400 μL) via acute vortex. The well-vortexed solution was then centrifuged at 16,000×*g* for 10 min to separate the organic layer and precipitation. The organic layer (300 μL) was then quantitatively transferred to a new EP tube and evaporated to dryness at 37°C under a gentle stream of nitrogen gas. The residue was re-dissolved in 500 μL of initial mobile phase (H_2O∶ACN=65∶35) by vigorously eddying and further centrifuged at 16,000×*g* for 20 min. Total 100 μL of the supernatant was transferred to the HPLC autosampler vial, and then 1 μL was injected into the LC-MS/MS system for analysis.

1.9 LC-MS/MS analysis of tolbutamide and 4-hydroxytolbutamide in plasma

Analysis of tolbutamide and 4-hydroxytolbutamide in plasma was performed on a HPLC coupled with tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system consists of an Agilent 1290 series HPLC and a 6460 triple quadrupole mass spectrometry (Agilent Technologies, USA). The 1290 HPLC was composed of a degasser, a binary pump, an autosampler and a thermostatic column compartment. The 6460 triple quadrupole mass spectrometry was equipped with an Agilent Jet Stream electrospray

ionization (ESI) source and operated with Agilent MassHunter software (version 5.0.280.1, Agilent Technologies). The chromatographic separation was performed by an Agilent Zorbax Eclipse Plus C_{18} Rapid Resolution HT column (2.1 mm×50 mm, 1.8 μm) protected by a Phenomenex C_{18} guard column (Torrance, USA). Mobile phase system of H_2O containing 1 mmol L^{-1} ammonium acetate and 0.01% acetate acid (v/v) (A) and acetonitrile (B) was utilized. The two compounds together with IS were eluted by HPLC gradient with a flow rate of 0.1 mL min^{-1} as follows: 0–0.5 min, 35% acetonitrile; 0.5–2.5 min, 35%–85% acetonitrile; 2.5–4 min, 85% acetonitrile; 4–4.2 min, 85%–35% acetonitrile; 4.2–6 min, 35% acetonitrile. The temperature of column oven was maintained at 40°C and the total run time for sample analysis was 6.0 min.

The mass spectrometer was operated in the negative ESI mode, and the detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 269.1 to the m/z 170.0 product ion for tolbutamide, m/z 285.1 to m/z 186.0 product ion for 4-hydroxytolbutamide, and m/z 275.0 precursor to m/z 189.8 product ion for IS, respectively. MS/MS conditions were optimized as follows: drying gas temperature, 300°C; gas flow, 10 L min^{-1}; nebulizer, 25 psi; sheath gas temperature, 350°C; sheath gas flow, 11 L min^{-1}; collision gas (N_2), 1.6 MPa. The fragment energy was set at 110 V for all of the three compounds tested. The collision energy was optimized to 12 eV for tolbutamide and 4-hydroxytolbutamide, and 14 eV for IS. A divert valve was used to divert the eluent to waste from 0 to 2 min, 5.5 to 6 min, and to MS from 2 to 5.5 min.

1.10 Western blotting assay of rat CYP2C11

A piece of rat liver tissue was homogenated in radio immunoprecipitation assay (RIPA) lysate buffer after 3 d CuE treatments. The homogenate was put into −80°C overnight for further dissociation. Frozen homogenate was thawed on ice, and then centrifuged at 13,000×*g* for 20 min at 4°C. The supernatant protein was collected and quantified using Micro BCA protein Assay Kit. For CYP2C11 immunoblot analysis, proteins (70 μg) from different treatment groups were resolved on 10% acrylamide gels and transferred to a nitrocellulose membrane. After blocking with bovine serum albumin (BSA, 5% w/v) in phosphate buffer with 0.1% tween-20 (PBST) for 1.5 h, the membrane was incubated with a primary antibody (1∶1,000) from Abcam (UK) for rat CYP2C11 at 4°C overnight. After washing, the membrane was incubated with a fluorescence-conjugated secondary antibody to rabbit IgG (1∶10,000) for 1.5 h. After washing, the blots on the membrane were visualized by the LI-COR Odyssey System and densitometry analysis performed with Odyssey 3.0 software (USA).

