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Assessment of the inhibition risk of shikonin on cytochrome P450 via cocktail inhibition assay

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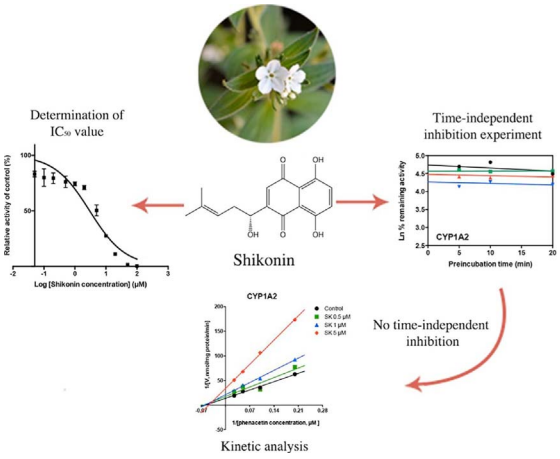
GRAPHICAL ABSTRACT



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ABSTRACT

Shikonin is a naphthoquinone pigment extracted from roots of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), and possesses various pharmaceutical activities, such as anti-inflammation and anti-cancer effects. In addition, shikonin as a natural red colorant for food garnishment and cosmetics ingredient is widely used in the world. However, the inhibition risk of shikonin on cytochrome P450 (CYP) remains unclear. The aim of this study was to investigate the potential inhibition of shikonin against CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1 and CYP3A2/4 activities in human and rat liver microsomes through cocktail approach *in vitro*. The results demonstrated that shikonin exhibited no time-dependent inhibition of CYP activities. In human liver microsomes, shikonin was not only a mixed inhibitor of CYP1A2, CYP2B6, CYP2C9, CYP2D6 and CYP3A4, but also a competitive inhibitor of CYP2E1, with K_i values no more than 7.72 μM. In rat liver microsomes, shikonin also exhibited the mixed inhibition on CYP1A2, CYP2B1, CYP2C11, CYP2D1, and the competitive inhibition on CYP2E1. Interestingly, shikonin presented an atypical kinetic inhibition of CYP3A2-mediated midazolam 1-hydroxylation in rats. In conclusion, the relatively low K_i values of shikonin would have a high risk

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potential to cause the possible toxicity, especially drug-drug or food-drug interactions based on the potent inhibition of CYP enzymes.

1. Introduction

Lithospermum erythrorhizon Sieb. et Zucc., known as Zicao, is documented in the "Bencao Gangmu" as an extensively-used Traditional Chinese Medicine for treating inflammation diseases in China. Shikonin (5,8-dihydroxy-2-[(1R)-1-hydroxy- 4-methyl-3-pentenyl]-1,4- naphthoquinone, Fig. 1) and its derivatives isolated from dried roots of *Lithospermum* are the principle components responsible for possessing therapeutic effects. With the exponentially increasing attention on shikonin, the major pharmacological properties of shikonin have been already elaborated in the previous studies (Andujar et al., 2013a), such as anti-inflammatory (Lee et al., 2010), antioxidant (Jin and Bai, 2012), antimicrobial (Miao et al., 2012) and wound healing activities (Andujar et al., 2013b).

In recent years, many studies have reported that shikonin exhibits potential anti-tumor effects on different kinds of cancers, such as myelogenous leukemia (Mao et al., 2008), breast cancer (Yin et al., 2016) and hepatocellular carcinoma (Song et al., 2016) through multiply molecular targets, thus suggesting that shikonin is a promising candidate for the development of antineoplastic drug. Moreover, in East Asia, especially in China, Japan and Korea, shikonin is specifically used as a colorant for food (Albreht et al., 2012; Ito et al., 2011; Kim et al., 2015) or a beneficial nature compound for cosmetics (Kim et al., 2015; Lee et al., 2008). For example, shikonin together with other components of *Lithospermum* are applied as pigments named "gromwell red", which are widely used in China as an additive for food and beverage.

Cytochrome P450 (CYP) enzymes are the major Phase I enzymes responsible for biotransformation of most drugs and other hydrophilic xenobiotics. In addition, CYP enzymes participate in cellular function through biosynthesis and metabolism of endogenous molecules (Nebert and Dalton, 2006). Actually, induction or inhibition of CYP will cause variation in drug pharmacokinetics, leading to decrease pharmacological efficacy or enhance the toxicity (Wang et al., 2010). Therefore, the U.S. Food and Drug Administration (FDA) has recommended investigating potential CYP inhibition of co-administration drugs, herbs or even food, which may avoid unnecessary attritions in drug development (Kerns and Di 2003; Sun et al., 2014). In particular, there is no related investigation between shikonin and CYP enzymes despite of its long-term application for Chinese herbal decoction and food garnishment.

The purpose of present study was to evaluate the inhibitory effects of shikonin on the major CYP enzymes, including CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1, CYP3A2/4, in both human and rat liver microsomes via a rapid six-in-one cocktail approach, which is a powerful tool for high-throughput inhibition risk screening *in vitro* (Dinger et al., 2014; Spaggiari et al., 2016). Enzyme inhibition kinetic analysis were also performed to study the mode of inhibition of shikonin on different CYP isoforms in human and rat liver microsomes using the CYP probe substrates.

2. Materials and methods

2.1. Chemicals and reagents

Shikonin (purity > 98%) was purchased from Dalian Meilun Biotech Co., Ltd (Dalian, China). Phenacetin, bupropion, tolbutamide, dextromethorphan, midazolam, 3-acetamidophenol (internal standard), chlorpropamide (internal standard), 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. (St. Louis, MO, USA). Chlorzoxazone was purchased from Alfa Aesar (Massachusetts, USA). 4-acetamidophenol, hydroxybupropion, 4-hydroxytolbutamide, dextrorphan, 6-hydroxychlorzoxazone and 1-hydroxymidazolam were obtained from Toronto Research Chemical (North York, Canada). Mebendazole (internal standard) was obtained from Aladdin Industrial Co. (California, USA). Pooled human liver microsomes (HLMs, n = 20) were obtained from Corning Gentest Corporation (Woburn, MA, USA) and stored at −150 °C until use. All the experimental procedures involving humans have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines. Acetonitrile and methanol (all HPLC grade) were obtained from Fisher Chemicals (Leicester, UK). Formic acid (HPLC grade) was purchased from TEDIA (Ohio, USA). Distilled water was purified in a Millipore system Milli Q (Millipore Corp., Bedford, MA, USA).

2.2. Animals

Male Sprague-Dawley rats (200–250 g) were supplied by National Rodent Laboratory Animal Resources, Shanghai Branch of China. Rats were kept in a specific pathogen-free facility and fed with standard food and water with 12 h light-dark cycles. All the methods in animals were carried out in accordance with the National Institutes of Health standards established in the 'Guidelines for the Care and Use of Experimental Animals'. All experimental protocols in animals were approved by the Ethics Committee on Animal Experimentation of East China Normal University (Shanghai, China).

2.3. Preparation of rat liver microsomes

The animals 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 homogenized in a 0.05 M Tris/KCl buffer (pH 7.4). The homogenate was centrifuged at 10,500 × *g* at 4 °C for 20 min, and the supernatant was centrifuged at 105,000 × *g* at 4 °C for 60 min. Then, the supernatant was discarded, and precipitate was further centrifuged at 105,000 × *g* at 4 °C for 60 min. The pellet was reconstituted with 0.05 M Tris/KCl buffer (pH 7.4) and stored at −150 °C until use. The protein concentration of the rat liver microsomes (RLMs) was determined by a protein quantitative assay using bicinchoninic acid (Sun et al., 2014).

