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In vivo inhibitory effects of puerarin on selected rat cytochrome P450 isoenzymes

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Puerarin, the major bioactive constituent in kudzu root, is used widely in China for the treatment of cardiovascular diseases and diabetes. The purpose of this study was to find out whether puerarin influences the effect on rat cytochrome P450 (CYP) enzymes (CYP2B6, CYP2C9 and CYP3A4) by using cocktail probe drugs *in vivo*. A cocktail solution at a dose of 5 mL/kg, which contained bupropion (20 mg/kg), tolbutamide (5 mg/kg) and midazolam (20 mg/kg), was given as oral administration to rats treated with 10 days oral administration of puerarin. Blood samples were collected at a series of time-points and the concentrations of probe drugs in plasma were determined by HPLC-MS/MS. The results showed that treatment with multiple doses of puerarin had inhibitory effects on rat CYP2B6, CYP2C9 and CYP3A4 enzyme activities. Therefore, caution is needed when puerarin is co-administered with CYP substrates, in view of herb-drug interactions.

1. Introduction

Chinese herbs are part of Traditional Chinese Medicine (TCM) and have been used for thousands of years in China (Leung 2006). Recently, Chinese herbs have become more popular in the Western world (Youns et al. 2010). Cancer patients use TCM treatments to improve general health, to strengthen immunity, to reduce side effects of conventional cancer therapies and to increase quality of life (Xu et al. 2006). Moreover, patients appreciate the more personal approach of TCM in contrast to most Western standard therapies (Xu et al. 2006; Youns et al. 2010). The application of TCM is not only restricted to cancer, but can also be found in other diseases such as rheumatoid arthritis, obesity and HIV/AIDS (Sui et al. 2012; Wang and Zou 2011).

When patients use Chinese herbs and Western drugs together, these herbs can cause unwanted pharmacodynamic (PD) interactions. For example, the concomitant use of warfarin and several Chinese herbs such as *Angelica sinensis* (dong quai), *Salvia miltiorrhiza* (dan shen), *ginseng* (ren shen) and *Ginkgo biloba* (yin xing) have been shown to cause an increased INR (international normalized ratio) and more extensive bleeding as a result of the anticoagulation and antiplatelet properties of these herbs (Izzo 2005).

In addition, Chinese herbs can cause pharmacokinetic (PK) interactions *via* drug metabolizing enzymes (Yu et al. 2011), such as the phase I metabolizing cytochrome P450 (CYP) enzyme family. CYP is considered the most important enzyme because it is responsible for the metabolism of most currently available drugs (Scripture and Figg 2006). Inhibition of CYPs can lead to increased plasma levels of drugs that are substrates for CYPs and thus can cause toxicity. In contrast, induction of this enzymes can result in decreased plasma levels of these drugs and consequently reduce their efficacy. Since numerous anticancer, immunosuppressive and antiviral drugs have a small therapeutic window, PK interactions between Chinese herbs and Western drugs can have dramatic consequences. Although Chinese herbs have the potential to generate PK and PD interactions, patients generally consider them to be safe because of their natural origin (Youns et al. 2010). Moreover, interactions between TCM and Western drugs are under-reported and little is known about the risks and effects of these interactions (Fasinu et al. 2012).

Puerarin (7,4′-dihydroxy-8-β-D-glucosylisoflavone, $C_{21}H_{20}O_9$), the major bioactive constituent in kudzu root, is used widely in China for the treatment of cardiovascular diseases and diabetes (Wong et al. 2011; Yao et al. 2012). In addition, previous studies suggest that puerarin possesses anti-oxidant (Liu et al. 2011), anti-platelet (Choo et al. 2002), anti-inflammatory (Huang et al. 2012), anti-arrhythmic (Zhang et al. 2011), and anti-apoptotic properties (Liu et al. 2012). It is also reported that puerarin could promote neovascularization in the myocardium of rats suffering from heart failure induced by coronary artery ligation (He et al. 2008). Two recent studies indicated that puerarin may have the ability to reduce cardiac expression of transforming growth factor β 1 (TGFβ 1) in isoprenaline-treated mice or spontaneous hypertensive rats (Chen et al. 2012). However, the effect of puerarin on CYP activities still needs to be clarified.

