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In Vitro Inhibitory Effect of Licoricidin on Human Cytochrome P450s

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Abstract: Licoricidin isolated from *Glycyrrhiza uralensis* is known to have anticancer, anti-nephritic, anti-*Helicobacter pylori*, and antibacterial effects. In this study, a cocktail probe assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used to investigate the modulating effect of licoricidin on cytochrome P450 (CYP) enzymes in human liver microsomes. When licoricidin was incubated at 0-25 μM with CYP probes for 60 min at 37°C, it showed potent inhibitory effects on CYP2B6-catalyzed bupropion hydroxylation and CYP2C9-catalyzed diclofenac 4'-hydroxylation with half maximal inhibitory concentration (IC_{50}) values of 3.4 and 4.0 μM, respectively. The inhibition mode of licoricidin was revealed as competitive, dose-dependent, and non-time-dependent, and following the pattern of Lineweaver-Burk plots. The inhibitory effect of licoricidin has been confirmed in human recombinant cDNA-expressed CYP2B6 and 2C9 with IC_{50} values of 4.5 and 0.73 μM, respectively. In conclusion, this study has shown the potent inhibitory effect of licoricidin on CYP2B6 and CYP2C9 activity could be important for predicting potential herb-drug interactions with substrates that mainly undergo CYP2B- and CYP2C9-mediated metabolism.

Key words: Licoricidin, LC-MRM, cytochrome P450, inhibition

Introduction

The cytochrome P450 (CYP) subfamily is one of the most important groups for biotransformation of xenobiotics and endogenous compounds.[1] The regulation of drug-metabolizing enzymes is a major cause of numerous drug-drug and herb-drug interactions.[2] CYP modulation is of considerable clinical importance and is known to occur through both enzyme induction and direct inhibition. Therefore, various assays have been developed to determine CYP activities in CYP enzyme sources such as liver microsome, recombinant enzyme, and hepatocyte.[3] Over the last decade, *in vitro* cocktails have been used successfully, coupled with liquid chromatography-mass spectrometry (LC-MS). Many substrates of CYP isoforms have been applied to assess multiple CYP activities simultaneously within a single experiment.[3] This strategy has been widely used to predict drug-drug or herb-drug interaction.

Licorice roots of the *Glycyrrhiza* species have been used since ancient times in traditional oriental medicine. Licorice contains several bioactive ingredients such as glycyrrhizin, glabridin, licochalcone A, licoricidin, and licorisoflavan A.[4] In the isoflavonoid class, licoricidin isolated from *Glycyrrhiza uralensis* is known as the active component for its anticancer, anti-nephritic, anti-*Helicobacter pylori*, and antibacterial effects.[5,6] Licoricidin has shown antibacterial activity against upper airway respiratory tract bacteria such as *Streptococcus pyogenes*, *Haemophilus influenzae,* and *Moraxella catarrhalis*.[7] In relation to anti-nephritic effects, licoricidin has shown scavenging activity against superoxide anion radical.[8] Moreover, it has been shown to inhibit the secretion of interleukin-6 and chemokine ligand 5 associated with reduced activation of NF-κB p65, and is a potential novel strategy for the treatment of cytokine and/or matrix metalloproteinase (MMP)-mediated disorders such as periodontitis.[9] Although several studies have been conducted on the pharmacological effects of licoricidin, its effects on CYP enzymes *in vitro* have not been previously examined. In this study, we investigated, for the first time, the modulating effects of licoricidin on seven hepatic CYPs in human liver microsomes (HLMs) by using cocktail approach coupled with LC-MS.

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Experimental

Materials

The licoricidin used in this study was isolated from the roots of *Glycyrrhiza uralensis* as previously described (Figure 1).[6] Pooled HLMs (BD UltraPoolTM HLM 150®, mixed gender, 20 mg/mL) and human recombinant cDNA-expressed CYP 2B6 and 2C9 (1 nmole/mL) were obtained from Corning Gentest (Woburn, MA). Glucose 6-phosphate, â-nicotinamide adenine dinucleotide phosphate, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade and were used as received.