2.4. Assays of CYP enzymes activities

According to the guideline of FDA, the activities of CYP enzymes were assessed by the formation of 4-acetamidophenol from Phenacetin (CYP1A2), hydroxybupropion from bupropion (CYP2B1/6), 4-hydroxytolbutamide from tolbutamide (CYP2C6/11), dextrorphan from

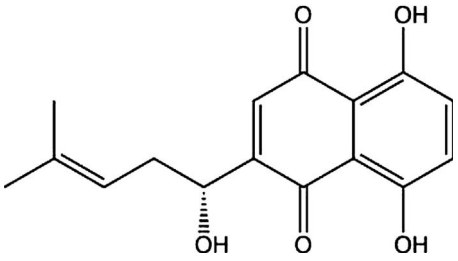

Fig. 1. Chemical structure of shikonin.

dextromethorphan (CYP2D1/6), 6-hydroxychlorzoxazone from chlorzoxazone (CYP2E1), and 1-hydroxymidazolam from midazolam (CYP3A2/4), respectively (Chen et al., 2016). The oxidation metabolism of probe substrates was carried out in microsomal incubation system at 37 °C. The incubation mixture (final volume of 200 μl in 0.05 M Tris/HCl buffer, pH 7.4) consisted of 0.5 mg/ml of HLMs or 1 mg/ml pooled RLMs with an NADPH-regenerating system including $MgCl_2$ (5 mM), G6P (10 mM), G6PDH (0.4 U/ml) and NADP (1 mM). Six probe substrates were added into the incubation mixture at close to each K_m with final concentrations (10 μM phenacetin, 20 μM bupropion, 20 μM tolbutamide, 5 μM dextromethorphan, 20 μM chlorzoxazone and 10 μM midazolam). The proportion of organic solvent was not higher than 1% (v/v) in the incubation mixture. After pre-incubation for 5 min, the reaction was initiated by addition of NADP. To determine the IC_{50} value for inhibition, the reaction mixtures in the presence of shikonin (0.2–20 μM) were performed for 20 min, stopped by adding 200 μl ice-cold acetonitrile, and then internal standard solution (20 μl) was added (3-acetamidophenol 2 μg/ml, chlorpropamide 2 μg/ml and mebendazole 2 μg/ml). The samples were mixed for 3 min and centrifuged for 15 min at 16,000 × *g* at 4 °C. The supernatant (60 μl) was transferred to autosampler vials, and 2 μl was injected into LC–MS/MS system.

2.5. Kinetic analysis of shikonin on CYPs activities in HLMs and RLMs

To investigate whether the inhibition effects by shikonin are time and/or concentration independent, the single-point inactivation experiments containing HLMs (0.5 mg/ml) or RLMs (1 mg/ml), $MgCl_2$ (5 mM), G6P (10 mM), G6PDH (0.4 U/ml) and 0.05 M Tris/HCl buffer (pH 7.4) with shikonin (0–5 μM) were performed for 0, 5, 10 and 20 min at 37 °C. After the inactivation incubation, the microsomal mixtures were diluted 10-fold, then NADP (1 mM) and probe substrates were added and further incubated for 20 min. The final concentrations of inhibitor were usually close to the IC_{25} after dilution, because the IC_{25} was the most sensitive concentration for detecting the time-dependent effects (Obach et al., 2007). To further confirm the reversible inhibition of shikonin on CYPs, the IC_{50} shift experiments containing HLMs (0.5 mg/ml) or RLMs (1 mg/ml), $MgCl_2$ (5 mM), G6P (10 mM), G6PDH (0.4 U/ml) and 0.05 M Tris/HCl buffer (pH 7.4) with shikonin (1–200 μM) were carried out under three different conditions: 0 min pre-incubation, 20 min pre-incubation plus NADPH, 20 min pre-incubation minus NADPH. All samples were treated with the same method mentioned above.

In enzyme kinetic studies, the types of inhibitory effects of shikonin on CYPs were carried out with various substrate concentrations in the presence of inhibitor or not. HLMs (0.5 mg/ml) with shikonin (1 μM, 2 μM, 5 μM, 10 μM) or RLMs (1 mg/ml) with shikonin (0.5 μM, 1 μM, 2 μM, 5 μM) in 0.05 M Tris/HCl buffer (pH 7.4) was incubated for 20 min at 37 °C. The following concentrations for cocktail probe substrates were used: 5–40 μM phenacetin for CYP1A2; 10–80 μM bupropion for CYP2B1/6; 20–160 μM tolbutamide for CYP2C9; and 10–80 μM tolbutamide for CYP2C11; 2–20 μM dextromethorphan for CYP2D1/6; 10–80 μM chlorzoxazone for CYP2E1; 2.5–80 μM midazolam for CYP3A2; and 0.625–5 μM midazolam for CYP3A4. To confirm the inhibition assays, the specific CYP inhibitors were also used as positive controls. In this study, the positive controls included furafylline for CYP1A2, thiotepa for CYP2B1/6, sulfaphennazole for CYP2C9/11, quinidine for CYP2D1/6, sodium diethyl-dithiocarbamate for CYP2E1, and ketoconazole for CYP3A2/4.

2.6. LC–MS/MS analysis

An Agilent 1290 LC system, consisting of a degasser, a binary pump, an autosampler and a thermostatic column compartment, was coupled with a 6460 triple-quadrupole mass spectrometer (Agilent Technologies, USA), which was equipped with an Agilent Jet Stream

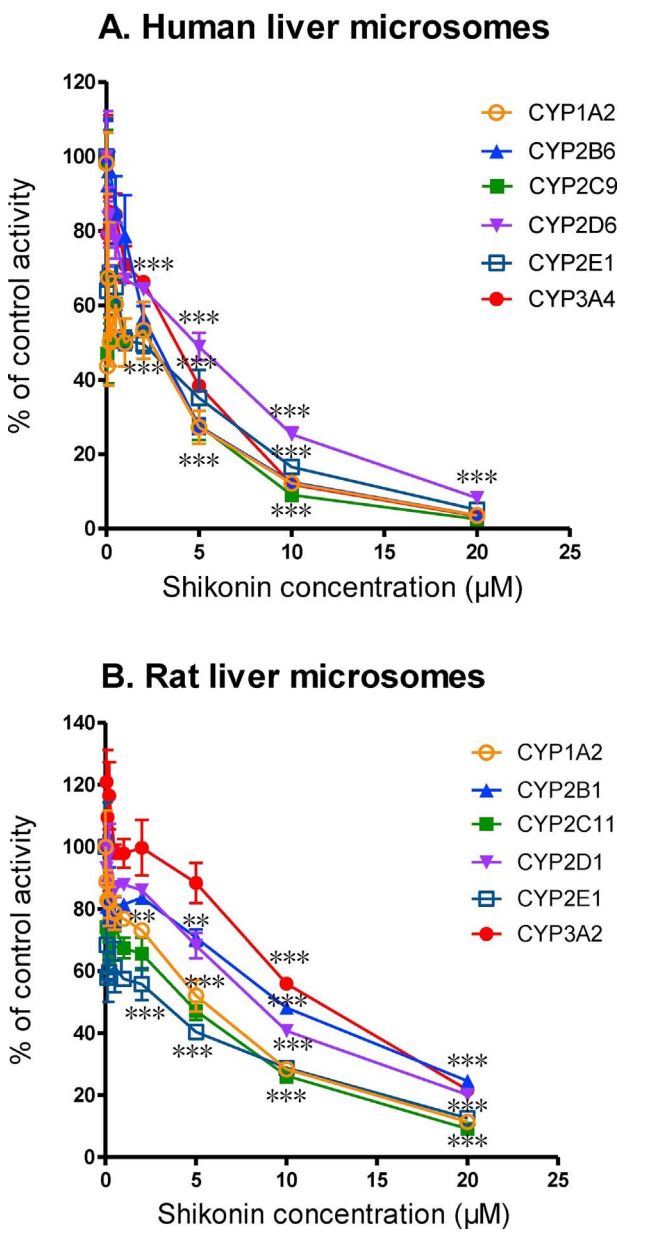

Fig. 2. Inhibitory effect of shikonin (0.2–20 μM) on the CYPs in human and rat liver microsomes. Values are expressed as mean ± SD (n = 4). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control (no shikonin).