Puerarin, one of the most important herbal medicines in traditional Chinese medicine, is widely consumed concomitantly with prescribed drugs in Chinese hospitals. However, no systematic study has been reported emphasizing the impact of

Table 1: Main pharmacokinetic parameters of bupropion in rats (n = 6, Mean ± SD)

Parameter	CG	TG
$t_{1/2}$ (h)	25.528 ± 6.874	28.037 ± 5.713
T_{max}(h)	1.300 ± 0.570	1.750 ± 0.378**
C_{max} (ng/mL)	232.810 ± 45.536	305.025 ± 77.135**
$AUC_{(0-\infty)}$(μg·h/L)	13288.767 ± 2575.392	17797.738 ± 3556.387**
$MRT_{(0-\infty)}$(h)	29.810 ± 7.850	39.954 ± 6.255*
CL (L/h/kg)	4.510 ± 0.630	3.419 ± 0.802*

*Significantly different from control, $P < 0.05$;** Significantly different from control, $P < 0.01$.

puerarin on hepatic CYP enzyme activities up to now. With the aim of avoiding possible side effects induced by herb-drug interactions, we evaluated the effect of puerarin on the activities of CYP2B6, CYP2C9 and CYP3A4 enzymes in rats. We used a new three-probe drug cocktail (containing bupropion for CYP2B6, tolbutamide for CYP2C9 and midazolam for CYP3A4) based on a developed and validated HPLC-MS/MS method to assess CYP activities by comparing pharmacokinetics of the three substrates between control and treatment groups *in vivo*. We predict that the results may be useful for the clinical safety evaluation of herb-drug interactions involving puerarin.

2. Investigations and results

A validated HPLC-MS/MS method was used to determine the levels of the three probe drugs (bupropion for CYP2B6, tolbutamide for CYP2C9, and midazolam for CYP3A4) in rat plasma after oral administration of puerarin for 10 days.

2.1. *Effect of puerarin on the activity of CYP2B6 in rats*

CYP2B6 activity was evaluated by comparing pharmacokinetic behavior of bupropion between the control group (CG) and the test group (TG). The pharmacokinetic profiles of bupropion before and after oral administration of puerarin for ten days are shown in Table 1 and Figure 1. Compared with CG, the $T_{1/2}$ of bupropion changed little, the T_{max} was significantly increased, the C_{max} increased, the $AUC_{(0-\infty)}$ significantly increased, and the CL significantly decreased by 24.2%. These results indicated that CYP2B6 activity was significantly inhibited by puerarin after multiple oral administrations in rats.

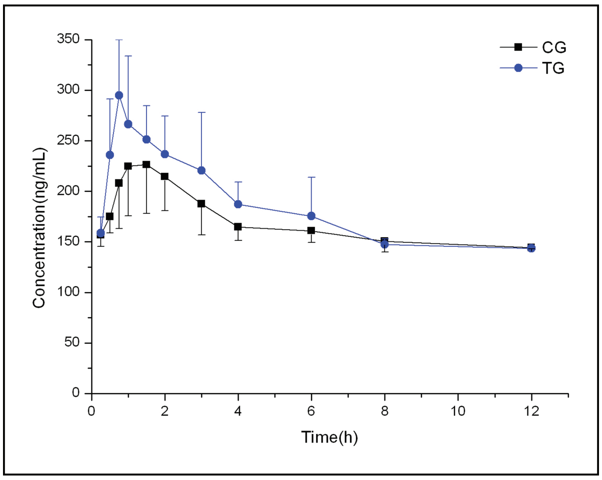

Fig. 1: Mean plasma concentration-time curves of bupropion in rats.