Inhibition of CYP2B6 and CYP2C9 in HLMs by licoricidin

The inhibitory effects of licoricidin on the metabolism of the following seven CYP-specific substrates were examined: 2 μM phenacetin for CYP1A2, 50 μM coumarin for CYP2A6, 10 μM bupropion for CYP2B6, 10 μM diclofenac for CYP2C9, 5 μM dextromethorphan for CYP2D6, 50 μM chlorzoxazone for CYP2E1, and 2.5 μM midazolam for CYP3A.[3,10] All incubations were performed in duplicate, and the data are presented as means. To investigate the inhibitory effect of licoricidin on the activity of seven CYPs, each reaction was performed with 0.5 mg/mL pooled HLMs in a final incubation volume of 0.1 mL. The incubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4), cocktail probe substrates, licoricidin, and an NADPH-generating system (NGS) containing 0.1 M glucose 6-phosphate, 10 mg/mL β-$NADP^+$, and 1.0 U/mL glucose-6-phosphate dehydrogenase. A mixture with licoricidin at final concentrations of 0-25 μM was prepared by adding NGS for 60 min without preincubation, or with preincubation for 5 min without cocktail probe substrates. After incubation, the reaction was stopped by adding 100 μL of acetonitrile containing 0.1% formic acid and 5 μL of internal standard solution (10 μM reserpine) in methanol. After mixing and centrifugation at 13,000 g for 10 min, a 10-μL aliquot was injected onto a C18 column for LC-MS/MS analysis. Furthermore, to characterize the mode of inhibition of CYP2B6 and 2C9 by licoricidin, 0.5 mg/mL HLMs were incubated with licoricidin at 0-8 μM in 0.1 M potassium phosphate buffer (pH 7.4) for 60 min at 37°C. Bupropion was used at a probe substrate at 25, 50, and 100 μM, and the corresponding values for diclofenac were 5, 10, and 20 μM.

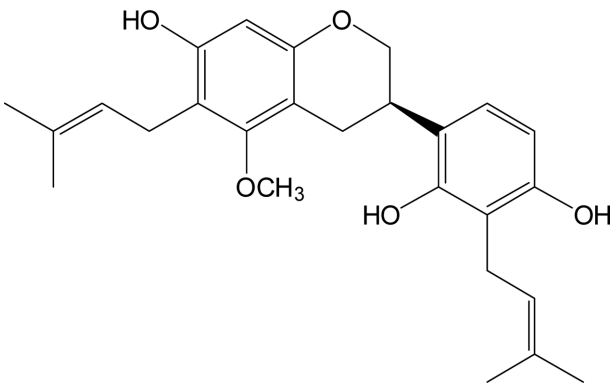

Figure 1. Chemical structure of licoricidin.

Inactivation of human recombinant cDNA-expressed CYP2B6, and CYP2C9 by licoricidin

To confirm the selective inhibition of CYP2B6 and 2C9 isoforms by licoricidin, 10 pmol of human recombinant cDNA-expressed CYP2B6 or 2C9 was incubated with 0.1-25 μM licoricidin and NGS for 60 min at 37°C after the addition of 50 μM bupropion and 10 μM diclofenac as selective CYP2B6 and 2C9 substrates, respectively.

LC-MS/MS analysis

LC-MS/MS assays were performed in the multiple reaction monitoring mode (MRM), and an Accela™ LC system coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., USA) equipped with a HESI-II Spray source was used. Electrospray ionization was performed in the positive mode at a spray voltage of 3,500 V (except for the detection of hydroxyl chlorzoxazone). Nitrogen was used as a sheath and auxiliary gas at optimum values of 45 and 20 (arbitrary units), respectively. Vaporizer and capillary temperatures were 150 and 300°C, respectively. For LC analysis, an Inertsil® ODS-2 column (3 μm, 2.1 × 150 mm, GL science) was used. The mobile phases consisted of LC-grade water containing 0.1% formic acid (A) and LC-grade acetonitrile containing 0.1% formic acid (B). The initial composition was increased to 95% solvent (B) over 10 min. A gradient program was used for HPLC at a flow rate of 220 μL/min. Multiple reaction monitoring was used for the detection of the CYP isozyme-specific marker metabolites. The precursor-product ion pairs used for monitoring the metabolites generated were as follows: *m/z* 152 → m/z 110 for CYP1A2 (4-acetamidophenol, CE 15), m/z 163 → m/z 107 for CYP2A6 (hydroxycoumarin, CE 21), m/z 256 → m/z 238 CYP 2B6 (hydroxybupropion, CE 15), m/z 312 → m/z 230 for CYP2C9 (4-hydroxydiclofenac, CE 15), m/z 258 → m/z 157 for CYP2D6 (dextrophan. CE 37), m/z 342 → m/z 203 for CYP3A4 (1-hydroxymidazolam, CE 15) and m/z 609 → m/z 174 (IS, reserpine) in positive mode and m/z 184 → 120 (hydroxychlorzoxazone) in negative mode.