1.11 Data analysis

All data were expressed as the mean±standard error of mean ($\bar{x}$ ±SE). Statistical analysis of the data was carried out by one-way ANOVA, and the P values less than 0.05 were considered statistically significant. Enzyme kinetic parameters were fitted by non-linear regression analysis for calculation of IC_{50} (concentration of inhibitor to cause 50% inhibition of original enzyme activity) value using GraphPad Prism 5.0 (GraphPad Software Inc., USA). A Lineweaver-Burk Plot is a double reciprocal plot in which varying substrate concentrations are plotted against reaction velocities to obtain linear transformation. The enzyme parameter Michaelis constant K_m and V_{max} values were obtained from Lineweaver-Burk Plot. The inhibition constant (K_i) was obtained by a secondary plot using the slope of the primary Lineweaver-Burk Plot (K_m/V_{max} versus inhibitor concentration).

Pharmacokinetic parameters such as half-time ($T_{1/2}$), initial plasma concentration extrapolated to time zero ($C_{initial}$), area under the plasma concentration-time curve during the period of observation ($AUC_{0\text{-}T}$), area under the plasma concentration-time curve from zero to infinity ($AUC_{0\text{-}\infty}$), clearance (CL), apparent volume of distribution (V_d), and the mean residence time (MRT) were calculated by WinNonlin software version 5.2.1 (Pharsight Corporation, USA) based on noncompartmental analysis.

2 Results

2.1 LC-MS/MS method validation for analysis of tolbutamide and 4-hydroxytolbutamide in rat plasma

Six different lots of blank rat plasma were checked for any false positive MS responses. As a result, no obvious interferences from endogenous plasma substances were observed and a good separation of the tolbutamide, 4-hydroxytolbutamide and chlorpropamide (IS) was achieved under the current LC-MS/MS conditions (Figure 2). The retention time of tolbutamide, 4-hydroxyltolbutamide and IS was 4.3, 3.1, and 4.1 min, respectively (Figure 2). Calibration curves were confirmed by plotting the peak area ratio (y) of each analyte to IS versus plasma concentration (x) using weighed least squares regression analysis ($1/x^2$), with good linearity (R^2>0.98 for tolbutamide and R^2>0.99 for 4-hydroxytolbutamide) over the concentration ranges (10–100 μg mL^{-1} for tolbutamide and 50–2000 ng ml^{-1} for 4-hydroxytol- butamide, respectively). The accuracy and precision were evaluated by assaying quality control (QC) samples at low, medium and high concentrations on the same day and three consecutive days, according to the Food and Drug Administration (FDA) acceptance criteria. The accuracy was between 86.4%–101.7% for tolbutamide and 93.1%–111.3% for 4-hydrox-ytolbutamide, respectively. The intra- and inter-day precision was within 6.41% for tolbutamide and