Table 2: Main pharmacokinetic parameters of tolbutamide in rats (n = 6, Mean ± SD)

Parameter	CG	TG
$t_{1/2}$ (h)	3.978 ± 1.063	6.670 ± 1.723**
T_{max}(h)	2.167 ± 0.408	3.014 ± 0.528**
C_{max} (ng/mL)	12090.859 ± 882.386	12274.311 ± 671.091
$AUC_{(0-\infty)}$(μg·h/L)	62192.708 ± 971.129	99714.429 ± 1056.162**
$MRT_{(0-\infty)}$(h)	6.448 ± 1.051	10.473 ± 1.605**
CL(L/h/kg)	0.098 ± 0.043	0.055 ± 0.017*

*Significantly different from control, $P < 0.05$;** Significantly different from control, $P < 0.01$.

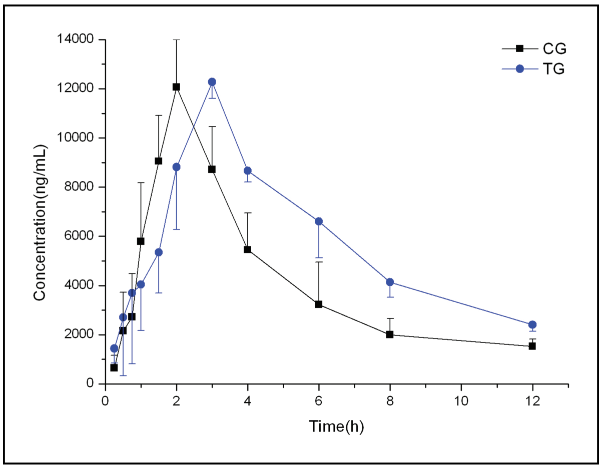

Fig. 2: Mean plasma concentration-time curves of tolbutamide in rats.

2.2. *Effect of puerarin on the activity of CYP2C9 in rats*

Pharmacokinetic profiles of tolbutamide after puerarin treatment were used to describe the activity of CYP2C9. The effects of puerarin on pharmacokinetic parameters of tolbutamide in rats are presented in Table 2. Mean plasma concentration-time curves of tolbutamide in different groups are presented in Figure 2. Compared with CG, and T_{max} $t_{1/2}$ of tolbutamide increased C_{max} increased, the $AUC_{(0-\infty)}$ significantly increased, and the CL significantly decreased by 43.9%. According to this data, CYP2C9 activity was significantly inhibited by puerarin after multiple oral administrations in rats.

2.3. *Effect of puerarin on the activity of CYP3A4 in rats*

As shown in Table 3 and Figure 3, compared with pre-administration, the $t_{1/2}$ of midazolam changed little, the T_{max} extended, the C_{max} and $AUC_{(0-\infty)}$ increased, and CL decreased.

Table 3: Main pharmacokinetic parameters of midazolam in rats (n = 6, Mean ± SD)

Parameter	CG	TG
$t_{1/2}$ (h)	3.922 ± 0.106	4.201 ± 0.872
T_{max}(h)	1.400 ± 0.274	1.750 ± 0.224*
C_{max} (ng/mL)	787.309 ± 134.767	898.509 ± 222.078*
$AUC_{(0-\infty)}$(μg·h/L)	6771.994 ± 328.676	7366.848 ± 188.644*
$MRT_{(0-\infty)}$(h)	4.753 ± 0.774	5.444 ± 0.685*
CL (L/h/kg)	1.875 ± 0.169	1.737 ± 0.199

*Significantly different from control, $P < 0.05$;** Significantly different from control, $P < 0.01$.

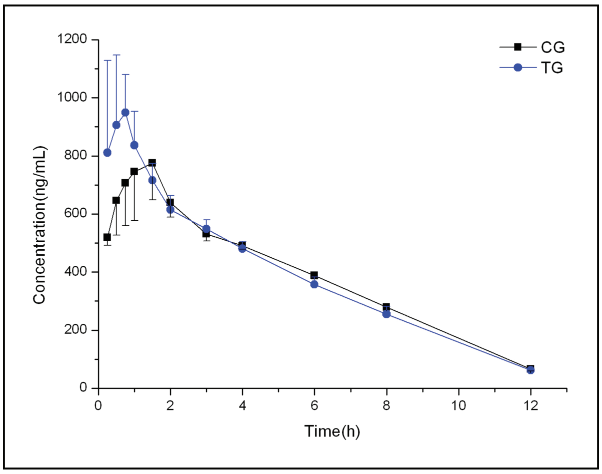

Fig. 3: Mean plasma concentration-time curves of midazolam in rats.