Table 1
The IC_{50} value, K_i value, αK_i value, α value and inhibition type of shikonin on CYPs in HLMs and RLMs. Each data are the means of quadruplicate determinations.

	CYP isoform	IC_{50} (μM)	K_i (μM)	αK_i (μM)	α	Inhibition type
HLMs	CYP1A2	5.32	1.25	2.91	2.33	Mixed
	CYP2B6	2.20	0.41	2.68	6.54	Mixed
	CYP2C9	1.01	0.54	1.58	2.93	Mixed
	CYP2D6	2.88	2.66	5.09	1.91	Mixed
	CYP2E1	1.21	1.68	–	–	Competitive
	CYP3A4	2.57	7.72	6.27	0.81	Mixed
RLMs	CYP1A2	4.19	2.62	4.14	1.58	Mixed
	CYP2B1	8.23	8.08	13.77	1.70	Mixed
	CYP2C11	2.36	3.55	5.48	1.54	Mixed
	CYP2D1	7.66	4.14	15.07	3.59	Mixed
	CYP2E1	1.47	5.79	–	–	Competitive
	CYP3A2	8.80	–	–	–	Atypical kinetics

electrospray ionization (ESI) source and operated with Agilent MassHunter version 5.0.280.1 sofware (Agilent Technologies, USA). Chromatography separation was performed on a Phenomenex Kinetex XB-C18 column (100 × 3.00 mm, 2.6 μM) protected by a Phenomenex C18 guard column (Torrance, CA, USA). To detect the concentrations of several CYP substrates and metabolites, the mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) using gradient elution at a flow rate of 0.3 ml/min. The optimum condition for elution was as followed: 0–2.2 min, 10–11% B; 2.2–8.5 min, 11–90% B; 8.5–9 min, 90–92% B; 9–9.3 min, 92% B; 9.3–9.6 min, 92–10% B; 9.6–11.5 min, 10% B. The chromatograms of monitoring analytes have been presented previously (Chen et al., 2016).

2.7. Data analysis

All data were presented as mean ± SD. One-way analysis of variance was used to estimate the significance of differences. A p-value less than 0.05 was considered to indicate statistical significance. The IC_{50} values (concentration of inhibitor to cause 50% inhibition of original

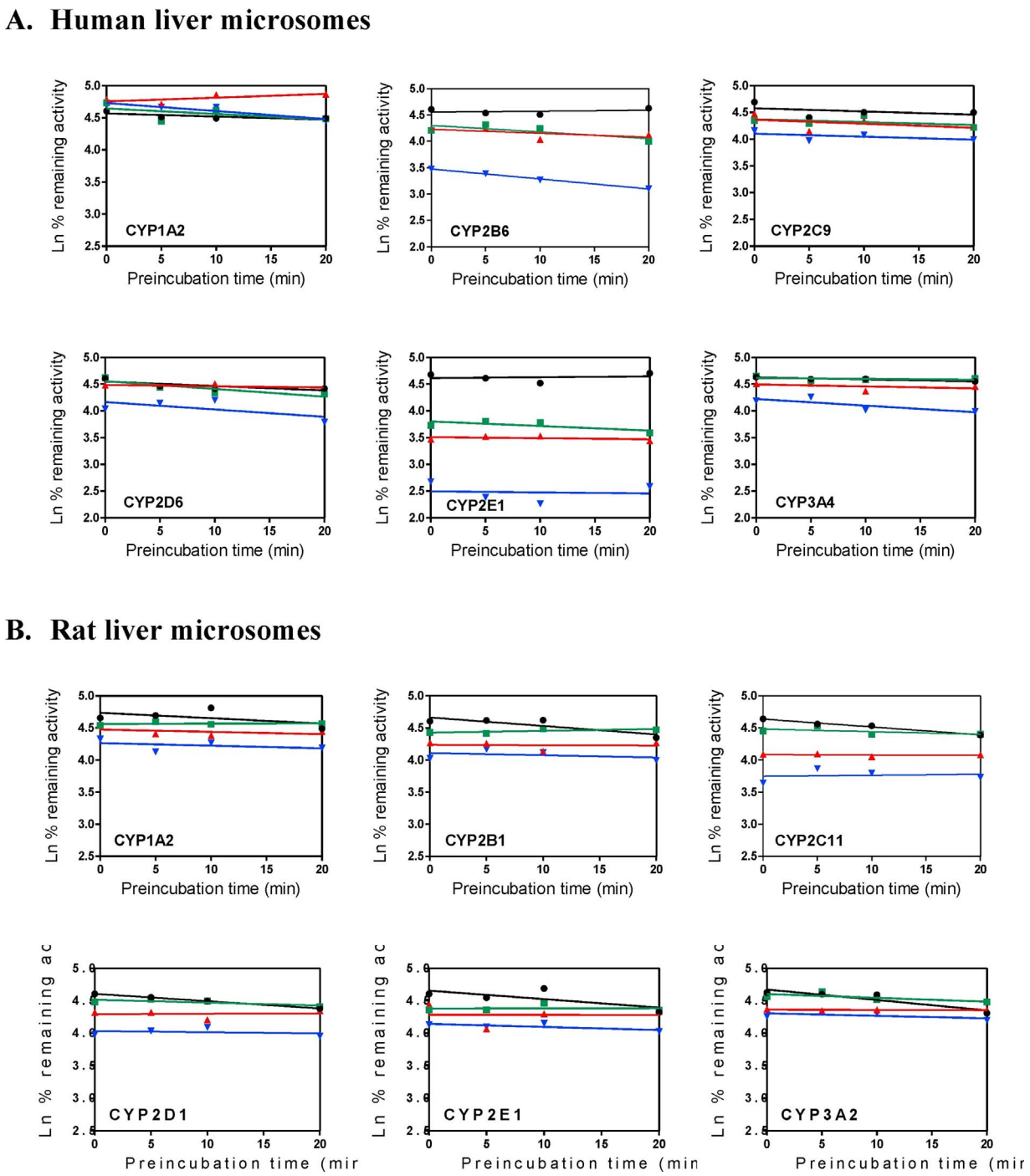

Fig. 3. Single point inactivation experiments of shikonin on human and rat liver microsomal CYPs. Microsomal reaction mixtures were pre-incubated for 20 min without (●) or with shikonin at concentration at 0.1 μM (■), 0.2 μM (▲), 0.5 μM (▼). Each data point represents the mean of quadruplicate determinations.
A. Human liver microsomes.
B. Rat liver microsomes.