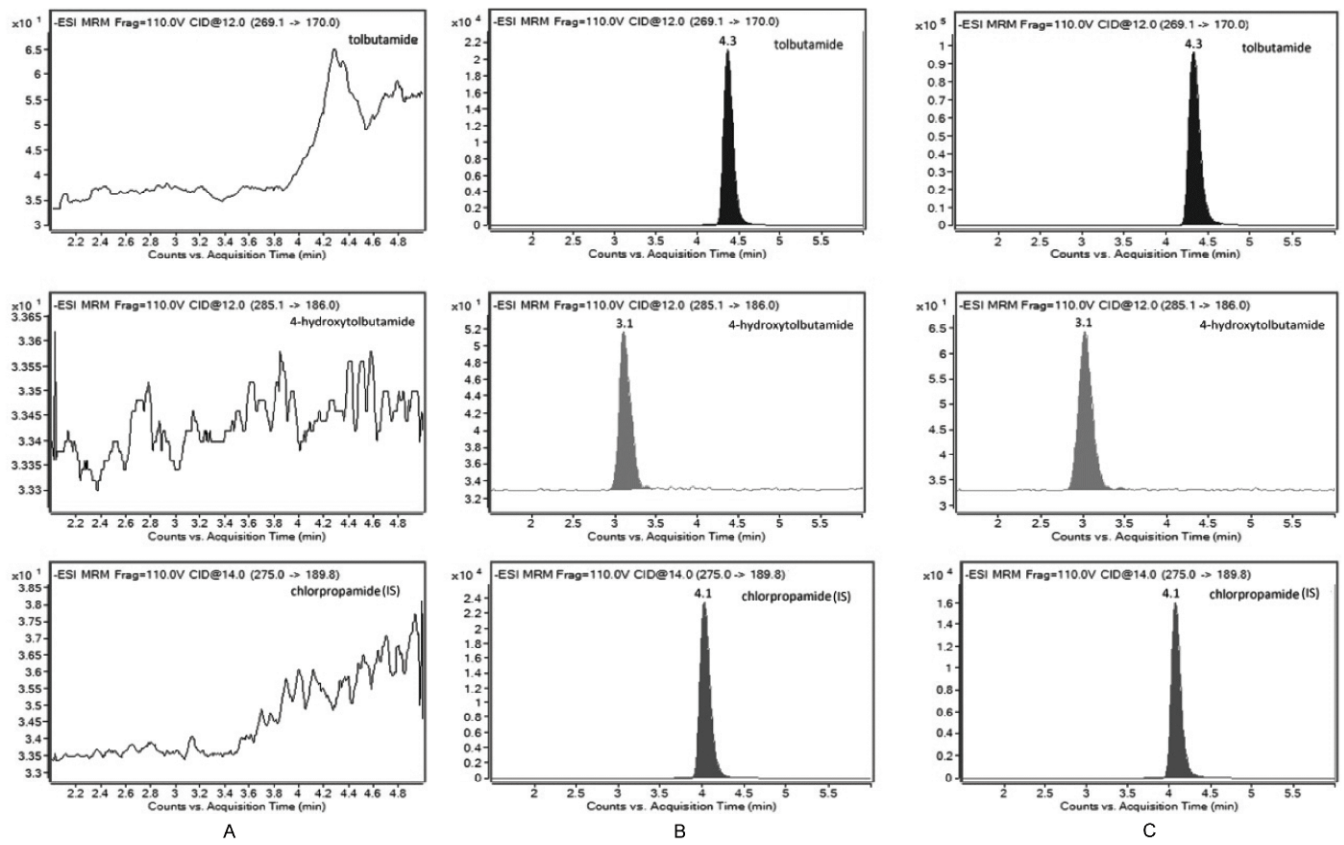

Figure 2 Representative chromatograms after multiple reaction monitoring (MRM) of tolbutamide and 4-hydroxytolbutamide in rat plasma. A, A blank plasma sample. B, A blank plasma spiked with tolbutamide, 4-hydroxytolbutamide and IS at lower limit of quantification (LLOQ) sample. C, A plasma sample collected at 60 min after caudal intravenous administration of 10 mg kg^{-1} tolbutamide in rats.

7.68% for 4-hydroxytolbutamide, respectively. Therefore, the high selectivity and sensitivity of the LC-MS/MS assay was validated with short analytical time and easy sample treatment, and consequently would be suitable for pharmacokinetic studies.

2.2 Effects of CuE on CYP2C11 (tolbutamide 4-hydroxylase) activity in rat liver microsomes *in vitro*

To evaluate the effect of CuE on CYP2C11 activity, tolbutamide 4-hydroxylase activities with or without CuE were determined by using rat liver microsomes. CuE inhibited the formation of 4-hydroxytolbutamide (IC_{50}=51.7 μmol L^{-1}) concentration-dependently with decreases in the 4-hydroxytolbutamide/tolbutamide ratios (Figure 3). Enzyme kinetics studies were carried out with various tolbutamide concentrations in the presence or absence of CuE and sulfaphenazole (positive control for rat CYP2C11 inhibition). The Lineweaver-Burk transformation of the enzyme velocities versus substrate concentration showed that CuE was a competitive inhibitor (Figure 4A). As shown in Figure 4B, the K_i value for CuE was 55.5 μmol L^{-1} which was determined by a secondary plot using the slope of the primary Lineweaver-Burk plot. The inhibitory effect of CuE on tolbutamide 4-hydroxylation was comparable to sulfaphenazole, a specific rat CYP2C11 inhibitor, with the K_i value at 68.5 μmol L^{-1}. Moreover, time-dependent inhibition experiments were also performed with 50 μmol L^{-1} (approaching IC_{50}) CuE to determine whether the inhibition of CYP2C11 activity by CuE was time-dependent. Results showed that no time-dependent inhibition was observed, indicating reversible inhibition.

2.3 Effects of CuE treatment on 4-hydroxylation and pharmacokinetics of tolbutamide

Figure 5A and B showed the plasma concentration-time profiles of tolbutamide and 4-hydroxytolbutamide, respectively, after single dose treatment with CuE (50–200 μg kg^{-1}, i.p.). No significant difference (P<0.05) in tolbutamide pharmacokinetics was observed with the single administration although the $T_{1/2}$ and AUC were increased and the clearance was decreased (Table 1). The enzyme inhibitor fluconazole (positive control) significantly delayed the elimination of tolbutamide starting from 120 min (Figure 5A) by increasing the $T_{1/2}$, AUC and MRT, and decreased the CL by 60.8% (Table 1).

