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ORIGINAL ARTICLE

Species Difference of Asarinin Metabolism *in vitro* and its Effect on the Activity of Cytochrome P450 Enzymes

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ABSTRACT

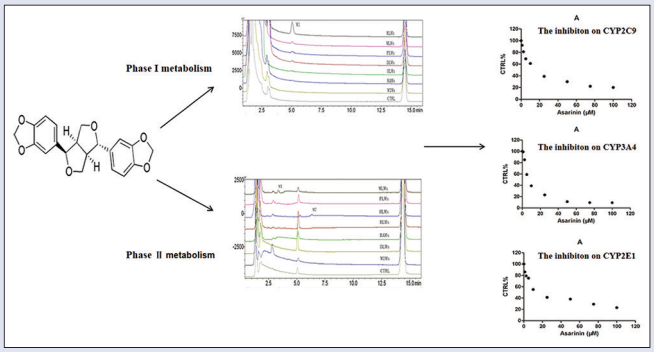
Aim: To investigate the metabolic behaviors of asarinin in different species and found animal models with metabolic pathways similar to those in humans and to study the effect (inhibition or induction) between asarinin and cytochrome P450 enzymes (CYP450s). **Materials and Methods:** The interspecies difference in asarinin metabolism was studied using liver microsomes (LMs) from humans and six mammals including rabbits, mini pigs, dogs, monkeys, rats, and mice. The potential drug–drug interactions (DDIs) between asarinin and cytochrome P450 enzymes was studied by incubating with six CYP enzymes in human liver microsomes (HLMs). **Results:** The metabolic profiles revealed substantially different metabolism of asarinin among the seven species. Moreover, the potential DDIs between asarinin and cytochrome P450 enzymes were studied *in vitro* by incubating with six CYP enzymes in HLMs, it was found that asarinin inhibited CYP2C9, CYP3A4, and CYP2E1 activity. **Conclusion:** The interspecies comparison can help find other species that have similar metabolic pathways to humans. There were potential DDIs between asarinin and some drugs metabolized by CYP2C9, CYP2E1, and CYP3A4.

Key words: Asarinin, cytochrome p450, inhibition, liver microsomes, metabolism

SUMMARY

- There are significant differences in the metabolism of asarinin among the seven species. Asarinin can inhibit the activity of CYP2C9, CYP3A4 and CYP2E1.

Abbreviations used: MIMs: Mice microsomes; PLMs: Mini pig microsomes; RLMs: Rat microsomes; RAMs: Rabbit microsomes; MLMs: Monkeys microsomes; DLMs: Dog microsomes; DDIs: Drug–drug interactions; HLMs: Human liver microsomes; HPLC: High-performance liquid chromatography.



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INTRODUCTION

Asarinin [Figure 1] has shown a variety of pharmacological and biological activities. Asarinin is one of the major effective chemical components isolated from asarum, a traditional Chinese medicine,[1] which has antiviral and antituberculous effects, and has certain curative effects on trachitis in clinical observation.[2] In addition, it has the functions of warming meridians, dispersing cold, removing wind, and relieving pain.[3] In recent years, studies on the pharmacological action of asarum have found that asarinin also has anti-immune effect.[4] However, drug–drug interaction (DDI) is always a potential problem during the development of the new drug, which is the major factor that influences pharmaceutical effect and pharmacokinetics, especially in Chinese traditional medicine.[5]

Animal models are crucial tools for predicting pharmacokinetics and toxicity in the human body.[6] The content and activity of metabolic enzymes in the liver vary greatly in different species.[7] However, many studies have reported that there were significant species differences between experimental animals and humans in drug effects and toxicities.[8] Therefore, comparing metabolic characteristics among different species can help search other species that have metabolic pathways similar to humans, the species may be used *in vivo* studies in future.[9]

Cytochrome P450 (CYP) enzymes are membrane-associated heme-containing proteins, which mainly exist in liver microsomes (LMs) and play significant roles in the biotransformation of xenobiotics.[10] In addition, inhibition or induction of CYP enzymes activity is the main factor affecting the metabolism of drugs and DDI.[11] The study of metabolic enzymes and the influence of various drugs on CYP enzymes activity has guiding significance for coadministration of drugs and clinical usage.[12]

The study investigated the metabolic characteristics and species differences of asarinin *in vitro*. We identified the metabolic enzymes involved in the biotransformation of asarinin using high-performance liquid chromatography (HPLC) by incubating the recombinant human

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CYPs and LMs with asarinin. The effect of asarinin on CYP enzymes activity was studied, which could enhance our knowledge regarding the clinical application of asarinin.