According to these data, CYP3A4 activity may be inhibited by puerarin after multiple oral administrations in rats.

3. Discussion

CYPs are a superfamily of mixed function oxidases that are responsible for the metabolism of many drugs and represent the major site for drug-drug and herb-drug interactions. Inhibition of CYPs can lead to clinically relevant increases in the exposure of the affected drug and thus to increased toxicity (Izzo and Ernst 2009; Pelkonen et al. 2008). Further, some of the CYP isozymes are also subject to induction by xenobiotics *via* activation of nuclear receptors, with a consequent result of decreased exposure of the affected compound leading to therapeutic failure or toxicological implications due to higher levels of a toxic metabolite (Izzo and Ernst 2009). Moreover, many carcinogens are metabolized by CYPs to either biological inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA resulting in carcinogenicity. Since many chemical carcinogens are metabolized by CYPs to both inactive, as well as to carcinogenic metabolites, the effects of inducers of these enzymes on the carcinogenicity of a chemical will depend on the inducer's effects on the different metabolic pathways.

A number of natural products have been demonstrated to modulate CYPs, including the induction of specific CYP isoforms, and the activation or inhibition of these enzymes (Saxena et al. 2008). For a new molecular entity, it is important to assess its possible inhibitory or inductive effects on CYP enzymes.

Chinese medicine has used many plants to safeguard public health for over two thousand years. Among these, puerarin is one of the most popular drugs. The use of puerarin is recorded in the classical medical book Shang Han Lun ("Treatise on fever"), composed more than 1800 years ago. Up to now, puerarin is effective in the treatment of myocardial and cerebral ischemia, coronary artery and sudden deafness in clinical setting in China (Gao et al. 2009). Its opportunities applied with other drugs are also increasing, drug interactions should be of concern. CYPs expressed widely in organisms are known to play an important role in the biotransformation of many endogenous and exogenous substances. Effect of drugs on CYPs is one of the most important mechanisms of drug interactions. However, the effect of puerarin on CYPs is still not very clear.

CYP2B6 is mainly expressed in the liver, accounting for 6% of total microsomal CYPs (Stresser and Kupfer 1999), and various extrahepatic tissues, including the kidney, skin, brain, intestine, and lung (Gervot et al. 1999). CYP2B6 can metabolize ~8% of all pharmaceutical drugs, to some extent. These include cyclophosphamide, ifosfamide, tamoxifen, ketamine, artemisinin, nevirapine, and so on (Zhou et al. 2009). In our study, we found a significant difference in the pharmacokinetic parameters of bupropion before and after administration of puerarin. This suggests that puerarin has an inhibitory effect on the activity of CYP2B6 after multiple oral administrations in rats. In views of the widespread use of puerarin, people should pay more attention to its side effects caused by herb-drug interactions especially when the drug is administered togethers with substrates of CYP2B6.

CYP2C9, the major member of the CYP2C subfamily in human liver, metabolizes more than 16% of clinically used drugs, including hypoglycemic agents tolbutamide and glipizide, anticonvulsant phenytoin, anticoagulant warfarin, nonsteroidal anti-inflammatory drugs such as fluriprofen, diclofenac as well as some newly developed drugs such as the antihypertensive losartan (Schwarz 2003). Therefore, the induction or inhibition on activity of CYP2C9 may lead to undesirable effects. According to our results, CYP2C9 activity could be significantly inhibited by puerarin after multiple oral administrations in rats.