For the multiple-pretreatment study, the mean plasma

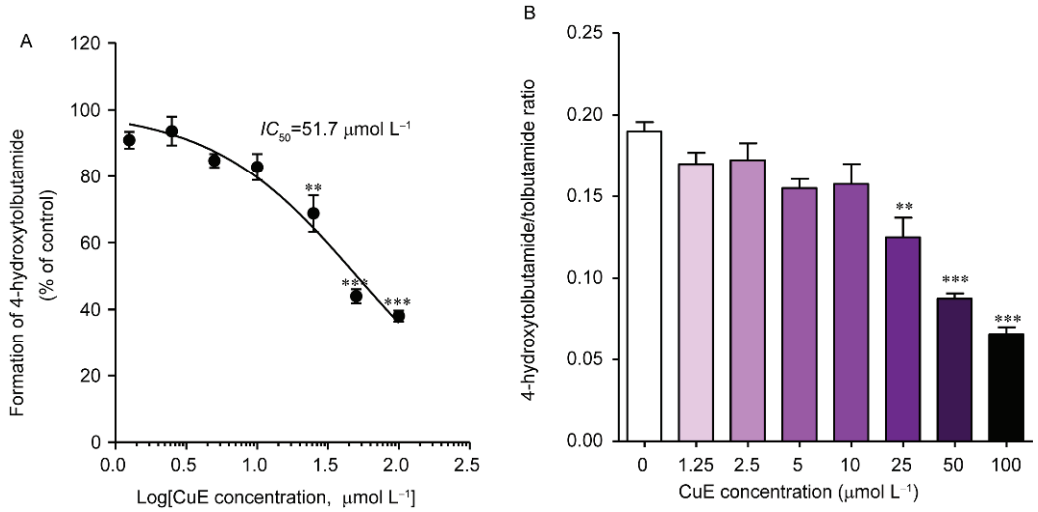

Figure 3 (color online) Inhibition of CYP2C11 activity by CuE in rat liver microsomes, presented as 4-hydroxytolbutamide/tolbutamide ratios. Results were $\bar{x}$ ±SE of six rats. Statistical significance was determined by analysis of variance. **: P<0.01; ***: P<0.001.

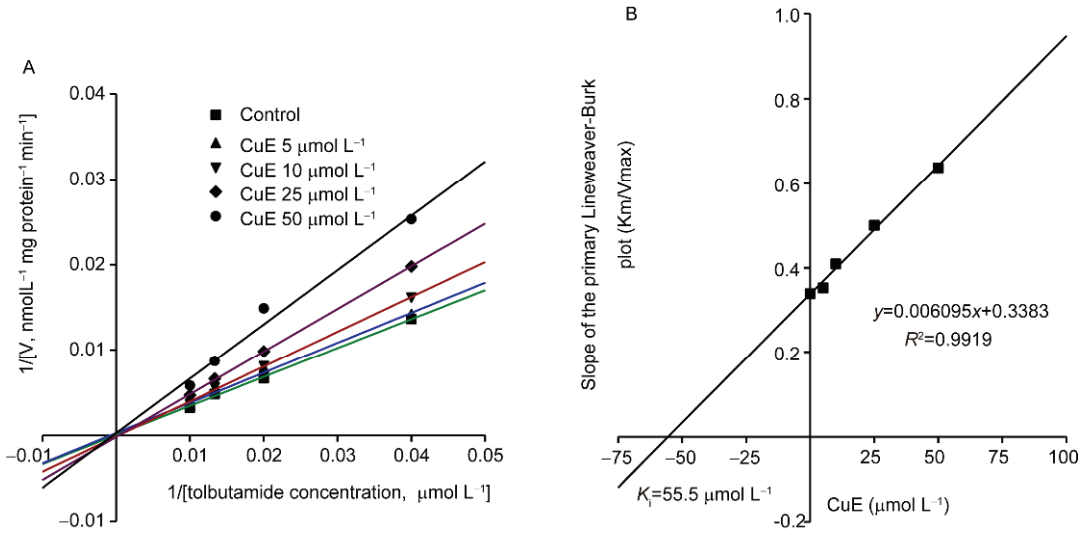

Figure 4 (color online) Primary Lineweaver-Burk plot (A) and the secondary plot for K_i (B) in the inhibition of CYP2C11-mediated tolbutamide 4-hydroxylase by various concentrations of CuE (5–50 μmol L^{-1}) in rat liver microsomes. Tolbutamide was used at concentrations of 25, 50, 75 and 100 μmol L^{-1}. Each data point represents the mean of six rats.