MATERIALS AND METHODS

Chemicals and reagents

Asarinin (>98%) was obtained from Aifa Corp in Sichuan, China. Table 1 lists the probe substrates, metabolites, and positive inhibitors that were procured from Sigma-Aldrich (MO, USA). D-glucose-6-phosphate, uridine5'-diphosphoglucuronic acid (UDPGA), Tris-HCl, and dehydrogenase (G-6-PD) were provided by Sigma Chemical Co (MO, USA). Recombinant human supersomes were obtained from BD Gentest Corp. (MA, USA). Methanol, formic acid, and MeCN were obtained from Merck (Darmstadt, Germany). The remaining solvents and chemicals were of analytical reagent grade.

Liver microsomes

The Research Institute for Liver Disease Corp (Shanghai, China) provided seven different LMs (0.2 mg/mL). Human Liver Microsomes (HLMs), Dogs microsomes (DLMs), Mini pigs microsomes (PLMs), Monkeys microsomes (MLMs), Rabbits microsomes (RAMs), Mice microsomes (MIMs), and rat microsomes (RLMs) were prepared; these LMs from different donors were usually pooled to imitate the standard proportion of enzymes in human/animal livers. Thirteen recombinant human CYP isoforms (0.2 mg/mL), including CYP1A1, CYP1B1, CYP1A2, CYP3A4, CYP3A5, CYP2C8, CYP2B6, CYP2C9, CYP2D6, CYP2A6, CYP2C19, CYP2E1, and CYP4F2 that were provided from corning life sciences (MA, USA). The procedures involved in animal experiments comply with the regulations on the administration of experimental animals. The medication history of the donor of human LMs was unknown, which was in line with the regulations of the government and the Declaration of Helsinki. We stored all recombinant human CYP isoforms and different LMs-80°C to ensure the activity of enzymes.

Analytical instruments

The peak areas of asarinin and its metabolites were detected using an HPLC system (Shimadzu, Japan). A C_{18} column (4.6 mm, 150 mm, 5 μm Kromasil; temperature: 40°C) was used for the chromatographic separation of asarinin and its metabolites. We used H_2O + 0.1% formic acid (A) and LC grade methanol (B) as the mobile phases at the flow rate of 1 mL/min with the following gradient elution pattern: 0–12 min: 55% B, 12–17 min: 65% B, 17–19 min: 95% B, 19–20 min: 95% B, 20–23 min: 55% B. The metabolism of asarinin was detected by HPLC at 295 nm.

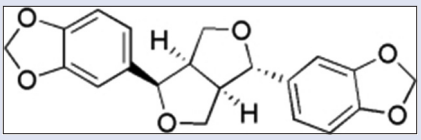

Figure 1: The structure of asarinin

Incubation systems

The CYP reaction system contained 100 mM potassium phosphate buffer (pH = 7.4), NADPH-generating system (4 mM $MgCl_2$, 1 mM NADP+, 10 mM glucose-6-phosphate), recombinant human supersomes and other LMs, the final volume of incubation system was 200 μL. Before adding NADPH to start the reaction, it was necessary to preincubate for 3 min at 37°C to ensure the production of metabolites. The incubation time was 60 min, and then 200 μL ice-cold MeCN was added to terminate the reaction, followed by centrifugation (20 min, 20,000 g, 4°C), 10 μL of the supernatants were remained at-20°C and analyzed by HPLC.