Data Analysis

All incubations were performed in duplicate, and data are presented as means. Half maximal inhibitory concentration (IC_{50}) values were obtained using percent activity versus log[I] concentration plots. Kinetic parameters were estimated by curve fitting using SigmaPlot (version 12.0, Systat Software, Inc.).

Results and Discussion

In this study, we used the cocktail probe to determine the activity of seven CYPs simultaneously; phenacetin for CYP1A2, coumarin for CYP2A6, bupropion for CYP2B6, diclofenac for CYP2C9, dextromethorphan for CYP2D6, chlorzoxazone for CYP2E1, and midazolam for CYP3A4 (Table 1). LC-MS/MS system in MRM mode was optimized for the detection for each metabolite. When licoricidin was incubated at 0-25 μM with CYP probes for 60 min at 37°C, licoricidin showed potent inhibitory effects on CYP2B6-catalyzed bupropion hydroxylation and CYP2C9-catalyzed diclofenac 4'-hydroxylation with IC_{50} values of 3.4 and 4.0 μM, respectively (Table 1). The IC_{50} values of licoricidin for CYP2D6 and 3A4 activities were higher than 20 μM, indicating a weak inhibitory effect. Other CYPs, including CYP1A2, 2A6, and 2E1, were not significantly inhibited by licoricidin. When the metabolic stability of licoricidin was evaluated in HLMs in the presence of NGS, the initial concentration of licoricidin diminished by less than 10% after incubation for 90 min, suggesting that licoricidin is metabolically stable and not a suicide inhibitor of CYP2B6 and 2C9.

To investigate the mechanism underlying licoricidin's inhibition of CYP2B6-catalyzed bupropion hydroxylation and CYP2C9-catalyzed diclofenac 4'-hydroxylation, the inhibitory activities (IC_{50} values) were determined both with and without pre-incubating microsomal incubation mixtures for 15 min at 37°C in HLMs. The IC_{50} value of CYP2B6-catalyzed bupropion hydroxylation after preincubation was increased and it of CYP2C9-catalyzed diclofenac 4'-hydroxylation was not changed by preincubation. The pattern of IC_{50} shift showed the typical competitive inhibition. In addition, Figure 2 shows strong and dose-dependent inhibition, but not time-dependent inhibition, by licoricidin in HLMs.

To investigate the mode of CYP2B6 and CYP2C9 inhibition by licoricidin in HLMs, Lineweaver-Burk plots were constructed by kinetic study of CYP2B6-catalyzed bupropion hydroxylation and CYP2C9-catalyzed diclofenac 4'-hydroxylation in the presence of 0-6.8 and 0-8 μM licoricidin, respectively. The Lineweaver-Burk plots collected on the y-axis and secondary plots were linear (Figures. 3A and B) indicating competitive inhibition of licoricidin with a K_i value of 0.67 μM for CYP2B6 and 1.7 μM for CYP2C9 in HLMs, respectively. To confirm the inhibitory effects of licoricidin on CYP2B6 and 2C9, licoricidin was incubated with human recombinant cDNA-expressed CYP2B6 and

Table 1. Inhibitory effects of licoricidin on the activities of hepatic CYPs in human liver microsomes