Table 1 Pharmacokinetics of tolbutamide (10 mg kg^{-1}, i.v.) in rats after a single administration of CuE (50–200 μg kg^{-1}, i.p.)[a)]

Parameters	Control	Fluconazole 60 mg kg^{-1}	CuE treatment		
			50 μg kg^{-1}	100 μg kg^{-1}	200 μg kg^{-1}
$T_{1/2}$ (min)	217.36±18.97	544.19±19.32***	245.23±11.21	242.43±25.34	263.32±12.65
$C_{initial}$ (μg mL^{-1})	80.19±6.99	75.47±2.18	74.29±5.15	69.64±4.20	76.95±4.08
AUC $_{0\text{-}T}$ (min μg mL^{-1})	13,867.31±782.34	18,701.70±754.69**	14,680.75±370.82	14,622.76±616.34	14,111.75±385.60
AUC $_{0\text{-}\infty}$ (min μg mL^{-1})	20,208.64±1,725.75	51,559.50±2,291.20***	23,176.09±784.79	23,549.27±2,430.00	23,216.93±782.26
V_d (mL kg^{-1})	153.90±5.19	152.86±6.61	152.44±3.59	148.51±3.29	163.74±6.20
CL (mL min^{-1} kg^{-1})	0.51±0.04	0.20±0.01***	0.43±0.01	0.44±0.04	0.43±0.01
MRT (min)	139.23±2.84	164.17±0.95***	145.31±1.84	144.77±2.62	146.11±1.07

a) Fluconazole (60 mg kg^{-1}, i.p.) was used as a positive control for enzyme inhibition studies. Results were $\bar{x}$ ±SE of 5–8 animals. **: P<0.01; ***: P<0.001.

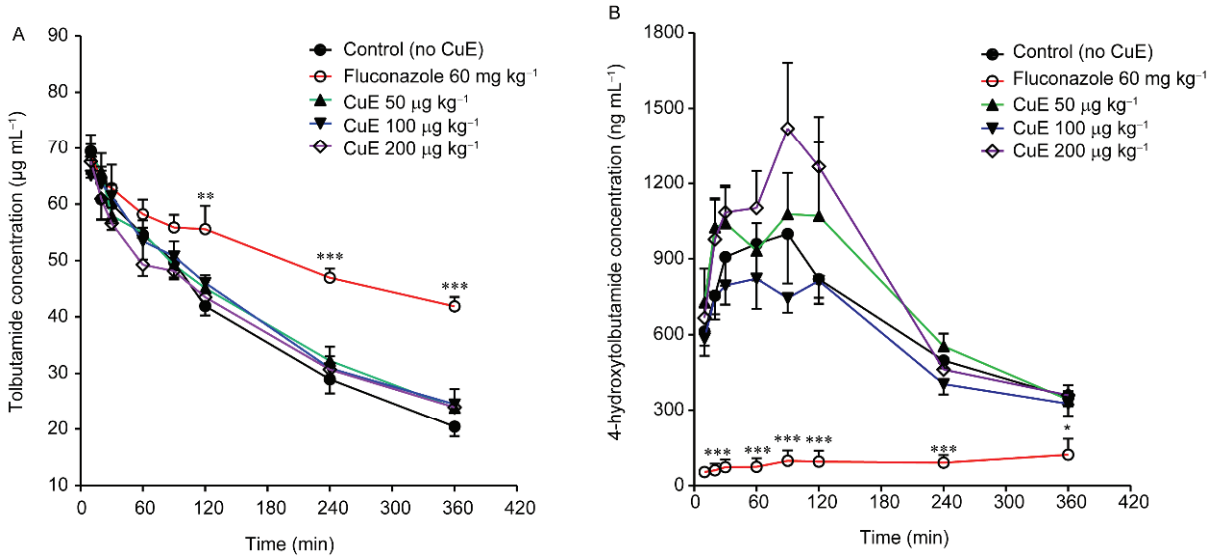

Figure 5 (color online) Concentration-time profiles of (A) tolbutamide and (B) 4-hydroxytolbutamide after a single administration of CuE (50–200 μg kg^{-1}, i.p.) after a standard dose of tolbutamide (10 mg kg^{-1}, i.v.). Results were $\bar{x}$ ±SE of 5–8 rats. *: P<0.05; **: P<0.01; ***: P<0.001.