The UGT reaction system (200 μL) contained UDPGA (4 mM), $MgCl_2$ (5 mM), Tris-HCl buffer (pH 7.4; 50 mM), and alamethicin (20 μg mL^{-1}). First, UDPGA (20 μg mL^{-1}) was added after preincubation for 3 min at 37°C. The mixtures were incubated for 60 min, followed by the addition of 200 μL of cold MeCN to quench the reaction. The incubation solution was kept on ice plate for 20 min. After centrifugation for 20 min (×20,000g, 4°C), 10 μL of the supernatants were analyzed by HPLC.

Metabolic characteristics of asarinin

Asarinin (100 μM) was incubated with seven LMs, including HLMs, PLMs, MLMs, RAMs, DLMs, RLMs, and MIMs in phase I metabolism and phase II glucuronide conjugative metabolism. The control group was incubated without NADPH or UDPGA. The incubation conditions were described in Section 2.4. The new peaks were detected by HPLC.

The metabolic features of asarinin in seven different LMs were evaluated.

Metabolic stability assessment with different microsomes

Asarinin (100 μM) was incubated with seven LMs, including HLMs, DLMs, PLMs, MLMs, RAMs, RLMs, and MIMs in phase I and phase II metabolisms. The total volume of the incubation system was 600 μL, and the reaction conditions were described in Section 2.4. Before adding NADPH or UDPGA to start the reaction, there were 3 min preincubation at 37°C. When the incubation time was 0, 30, 60, and 90 min, 100 μL of samples were mixed with cold MeCN (100 μL) to terminate the reaction, followed by centrifugation (20 min, 20,000 g, 4°C). Subsequently, 10 μL of the supernatants were used for HPLC analysis. The concentration of asarinin with the reaction time of 0 min was set to 100%, and the remaining concentration percentage was calculated. The half-life *in vitro* was calculated based on the slope of the linear regression of the natural log of the parent remaining percentage versus incubation time. The half-life was obtained by the formula $T_{1/2} = 0.693/k$.

Screening the metabolic enzymes of asarinin

Asarinin (100 μM) was incubated with thirteen recombinant human CYP isoforms (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP3A4, CYP2C19, CYP3A5, CYP1B1, CYP2C9, CYP2D6, CYP2E1, and CYP4F2) in phase I reaction system. We screened the isoforms

Table 1: Specific substrate and inhibitor concentrations

CYP450 isoforms	Substrates	Specific probe reaction	Specific inhibitors
CYP1A2	Phenacetin (50 μM)	o-diethylation	Furafylline (10 μM)
CYP2A6	Coumarin (10 μM)	7-hydroxylation	Xanthotoxin (2.5 μM)
CYP2C8	Paclitaxel (10 μM)	6α-hydroxylation	Quercetin (10 μM)
CYP2C9	Diclofenac (10 μM)	4 '- hydroxylation	Sulfaphenazole (10 μM)
CYP2E1	Chlorzoxazone (120 μM)	6-hydroxylation	Chlormethiazole (50 μM)
CYP3A4	Testosterone (80 μM)	6 β - hydroxylation	Ketoconazole (1 μM)

involved in the asarinin metabolism. In addition, asarinin was incubated at 37°C for 60 min and centrifuged for 20 min (×20,000g, 4°C), the supernatants were collected and analyzed by HPLC.

CYP 450 enzymes inhibition experiments

CYP isoforms (CYP1A2, CYP3A4, CYP2C9, CYP28, CYP2A6, and CYP2E1) were key factors in the pharmacokinetics of most drugs, so these CYPs were used to study the inhibition of asarinin. The probe substrates were phenacetin O-de-ethylation (CYP1A2), testosterone 6 β-hydroxylation (CYP3A4), diclofenac 4'-hydroxylation (CYP2C9), Coumarin 7-hydroxylation (CYP2C8), coumarin (CYP2A6), chlorzoxazone 6-hydroxylation (CYP2E1). The incubation condition is described in Section 2.4. The type of inhibition could be judged by the Lineweaver–Burk and Dixon plots. The IC_{50} and K_i were calculated based on the slope of the Lineweaver–Burk plots versus the concentration of asarinin.