Substrate reaction probes	CYP450 iso-forms	Substrate Conc. (μM)	IC_{50} (μM)	
			Without Preincubation	With Preincubation
Phenacetin *O*-deethylation	CYP1A2	40	>50	46.0
Coumarin 7-hydroxylation	CYP2A6	2.0	>50	>50
Bupropion hydroxylation	CYP2B6	50	3.4	13.0
Diclofenac 4'-hydroxylation	CYP2C9	10	4.0	2.0
Dextromethorphan *O*-deethylation	CYP2D6	5	28.0	13.0
Chlorzoxazone 6-hydroxylation	CYP2E1	50	>50	>50
Midazolam 1-hydroxylation	CYP3A4	2.5	24.3	31.0
Ticlopidine*	CYP2B6	-	0.14	-

To determine the inhibitory effects of licoricidin on the activities of CYPs, a cocktail probe was incubated with licoricidin at 0 to 25 mM in HLMs. The data shown represent the means of duplicate experiments. *, CYP2B6 inhibitor as positive control.

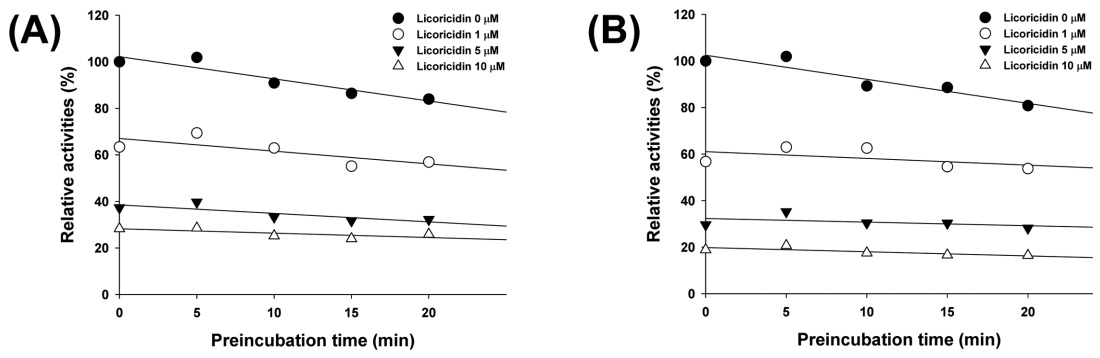

Figure 2. Time-dependent effect of licoricidin on CYP2B6-catalyzed bupropion hydroxylation (A) and CYP2C9-catalyzed diclofenac 4'-hydroxylation (B) in human liver microsomes (HLMs). The results shown are the means of duplicate experiments.