CYP3A4 is by for the most abundant CYP protein (up to 50% of total CYP content) in human liver (Danielson 2002), responsible for the metabolism of a wide variety of substrates including nifedipine, erythromycin, troleandomycin, quinidine, cyclosporine A, 17α-ethynylestradiol, lidocaine and diltiazem (Peyronneau et al. 1993). In the present study, we determined the activity of CYP3A4 by midazolam as probe substrate. According to Table 3 and Figure 3, CYP3A4 activity was significantly inhibited by puerarin after multiple oral administrations in rats. The results show that when puerarin is used in combination with other drugs metabolized by CYP3A4, the potential herb-drug interactions should be paid more attention so as to reduce adverse reactions due to high plasma concentrations.

In conclusion, the conspicuous effects of puerarin *in vivo* on probes of CYP2B6, CYP2C9 and CYP3A4 metabolism suggest that there are clinically relevant herb-drug interactions between the drugs metabolized by these enzymes and puerarin when they are used concomitantly. Further clinical studies are required to fully assess the safety of puerarin in terms of CYP.

4. Experimental

4.1. Chemicals and reagents

Puerarin (98% purity) was purchased from Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China). Bupropion (purity > 98.0%), tolbutamide (purity > 98.0%), midazolam (purity > 98.0%) and the internal standard carbamazepine (IS, purity > 98.0%) were also purchased from Sigma-Aldrich Company (St. Louis, USA). HPLC grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were of analytical grade and used without further purification. Ultra-pure water (resistance > 18.2 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA).

4.2. Apparatus

All analyses were performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

4.3. Animals

Male Sprague-Dawley rats with body weights of 220 ± 30 g were provided by the Animal Care and Use Committee of Wenzhou Medical College. They were housed into house cages at 23–25 °C and had free access to regular rodent diet and water. After an 1-week acclimatization period, the rats were used for experiments and all efforts were made to minimize any animal suffering. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals.

4.4. *Drug administration and sampling*

Twelve male Sprague-Dawley rats were randomly divided into 2 groups (n = 6): control group (CG) and test group (TG, 180 mg/kg), which were given vehicle or puerarin (dissolved in 0.5% CMC-Na solution) once daily. After oral administration for consective 10 days, a cocktail solution at a dose of 5 mL/kg, which contained bupropion (20 mg/kg), tolbutamide (5 mg/kg) and midazolam (20 mg/kg) in CMC-Na solution, was administered orally to all rats in each group. Blood samples of each rat were collected predose (0 h) and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 h after probe drugs administration and immediately separated by centrifugation at 8,000 rpm for 10 min to obtain plasma. From the 7th blood collection, the rats were treated by oral administration of normal saline of the same blood collection volume in order to restore blood capacity quickly. 100 μL plasma samples were transferred to another tube and stored frozen at –80 °C until analyzed.

4.5. *Sample preparation*

In a 1.5 mL centrifuge tube, an aliquot of 0.2 mL acetonitrile with carbamazepine (500 ng/mL) as the internal standard was added to 0.1 mL of collected plasma sample. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 13,000 rmp for 10 min. Next, the supernatant (10 μL) was injected into the HPLC-MS/MS system for analysis.

4.6. *Chromatographic conditions*

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 column (150 mm × 2.1 mm, 3.5 μm) with the column temperature set at 30 °C. The mobile phase consisted of (A) acetonitrile and (B) 0.1% formic acid in water, and a gradient elution of 10–85% A at 0–1.5 min, 85–85% A at 1.5–6.0 min, 85–10% A at 6.0–7.0 min and 10–10% A at 7.0–10.0 min was employed. The flow rate was 0.4 mL/min. The injection volume was 10 μL.

The quantification was performed by the peak-area method. The determination of target ions were performed in SIM mode (m/z 240 for bupropion, m/z 271 for tolbutamide, m/z 326 for midazolam and m/z 237 for IS) and positive ion electrospray ionization interface. Drying gas flow was set to 6 L/min and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 20 psi and 3,500 V, respectively.

4.7. *Statistical analysis*

The concentration-time profile of each probe drug was analyzed by DAS software (Version 3.0, Wenzhou Medical College, China) and statistic analyses were tested by t-test using SPSS (Version 13.0, Wenzhou Medical College, China). A value of $P < 0.05$ was considered to be statistically significant.

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