Enzymatic kinetic parameters

Asarinin (1–100 μM) was incubated with LMs (MLMs, DLMs, PLMs, HLMs, and RLMs) to determine the enzymatic kinetics parameters in the phase I system. The protein concentration of all LMs was 0.2 mg/mL. The kinetics parameters (K_m, V_{max}) of asarinin were calculated from the nonlinear regression using the Michaelis–Menten equation, and the results were expressed on Eadie–Hofstee plots. Intrinsic clearance (CL_{int}) refers to as the maximum activity of the liver (hepatocytes or microsomal proteins) to drugs in the absence of other physiological determinants ($CL_{int} = V_{max}/K_m$).[13] Finally, the standard curve of asarinin was used to quantitatively analyze the metabolites in the incubation mixture.

$$V = \frac{V_{max} \times [S]}{K_m + [S]}$$

V: The rate of reaction;

V_{max}: The estimated maximum velocity;

K_m: The affinity constant of substrate.

[S]: The concentration of substrate;

Prediction of *in vivo* hepatic clearance

The *in vivo* hepatic clearance of asarinin in HLMs, RLMs, and DLMs was predicted based on eqns.(1)-(3).[14]

$$\text{CL int in vitro} = \sum_{i=1}^{n} \frac{V_{max}\,(Mi)}{K_m\,(Mi)} \quad (1)$$

$$\text{CLint in vivo} = \text{CLint in vitro SF} \quad (2)$$

$$CL_H = \frac{Q_H\, f_u\, \text{CL int in vivo}}{Q_H + f_u\, \text{CL int in vivo}} \quad (3)$$

fu: The free fraction in blood (the value of *fu* was set to 1 due to the absence of available data for asarinin);

SF (scaling factor): The liver weight (g) multiplied by microsomal protein (mg/g of the liver);

CL_H: Hepatic clearance;

Q_H: The blood flow in the liver;

The liver weight of humans, rats, and dogs was 25.7, 40, and 32 g/kg body weight; the content of microsomal protein was 48.8, 44.8, and 77.9 mg/g of the liver; and the blood flow in the liver was 20.7, 55.2, and 30.9 ml/(min kg), respectively.[15,16]

RESULTS

Metabolic profiling of asarinin

The quantity of asarinin metabolites was detected by HPLC. The chromatographic conditions and mobile phases were described in section 2.3. After the incubation of asarinin with different LMs in the phase I reaction system, one new peak (M1) was observed in HLMs, DLMs, PLMs, MLMs, and RLMs. The new peak was not detected in the absence of asarinin, G-6-P or NADPH. Therefore, it was speculated that it was the metabolite of asarinin [Figure 2]. In the phase II system, metabolite peak M1 was detected in MLMs, PLMs, and RLMs, M2 was detected in HLMs [Figure 3].

Metabolic stability of asarinin

The metabolic stabilities of asarinin in phase I and II metabolisms were determined using different LMs, the half-life value ($T_1/_2$) of asarinin were calculated. The $T_1/_2$ value of asarinin in the CYP incubation system was 88, 59, 58, 54, 376, 50, and 92 min for humans, dogs, pigs, monkeys, rabbits, rats, and mice, respectively. The $T_1/_2$ value of asarinin in the UGT incubation system was 57, 46, 58, 45, 40, 51, 55 min for HLMs, DLMs, PLMs, MLMs, RAMs, RLMs, and MIMs, respectively.

Assays with recombinant human CYP isoforms

Thirteen recombinant human cytochrome P450 isoforms were used to test their activities in the biotransformation of asarinin to identify the CYP450 isoforms involved in the asarinin metabolism in the human body. As could be seen from Figure 4, asarinin was predominantly catalyzed by CYP3A5, CYP2C8, CYP1A1, and CYP4F2.

Kinetic analyses

The enzyme kinetics of asarinin in the phase I reaction system was studied, the kinetic parameter values [Table 2] were calculated using the metabolite with the new peak area. The metabolism profiles of asarinin (1–100 μM) in the phase I reaction system is shown in Figure 5.