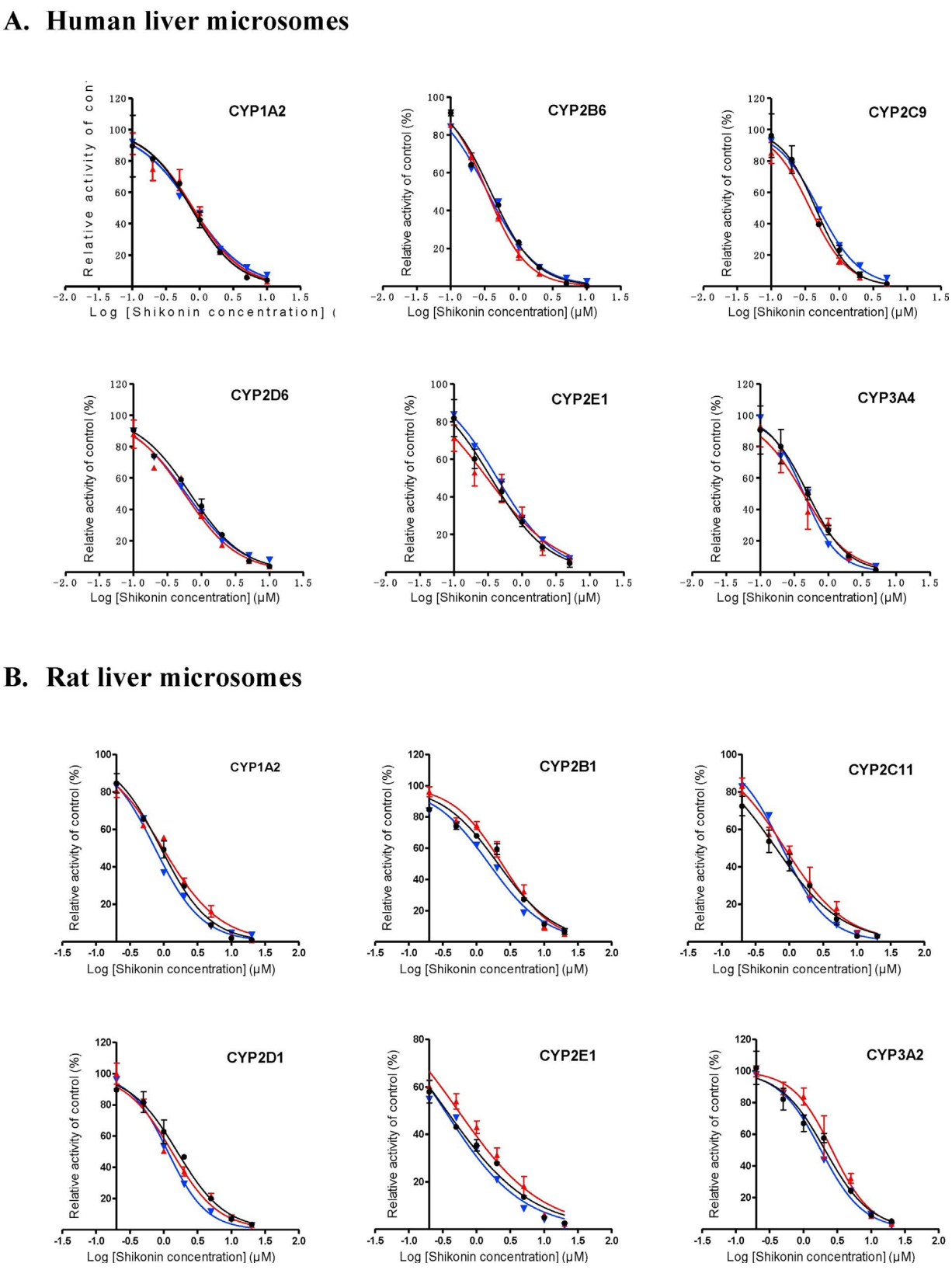

Fig. 4. IC_{50} shift experiments of shikonin on human and rat liver microsomal CYPs. Microsomal reaction mixtures were pre-incubated for three conditions: 0 min pre-incubation (●), 20 min pre-incubation with NADPH (▲) and 20 min pre-incubation minus NADPH (▼). Each data point represents the mean of quadruplicate determinations.
A. Human liver microsomes.
B. Rat liver microsomes.

enzyme activity) were determined by a nonlinear regression analysis of plotting relative activities over the logarithm of shikonin concentrations. The inactivation plots were performed by the natural logarithm of remaining enzyme activity *vs* pre-incubation time. Enzyme kinetics data were fitted with nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). The data were fitted to the Michaelis–Menten model and further analyzed using the Lineweaver-Burk plot (the reciprocal of reaction velocities *versus* the reciprocal of substrates concentrations). The secondary plot for K_i value was obtained by the slopes of the regression lines in the Lineweaver–Burk plot *vs* inhibitor concentration, while the secondary plot for αK_i was obtained by the y-intercepts of the regression lines in the Lineweaver–Burk plot *vs* inhibitor concentrations. The atypical kinetics of CYP3A2 was analyzed by Prism where appropriately using the Hill equation showed in Eq. (1) and Eadie-Hofstee plot.

$$v = \frac{V_{max} \times [S]^n}{{K_{half}}^n + [S]^n} \quad (1)$$

Here n is the Hill coefficient, which can be used as the index of cooperativity. K_{half} is the constant that produce half-maximal velocity, but when n ≠ 1, K_{half} is not equivalent to the Michaelis constant (K_m) due to the interaction factors (Tracy, 2006).

3. Results

3.1. Inhibitory effects of shikonin on CYP activities in human and rat liver microsomes

For assessing the direct inhibitory potential of shikonin on CYPs, the IC_{50} values were determined with specific concentrations of shikonin ranged from 0.2 μM to 20 μM. The results showed that in HLMs, shikonin potently inhibited CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 with IC_{50} value of 5.32 μM, 2.20 μM, 1.01 μM, 2.88 μM, 1.21 μM and 2.57 μM (Fig. 2A, Table 1), respectively. In RLMs, shikonin also exhibited potent inhibition effects on CYP1A2, CYP2B1, CYP2C11, CYP2D1, CYP2E1, and CYP3A2 with IC_{50} value of 4.19 μM, 8.23 μM, 2.36 μM, 7.66 μM, 1.47 μM and 8.80 μM (Fig. 2B, Table 1), respectively.