concentration-time profiles of tolbutamide and 4-hydroxytolbutamide after 3 d CuE pretreatment were shown in Figure 6A and B, respectively. As shown in Table 2, pretreatment of CuE (200 μg kg^{-1} d^{-1}, i.p.) for 3 d significantly decreased tolbutamide clearance by 25.0%, and significantly prolonged plasma half-life ($T_{1/2}$) from (182.26±18.24) to (250.29±21.85) min. Other pharmacokinetic parameters (C_{inital}, AUC, MRT) of tolbutamide also increased compared with the control. In addition, pretreatment of CuE (200 μg kg^{-1} d^{-1}, 3 d, i.p.) also decreased the formation of 4-hydroxytolbutamide and the $AUC_{0\text{-}T}$ of 4-hydroxytolbutamide ((186,130±18,066) min ng mL^{-1} vs. (147,991±12,382) min ng mL^{-1}) by 20.5% (P<0.05). These may reflect changes inhibited by CuE on the metabolism of tolbutamide to 4-hydroxytolbutamide, and may be related to CYP2C11 inhibition. The effects of carbamazepine, an enzyme inducer, were as expected in terms of its ability to alter tolbutamide 4-hydroxylation and cause changes in the pharmacokinetic of tolbutamide (Table 2).

2.4 Effects of CuE treatment on hepatic CYP2C11 expression

To explore whether a 3 d CuE administration can affect CYP2C11 protein expression, the western blot assay was used to study the CYP2C11 protein expression in rat liver. As shown in Figure 7, CuE administration (50–200 μg kg^{-1} d^{-1}, 3 d, i.p.) only slightly inhibited CYP2C11 protein expression (P>0.05) in rat liver while carbamazepine (positive control for CYP2C11 induction) produced an expected increase in CYP2C11 expression (P<0.05). This result was a part of the study complemented the *in vitro* and *in vivo* metabolic studies and clarified that CuE competitively inhibited the rat CYP2C11 activity without affecting the expression of CYP2C11 protein.

3 Discussion

The CYP2C subfamily in humans accounts for approximately 16% of the total hepatic P450 complement, and is responsible for the metabolism of 15% of drug oxidations [27]. CYP2C9 is a major CYP isoform to metabolize a wide range of therapeutic agents, including non-steroidal anti-inflammatory drugs, oral anticoagulants and oral hypoglycaemic agents, such as meloxicam, suprofen, tolbutamide, and S-warfarin [22]. Moreover, CYP2C9 also contributes to the metabolism of fatty acids, prostanoids and steroid hormones [21]. The rat forms of the human CYP2C9 equivalent CYP2C isoforms include CYP2C6 and CYP2C11, and CYP2C11 is a homologue of the human CYP2C9 with 77% homology [21,22]. Moreover, the CYP2C11 as the most abundantly expressed CYP enzymes in male SD rats is very important, in a way equivalent to CYP3A4 in humans. Until now, tolbutamide 4-hydroxylase has been a widely used index reaction for the measurement of CYP2C9 (human) and CYP2C11 (rat) activity [21,22,28].

Initial evidence that CuE may affect CYP-mediated metabolism came from our previous studies in which CuE reduced the clearance of warfarin *in vivo* in rats [15]. One of the objectives of this study was to delineate the CuE- warfarin interactions observed in rats, which may involve interaction with CYP1A2 and/or CYP3A and/or CYP2C11. In this study, it is clear from the *in vitro* inhibition kinetic studies that CuE can competitively inhibit rat tolbutamide 4-hydroxylase (CYP2C11) activity. Moreover, we also used the simultaneous nonlinear regression (SNLR) model [29] to make sure the competitive inhibition and get the K_i value