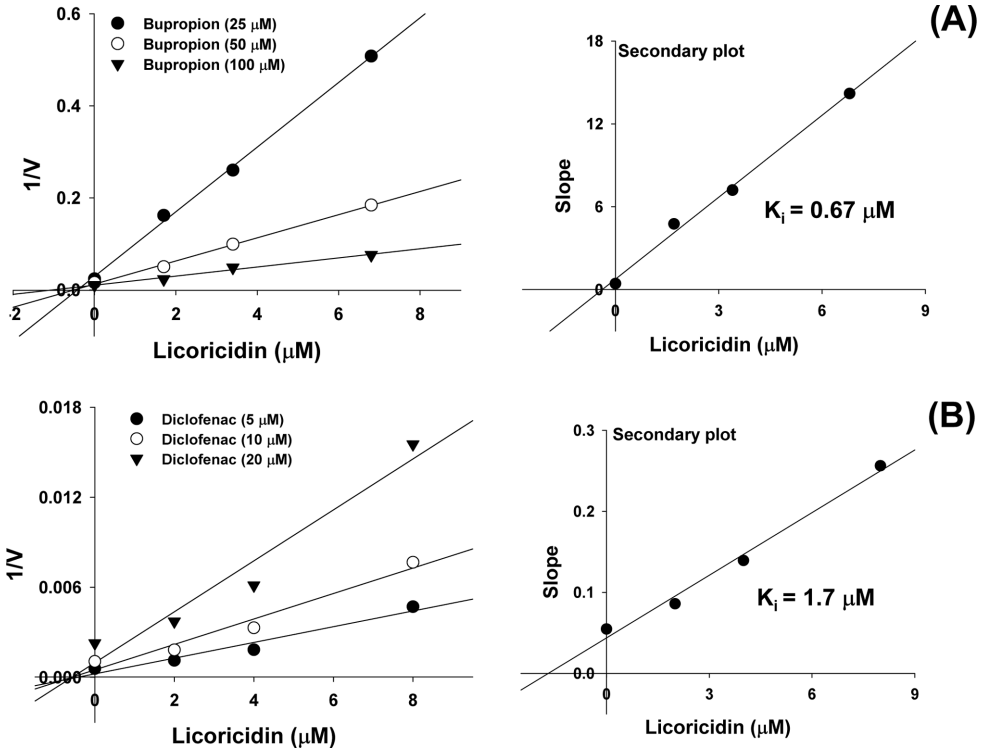

Figure 3. Dixon plots and double-reciprocal plots of licoricidin on CYP2B6-catalyzed bupropion hydroxylation (A) and CYP2C9-catalyzed diclofenac 4'-hydroxylation (B) in human liver microsomes (HLMs). Each data point represents the mean of duplicate experiments.

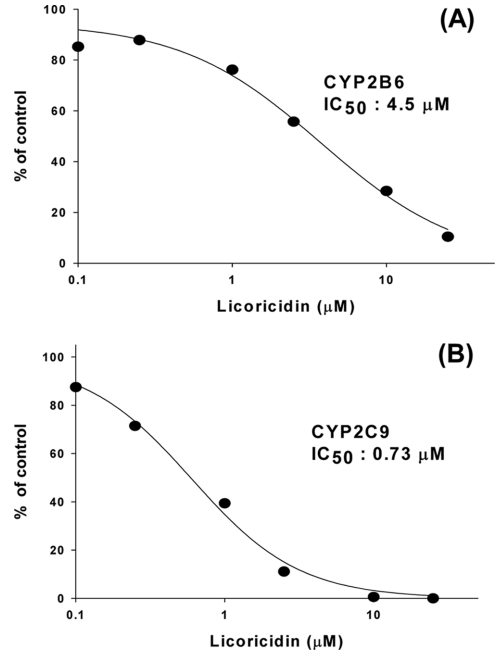

Figure 4. Inhibitory effects of licoricidin on the bupropion hydroxylation activity of human recombinant cDNA-expressed CYP2B6 (A) and diclofenac 4'-hydroxylation activities of human recombinant cDNA-expressed CYP2C9 (B). The results shown are the means of duplicate experiments.

2C9, respectively (Figure. 4). The IC_{50} values of licoricidin for CYP2B6-catalyzed bupropion hydroxylation and CYP2C9-catalyzed diclofenac 4'-hydroxylation were 4.5 and 0.73 μM, respectively.

Identification of a potent inhibitory effect of an herb compound is of importance for investigating potential herb-drug interaction. CYP2B6 has been estimated to represent approximately 1-10% of the total hepatic CYP content and metabolize approximately 8% of clinically used drugs (n > 60) and endogenous materials.[11,12] CYP2B6 is one of the CYP enzymes that bioactivates several procarcinogens and toxicants.[1] CYP2C9 is one of the most important CYP enzymes involved in approximately 20% of CYP-mediated drug metabolism.[13] Specifically, the substrate of CYP2C9 is clinically important to drugs with a narrow therapeutic index, such as warfarin and phenytoin.[14] Therefore, the accidental regulation of CYP2C9 activity could lead to severe toxicity. Furthermore, the potent inhibitory effect of licoricidin on CYP2B6 and CYP2C9 activity could be responsible for blocking CYP-related carcinogenesis and/or producing potential herb-drug interactions with substrates that mainly undergo CYP2B-and CYP2C9-mediated metabolism.

Conclusion

In this study, the selective and potent inhibitory effects of

licoricidin on CYP2B6 and 2C9 activity in HLMs were determined using a cocktail assay coupled with an LC-MRM strategy. CYP2B6 and CYP2C9 are the most important enzymes accounting for approximately 30% of CYP-originated drug metabolism. Therefore, the administration of herbal products including licoricidin or licorice roots could cause a toxic herb-drug interaction with CYP2B6 and CYP2C9 substrate drugs. A clinical study is recommended to further investigate the potential for interaction.

Acknowledgements

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