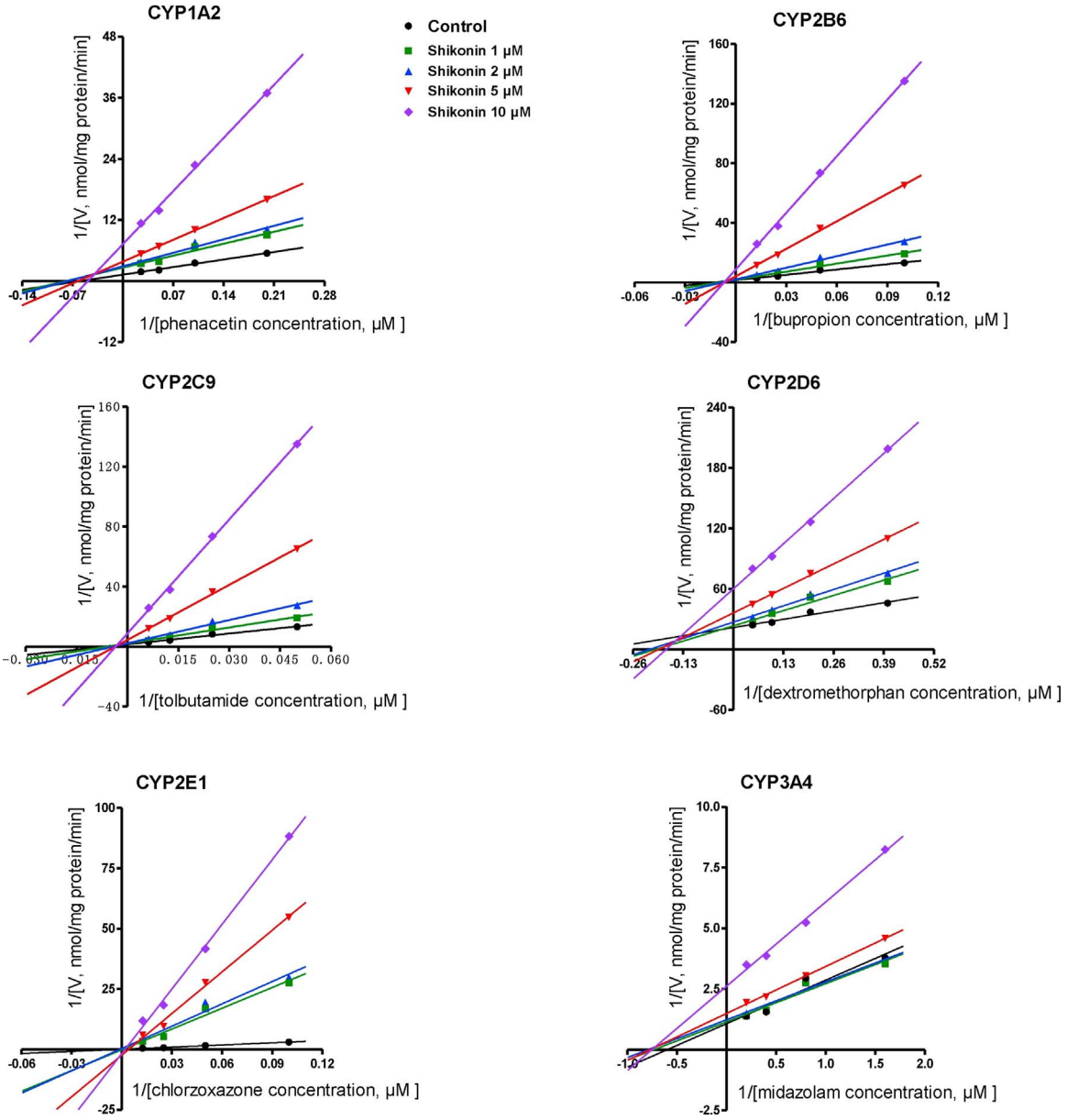

Fig. 5. Primary Lineweaver–Burk plot for six CYP-mediated probe substrates metabolism with 1 μM, 2 μM, 5 μM, 10 μM of shikonin in human liver microsomes.

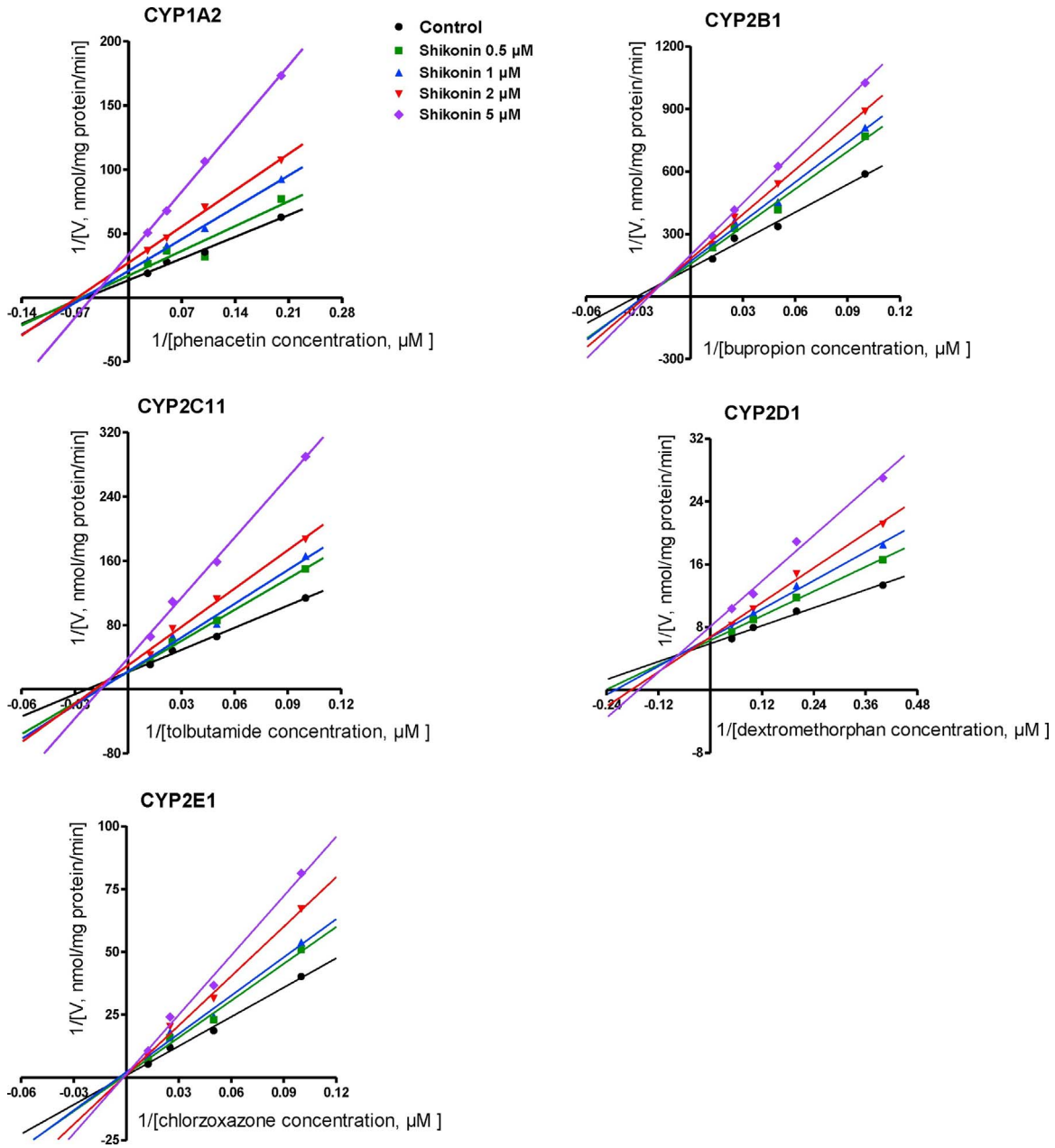

Fig. 6. Primary Lineweaver–Burk plot for five CYP-mediated probe substrates metabolism with 0.5 μM, 1 μM, 2 μM, 5 μM of shikonin in rat liver microsomes.

3.2. Time-dependent analysis for inhibition of CYPs by shikonin in HLMs and RLMs

To characterize the reversible or irreversible mechanism of CYPs inhibition by shikonin, the time and concentration independent experiments were used to determine whether it was both time-dependent and concentration-dependent. In the single point inactivation experiments, although a slight decrease in activity was observed on CYP2B6, CYP2D6 and CYP3A4 (Fig. 3), there were all less than 15%, which is a cut-off value for identifying time-dependent effects (Obach et al., 2007). Furthermore, in the IC_{50} shift profiles, no significant changes in IC_{50} values were observed among three groups (Fig. 4), thus suggesting shikonin was a reversible inhibitor in HLMs and RLMs.