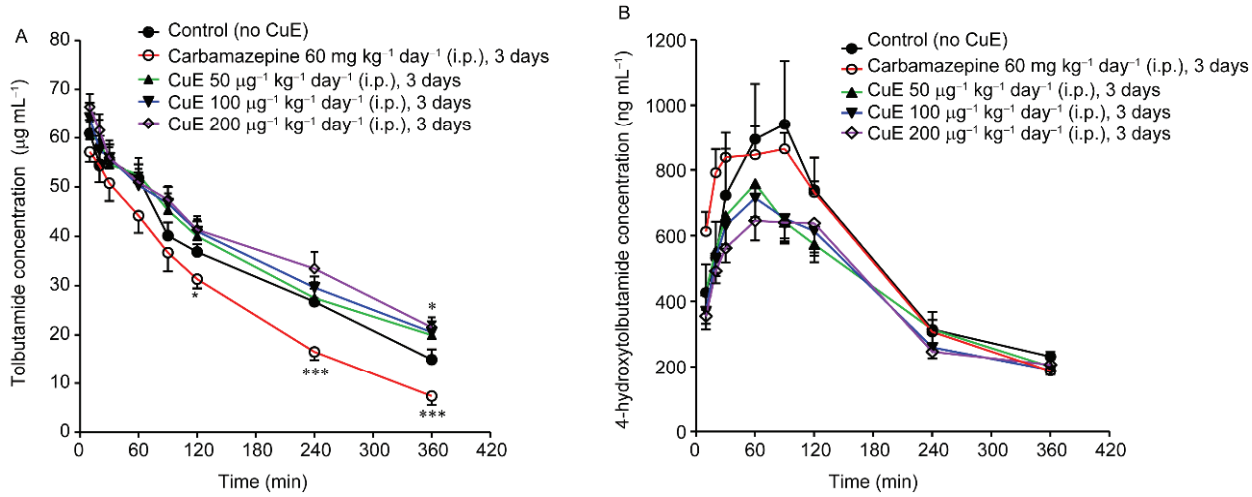

Figure 6 (color online) Concentration-time profiles of (A) tolbutamide and (B) 4-hydroxytolbutamide after a 3 d administration of CuE (50–200 μg kg^{-1} day^{-1}, i.p.) after a standard dose of tolbutamide (10 mg kg^{-1}, i.v.). Results were $\bar{x}$ ±SE of 5–8 rats. *: P<0.05; **: P<0.01; ***: P<0.001.

Table 2 Pharmacokinetics of tolbutamide (10 mg kg^{-1}, i.v.) in rats after multiple pretreatments with CuE (50–200 μg kg^{-1} d^{-1}, i.p.) for 3 d[a)]

Parameters	Control	Carbamazepine 60 mg kg^{-1} d^{-1}, 3 d	CuE treatment		
			50 μg kg^{-1} d^{-1}, 3 d	100 μg kg^{-1} d^{-1}, 3 d	200 μg kg^{-1} d^{-1}, 3 d
$T_{1/2}$ (min)	182.26±18.24	118.09±13.21*	215.60±19.55	242.49±21.74	250.29±21.85*
$C_{initial}$ (μg mL^{-1})	68.71±4.55	62.55±3.23	68.27±5.84	71.99±3.89	72.71±3.29
AUC $_{0-T}$ (min μg mL^{-1})	12,227.19±669.35	9,644.59±658.73*	13,056.35±1,079.53	13,463.53±817.00	14,111.86±920.50
AUC $_{0-\infty}$ (min μg mL^{-1})	16,484.26±1,546.06	11,011.48±996.78*	19,669.24±2,433.98	21,120.10±2,196.18	22,190.16±2,172.22
V_d (mL kg^{-1})	158.91±9.07	154.35±10.07	162.51±6.67	167.64±4.25	164.66±8.16
CL (mL min kg^{-1})	0.64±0.07	0.93±0.09**	0.56±0.07	0.51±0.06	0.48±0.04*
MRT (min)	136.00±4.79	118.00±3.37*	140.17±3.10	140.71±2.06	145.93±2.76

a) Carbamazepine (60 mg kg^{-1} d^{-1}, i.p., 3 d) was used as a positive control for enzyme induction studies. Results were $\bar{x}$ ±SE of 5–8 animals. *: P<0.05; **: P<0.01.

was 62.7 μmol L^{-1}, which was similar to 55.5 μmol L^{-1} determined by a secondary plot using the slope of the primary Lineweaver-Burk plot ($K_{m,app}$ method) [29]. Furthermore, the K_i value of CuE in rat liver microsomes was in the same range to that of sulfaphenazole, which was much more effective in inhibiting tolbutamide 4-hydrox- ylation in humans than in rats [20,28]. In addition, the present study also showed that the inhibition of CuE on CYP2C11 activity *in vitro* was only dose-dependent, and not time-dependent. Therefore, these results indicated that CuE is a reversible inhibitor of CYP2C11.