3.3. Enzyme inhibition kinetic analysis for inhibition of CYPs by shikonin in HLMs and RLMs

To further investigate the inhibitory modes of CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1 and CYP3A2/4 by shikonin, enzyme inhibition kinetic experiments were carried out with different concentrations of substrates. In HLMs, the Lineweaver-Burk plots showed that shikonin was not only a mixed inhibitor of CYP1A2, CYP2B6, CYP2C9, CYP2D6 and CYP3A4 with K_i values of 1.25 μM, 0.41 μM, 0.54 μM, 2.66 μM and 7.72 μM (Fig. 5, Fig. S1, Table 1), but also was a competitive inhibitor on CYP2E1 with K_i value of 1.68 μM (Fig. 5, Fig. S1, Table 1). In RLMs, the primary Lineweaver-Burk plots presented that CYP2E1 had a typical pattern of competitive inhibition with K_i value of 5.79 μM, but inhibited CYP1A2, CYP2B1, CYP2C11, and CYP2D1 in a mixed manner with K_i value of 2.62 μM, 8.08 μM, 3.55 μM, and 4.20 μM, respectively (Fig. 6, Fig. S2, Table 1). Moreover, the calculated α values of CYP1A2, CYP2B1, CYP2C11, CYP2D1 were not equal to 1 (Table 1), indicating the mixed inhibition type. Interestingly, the enzyme inhibition kinetics of CYP3A2 did not show conform to the classical Michaelis-Menten kinetics, but fit to the atypical kinetics and Hill equation (Fig. 7). At the same time, the Hill coefficient values were all greater than one (Table 2).

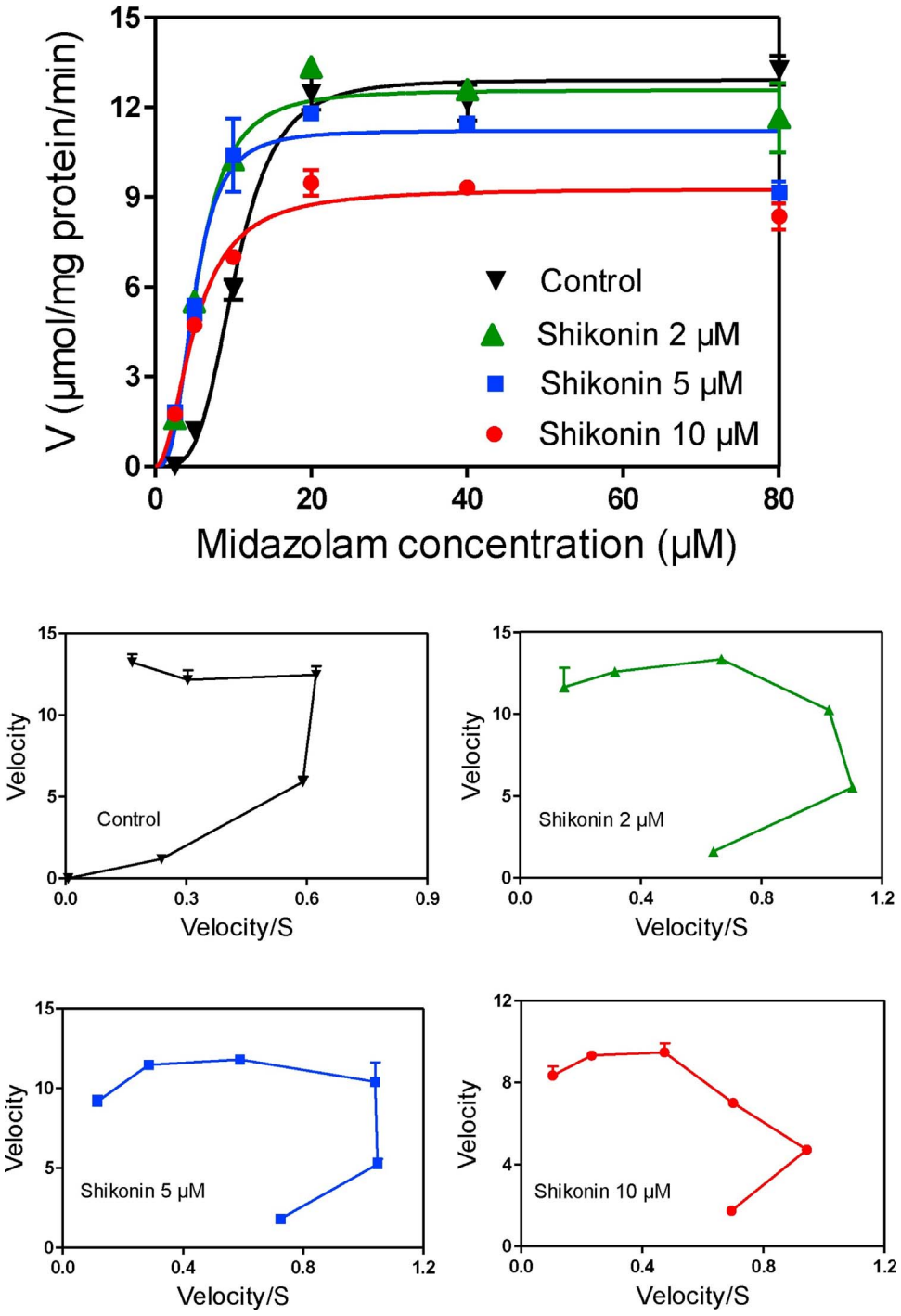

Fig. 7. Kinetics plot and corresponding Eadie-Hofstee plots of 1-hydroxymidazolam in rat liver microsomes. Midazolam was used at concentrations of 2.5, 5, 10, 20, 40 and 80 μM.

Table 2
Kinetic parameters of the shikonin on CYP3A2-mediated midazolam 1-hyroxylation. Data represent the mean ± SD (n = 4).

Inhibitor concentration (μM)	K_{half} (μM)	V_{max} (μmol/mg protein/min)	n
0	10.28 ± 0.27	12.91 ± 0.22	3.86 ± 0.20
2	5.45 ± 0.30	12.57 ± 0.35	2.71 ± 0.51
5	4.98 ± 0.25	11.21 ± 0.26	3.02 ± 0.30
10	5.08 ± 0.27	9.27 ± 0.21	2.04 ± 0.44

4. Discussion

According to the relevant significance of individual CYP isoform, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 are responsible for metabolizing a majority of clinical drugs (Zanger and Schwab, 2013). Thus, in this study we selected these CYP isoforms for monitoring the potential interaction using cocktail approach. CYP1A2 is one of the major numbers in CYP1A subfamily which plays a significant role in metabolism of several therapeutic agents such as acetaminophen, phenacetin, theophylline, clozapine and tacrine (Faber et al., 2005). Moreover, it is also capable of activating pro-carcinogens and maintaining hormone level steady (Adehin and Bolaji, 2015). The CYP2B subfamily constitutes about 4% of total content of hepatic CYPs,

but is highly inducible by many xenobiotics (Thorn et al., 2010). CYP2C9 could catalyze 10–20% of prescribe drugs including non-steroidal anti-inflammatory drugs, oral hypoglycemic agents and oral anticoagulants as well as endogenous substances like fatty acids, prostanoids and steroid hormones (Sun et al., 2015). While in male rats, CYP2C11 is far more important because the expression of CYP2C11 is more abundant than other CYPs, even in a way as the same value of CYP3A4 in humans (Sun et al., 2014). CYP2D6 is widely expressed in liver and other extrahepatic tissue, and it contributes to approximately 25% of clinical trials despite of its only accounting for 1.3-4.3% of total haptic CYPs content. Furthermore, a series of physiological, pathological and environmental factors are capable of modulating the activity and expression of CYP2D6 (He et al., 2015). CYP2E1 as one important enzyme also metabolizes many xenobiotics, while it is easy to be influenced by disease such as alcoholic fatty liver, obesity and diabetes (Wang et al., 2016). As the most predominant enzyme among CYP isoforms, CYP3A4 participates in metabolism of over 50% of market drugs (Lu et al., 2017).

To our knowledge, this study is the first time to investigate the effects of shikonin on the activities of six CYP isoforms in both human and rat liver microsomes. A comparison of the inhibitory effects of shiknoin on CYPs in HLMs with RLMs indicated that there were significant differences among species on the intensity of inhibition. In general, the inhibitory effects with lower K_i value of shikonin on CYPs in HLMs were stronger than those in RLMs. The variation in drug metabolism among species was attributed to the composition of different CYP isoforms, expression levels and catalyzed activities of CYPs (Martignoni et al., 2006). For examples, CYP2D6 is the only member of CYP2D subfamily in humans, while there were six isoforms (CYP2D1, CYP2D2, CP2D3, CYP2D4, CYP2D5, CYP2D18) identified in rats (Lewis et al., 1998). In this study, our results showed that the enzyme kinetics of CYP3A2 in RLMs disagreed with the classical Michaelis–Menten kinetics, but the enzyme kinetics of CYP3A4 in HLMs conformed to it when the concentration of midazolam was less than 5 μM. In fact, some studies have emphasized on the atypical enzyme kinetics, especially on CYP3A (Houston and Kenworthy, 2000). For example, our previous studies found that plumbagin also exhibited atypical inhibition kinetics of CYP3A2 (Chen et al., 2016). The central hypothesis of atypical kinetics has been proposed that multiply substrates can simultaneously bind to an activate site of the enzyme (Atkins 2005; Korzekwa et al., 1998). In the present study, the CYP3A2-mediated midazolam 1-hydroxylation displayed auto-activation kinetics (homotropic positive cooperativity), which fits to the Hill equation with the Hill coefficient value greater than one, and also fits to the sigmoidal Eadie-Hofstee plot (Hutzler and Tracy 2002; Houston and Galetin, 2005). In addition, shikonin also showed heterotropic activation on CYP3A2-meditaed midazolam 1-hyroxylation, which could explain the phenomenon of temporary induction happened under the low concentration of midazolam (< 5 μM). These results suggested that there were at least two active sites existing in CYP3A2, which exhibited positive cooperativity with respect to substrate binding (Goutelle et al., 2008). Since the mechanism of atypical kinetics has not yet been fully elucidated, further studies are required to investigate this issue and make sure whether the similar atypical kinetics could also appear *in vivo*.

The previous studies showed that shikonin exerts significantly antitumor activity against H22 tumors *in vivo* after a 7-day intravenous treatment with 4 or 8 mg/kg shikonin (Yang et al., 2009). Moreover, pharmacokinetic studies reported that after intravenous administration of 5 mg/kg shikonin in rats, the peak concentration (C_{max}) in plasma was 83.6 ng/ml (0.29 μM) (Huang et al., 2010), which is close to the K_i values determined in the present study. Therefore, shikonin could quite probably decrease the activities of CYPs, especially CYP2 B and CYP2C *in vivo*. Except for the classical drug-drug interactions, a growing number of studies have focused on the interactions between drugs and herbs, food as well as beverages. For example, the grapefruit juice-drug interactions have been reported and investigated (Ameer and Weintraub 1997; Bailey et al., 1998). In fact, the original component in food is an important factor to trigger the food-drug interactions. For examples, red yeast rice is a kind of additive applied for garnishment, which has been proved to inhibit CYP3A4 and P-glycoprotein (P-gp) activities and thereby has an impact on the absorption of verapamil (Fung et al., 2012). Currently, shikonin extracted from gromwell, as a colorant named “gromwell red” (CNS number: 08.140), is listed in the Chinese National Food Safety Standard for Food Additive Use (GB2760-2014), such as in frozen drinks (0.1 g/kg), pastries (0.9 g/kg), cookies (0.1 g/kg), baked food filling and topping syrups (1 g/kg), fruit and vegetable juice (0.1 g/kg), fruit-flavored drinks (0.1 g/kg) and fruit wine (0.1 g/kg). Therefore, if the shikonin or any shikonin-contained food and drinks are concomitantly consumed with market drugs, it may induce the unexpected toxic reaction based on the inhibition risk of shikonin on CYPs.

5. Conclusions

This study assessed the inhibition risk of shikonin on CYP enzymes *in vitro* via cocktail inhibition assay. Shikonin exhibited broad-spectrum and potent inhibitory effects toward CYP1A2, CYP2B1/6, CYP2C9/11, CYP2D1/6, CYP2E1 and CYP3A2/4 with lower K_i values in both human and rat liver microsomes. These results provided a useful guidance for safe and effective usage of shikonin. However, the significance of the interactions between shikonin and CYP should be confirmed with further human *in vivo* studies.

6. Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.09.014.

References

Adehin, A., Bolaji, O.O., 2015. Polymorphisms of CYP1A2 and CYP2A6 activity: phenotypes and the effect of age and sex in a Nigerian population. Drug Metab Pers Ther 30 (3), 203–210. http://dx.doi.org/10.1515/dmpt-2015-0001.

Albreht, A., Vovk, I., Simonovska, B., 2012. Addition of beta-lactoglobulin produces water-soluble shikonin. J. Agric. Food Chem. 60 (43), 10834–10843. http://dx.doi.org/10.1021/jf303153d.

Ameer, B., Weintraub, R.A., 1997. Drug interactions with grapefruit juice. Clin. Pharmacokinet. 33 (2), 103–121. http://dx.doi.org/10.2165/00003088-199733020-00003.

Andujar, I., Rios, J.L., Giner, R.M., Recio, M.C., 2013a. Pharmacological properties of shikonin – a review of literature since 2002. Planta Med. 79 (18), 1685–1697. http://dx.doi.org/10.1055/s-0033-1350934.

Andujar, I., Rios, J.L., Giner, R.M., Recio, M.C., 2013b. Shikonin promotes intestinal wound healing in vitro via induction of TGF-beta release in IEC-18 cells. Eur. J. Pharm. Sci. 49 (4), 637–641. http://dx.doi.org/10.1016/j.ejps.2013.05.018.

Atkins, W.M., 2005. Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions. Annu. Rev. Pharmacol. Toxicol. 45, 291–310. http://dx.doi.org/10.1146/annurev.pharmtox.45.120403.100004.

Bailey, D.G., Malcolm, J., Arnold, O., Spence, J.D., 1998. Grapefruit juice-drug interactions. Br. J. Clin. Pharmacol. 46 (2), 101–110.

Chen, A., Zhou, X., Tang, S., Liu, M., Wang, X., 2016. Evaluation of the inhibition potential of plumbagin against cytochrome P450 using LC–MS/MS and cocktail approach. Sci. Rep. 6, 28482. http://dx.doi.org/10.1038/srep28482.

Dinger, J., Meyer, M.R., Maurer, H.H., 2014. Development of an in vitro cytochrome P450 cocktail inhibition assay for assessing the inhibition risk of drugs of abuse. Toxicol.