The effects of CuE on CYP2C11 activity and expression have also been carried out *in vivo* to investigate any pharmacokinetic changes of tolbutamide as well as potential enzyme inducing effects on CYP2C11 in rats. The dose of CuE was selected on the basis of our previous studies on the CuE-warfarin interactions [15]. The method of pretreatment of rats has also been used in our previous studies [30,31]. After a single pretreatment with CuE (50–200 μg kg^{-1}, i.p.), the pharmacokinetics of tolbutamide in rats did not significantly change compared with the control. However, multiple pretreatments of CuE (200 μg kg^{-1} d^{-1}, 3 d, i.p.) resulted in a longer $T_{1/2}$ and lower CL of tolbutamide compared with the control group. The distinction of the effects of CuE on the pharmacokinetics of tolbutamide by two pretreatments may be due to the different doses of CuE in rats. In fact, multiple doses may maintain a lasting and stable blood concentration to inhibit CYP2C11 activity *in vivo*, while a single dose may contribute to clearance of CuE, so the inhibitory effect is not obvious.

On the other hand, CuE also showed strong hepatotoxicity after multiple doses administration (data not shown). Therefore, the hepatic injury which was attributed to the potent toxicity of CuE, may result in a longer $T_{1/2}$ and lower CL of tolbutamide. At the same time, western blot analysis of rat CYP2C11 in this study confirmed that CuE has no inducing effects on rat CYP2C11 expression after 3 d CuE administration. Taken together results from previous study with CuE-warfarin and the current study, CuE may alter the metabolism and pharmacokinetic of model CYP2C11 substrates.

In summary, co-administration of CuE or herbal products

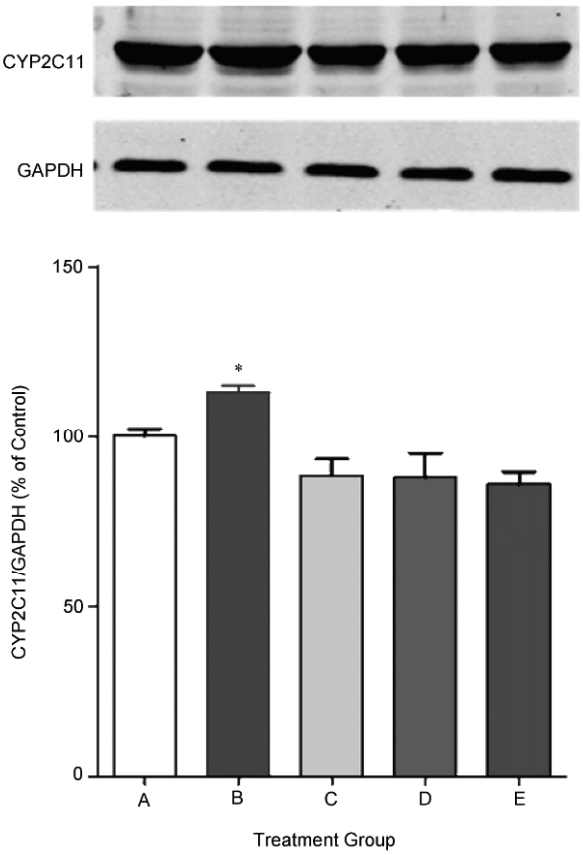

Figure 7 Effects of CuE on CYP2C11 expression in the rat. Rats received 3 d pretreatment according to the following groups. A, Control. B, Carbamazepine (60 mg kg^{-1} d^{-1}, i.p.). C, CuE (50 μg kg^{-1} d^{-1}, i.p.). D, CuE (100 μg kg^{-1} d^{-1}, i.p.). E, CuE (200 μg kg^{-1} d^{-1}, i.p.). Data were expressed as the $\bar{x}$ ±SE of six rats. *: $P<0.05$.

rich in CuE may affect the metabolism of CYP2C11 substrates though competitive inhibition and alter their clearance. In addition, CuE had no effect on CYP2C11 protein expression after multiple treatments and confirmed that CuE did not produce CYP2C11 enzyme induction in the Sprague-Dawley rat. This study offers a useful reference for the reasonable and safe use of herbal or natural products containing CuE to avoid unnecessary drug-drug interactions.

The authors declare that they have no conflict of interest.

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