Lett. 230 (1), 28–35. http://dx.doi.org/10.1016/j.toxlet.2014.08.004.
Faber, M.S., Jetter, A., Fuhr, U., 2005. Assessment of CYP1A2 activity in clinical practice: why, how, and when? Basic Clin. Pharmacol. Toxicol. 97 (3), 125–134. http://dx.doi.org/10.1111/j.1742-7843.2005. pto_973160.x.
Fung, W.T., Subramaniam, G., Lee, J., Loh, H.M., Leung, P.H., 2012. Assessment of extracts from red yeast rice for herb-drug interaction by in-vitro and in-vivo assays. Sci. Rep. 2, 298. http://dx.doi.org/10.1038/srep00298.
Goutelle, S., Maurin, M., Rougier, F., et al., 2008. The Hill equation: a review of its capabilities in pharmacological modelling. Fundam. Clin. Pharmacol. 22 (6), 633–648. http://dx.doi.org/10.1111/j.1472-8206.2008.00633.x.
He, Z.X., Chen, X.W., Zhou, Z.W., Zhou, S.F., 2015. Impact of physiological, pathological and environmental factors on the expression and activity of human cytochrome P450 2D6 and implications in precision medicine. Drug Metab. Rev. 47 (4), 470–519. http://dx.doi.org/10.3109/03602532.2015.1101131.
Houston, J.B., Galetin, A., 2005. Modelling atypical CYP3A4 kinetics: principles and pragmatism. Arch. Biochem. Biophys. 433 (2), 351–360. http://dx.doi.org/10.1016/j.abb.2004.09.010.
Houston, J.B., Kenworthy, K.E., 2000. In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. Drug Metab. Dispos. 28 (3), 246–254.
Huang, S., Yu, L., Shen, P., 2010. Simultaneous quantitative analysis of shikonin and deoxyshikonin in rat plasma by rapid LC–ESI–MS–MS. Chromatographia 72 (1-2), 63–69. http://dx.doi.org/10.1365/s10337-010-1599-5.
Hutzler, J.M., Tracy, T.S., 2002. Atypical kinetic profiles in drug metabolism reactions. Drug Metab. Dispos. 30 (4), 355–362.
Ito, Y., Onobori, K., Yamazaki, T., Kawamura, Y., 2011. Tigloylshikonin, a new minor Shikonin derivative, from the roots and the commercial root extract of lithospermum erythrorhizon. Chem. Pharm. Bull. (Tokyo) 59 (1), 117–119.
Jin, R., Bai, Y., 2012. Theoretical investigation of the radical scavenging activity of shikonin and acylshikonin derivatives. J. Mol. Model. 18 (4), 1401–1408. http://dx.doi.org/10.1007/s00894-011-1170-9.
Kerns, E.H., Di, L., 2003. Pharmaceutical profiling in drug discovery. Drug Discov. Today 8 (7), 316–323.
Kim, J.S., Seo, Y.C., No, R.H., Lee, H.Y., 2015. Improved cosmetic activity by optimizing the Lithospermum erythrorhizon extraction process. Cytotechnology 67 (1), 51–65. http://dx.doi.org/10.1007/s10616-013-9657-0.
Korzekwa, K.R., Krishnamachary, N., Shou, M., et al., 1998. Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. Biochemistry 37 (12), 4137–4147. http://dx.doi.org/10.1021/bi9715627.
Lee, H.Y., Kim, Y.J., Kim, E.J., Song, Y.K., Byun, S.Y., 2008. Red pigment from Lithospermum erythrorhizon by supercritical CO2 extraction. J. Cosmet. Sci. 59 (5), 431–440.
Lee, C.C., Wang, C.N., Lai, Y.T., et al., 2010. Shikonin inhibits maturation of bone marrow-derived dendritic cells and suppresses allergic airway inflammation in a murine model of asthma. Br. J. Pharmacol. 161 (7), 1496–1511. http://dx.doi.org/10.1111/j.1476-5381.2010.00972.x.
Lewis, D.F., Ioannides, C., Parke, D.V., 1998. Cytochromes P450 and species differences in xenobiotic metabolism and activation of carcinogen. Environ. Health Perspect. 106 (10), 633–641.
Lu, J., Shao, Y., Qin, X., et al., 2017. CRISPR knockout rat cytochrome P450 3A1/2 model for advancing drug metabolism and pharmacokinetics research. Sci. Rep. 7, 42922. http://dx.doi.org/10.1038/srep42922.
Mao, X., Yu CR, Li WH, Li, W.X., 2008. Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells. Cell Res. 18 (8), 879–888. http://dx.doi.org/10.1038/cr.2008.86.
Martignoni, M., Groothuis, G.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin. Drug Metab. Toxicol. 2 (6), 875–894. http://dx.doi.org/10.1517/17425255.2.6.875.
Miao, H., Zhao, L., Li, C., et al., 2012. Inhibitory effect of Shikonin on Candida albicans growth. Biol. Pharm. Bull. 35 (11), 1956–1963.
Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nat. Rev. Cancer 6 (12), 947–960. http://dx.doi.org/10.1038/nrc2015.
Obach, R.S., Walsky, R.L., Venkatakrishnan, K., 2007. Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions. Drug Metab. Dispos. 35 (2), 246–255. http://dx.doi.org/10.1124/dmd.106.012633.
Song, J., Zhao, Z., Fan, X., et al., 2016. Shikonin potentiates the effect of arsenic trioxide against human hepatocellular carcinoma in vitro and in vivo. Oncotarget 7 (43), 70504–70515. http://dx.doi.org/10.18632/oncotarget.12041.
Spaggiari, D., Daali, Y., Rudaz, S., 2016. An extensive cocktail approach for rapid risk assessment of in vitro CYP450 direct reversible inhibition by xenobiotic exposure. Toxicol. Appl. Pharmacol. 302, 41–51. http://dx.doi.org/10.1016/j.taap.2016.04.013.
Sun, M., Tang, Y., Ding, T., Liu, M., Wang, X., 2014. Inhibitory effects of celastrol on rat liver cytochrome P450 1A2, 2C11, 2D6, 2E1 and 3A2 activity. Fitoterapia 92, 1–8. http://dx.doi.org/10.1016/j.fitote.2013.10.004.
Sun, M., Tang, Y., Ding, T., Liu, M., Wang, X., 2015. Investigation of cytochrome P450 inhibitory properties of maslinic acid, a bioactive compound from Olea europaea L., and its structure-activity relationship. Phytomedicine 22 (1), 56–65. http://dx.doi.org/10.1016/j.phymed.2014.10.003.
Thorn, C.F., Lamba, J.K., Lamba, V., Klein, T.E., Altman, R.B., 2010. PharmGKB summary: very important pharmacogene information for CYP2B6. Pharmacogenet. Genom. 20 (8), 520–523.
Tracy, T.S., 2006. Atypical cytochrome p450 kinetics: implications for drug discovery. Drugs R. D. 7 (6), 349–363.
Wang, X., Cheung, C.M., Lee, W.Y., Or, P.M., Yeung, J.H., 2010. Major tanshinones of Danshen (Salvia miltiorrhiza) exhibit different modes of inhibition on human CYP1A2, CYP2C9, CYP2E1 and CYP3A4 activities in vitro. Phytomedicine 17 (11), 868–875. http://dx.doi.org/10.1016/j.phymed.2010.05.003.
Wang, X., Tang, Y., Lu, J., et al., 2016. Characterization of novel cytochrome P450 2E1 knockout rat model generated by CRISPR/Cas9. Biochem. Pharmacol. 105, 80–90. http://dx.doi.org/10.1016/j.bcp.2016.03.001.
Yang, H., Zhou, P., Huang, H., et al., 2009. Shikonin exerts antitumor activity via proteasome inhibition and cell death induction in vitro and in vivo. Int. J. Cancer 124 (10), 2450–2459. http://dx.doi.org/10.1002/ijc.24195.
Yin, S.Y., Efferth, T., Jian, F.Y., et al., 2016. Immunogenicity of mammary tumor cells can be induced by shikonin via direct binding-interference with hnRNPA1. Oncotarget 7 (28), 43629–43653. http://dx.doi.org/10.18632/oncotarget.9660.
Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol. Ther. 138 (1), 103–141. http://dx.doi.org/10.1016/j.pharmthera.2012.12.007.