Prediction of *in vivo* hepatic clearance

The hepatic clearance (CL_H) *in vivo* was calculated and the results were 16.50, 6.85, and 10.81 mL/(min·kg body weight) for rats, humans, and dogs, respectively. The hepatic clearance (% CL_H) versus hepatic blood flow (QH) for RLMs, HLMs, and DLMs was 29.9%, 33.1%, and 35.0%, respectively.

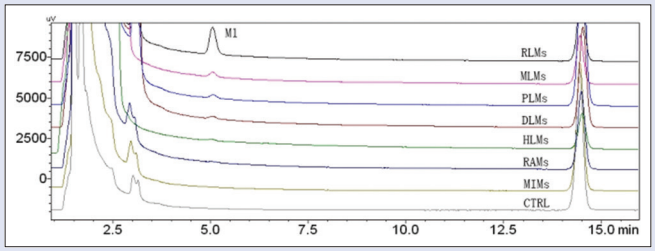

Figure 2: LIQUID chromatography profiles of asarinin and its metabolites in rat microsomes, monkeys microsomes, mini pig microsomes, dog microsomes, human liver microsomes, rabbit microsomes, and mice microsomes

Effect of asarinin on the activity of CYPs

Dixon plot [Figures 6b, 7b, 8b] and Lineweaver-Burk [Figures 6c, 7c, 8c] plot exhibited the inhibitory types of asarinin on CYP enzymes, asarinin inhibited CYP2C19 and 2E1 activities competitively and inhibited CYP3A4 activity noncompetitively. The K_i value of CYP3A4, CYP2C9, and 2E1 were 8.58, 10.81, and 16.33 μM, respectively [Figures 6d, 7d, 8d]. IC_{50} values were calculated, and their values were 8.97,13.80, and 18.04 μM for CYP2C9, CYP2E1, and CYP3A4, respectively [Figures 6a, 7a, 8a].

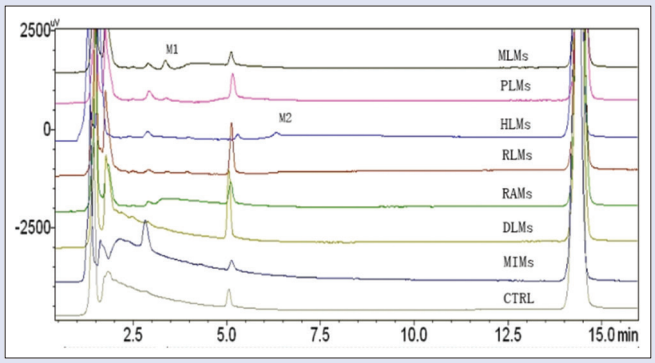

Figure 3: Liquid chromatography profiles of asarinin and its metabolites in monkeys microsomes, mini pig microsomes, human liver microsomes, rat microsomes, rabbit microsomes, dog microsomes, and mice microsomes

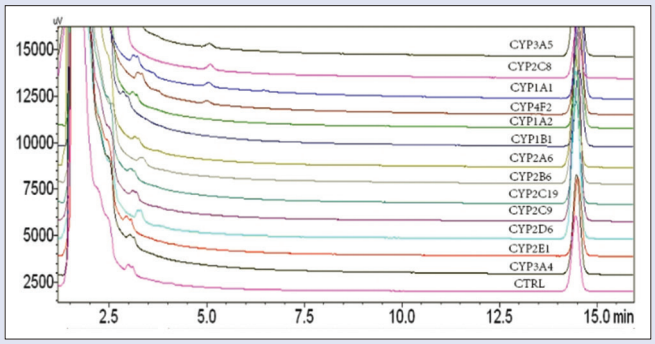

Figure 4: Liquid chromatography profiles of asarinin and its metabolites in recombinant CYP450 isoforms

DISCUSSION

Animal models are important tools for predicting kinetics and toxicity in humans. Thus, the species with similar metabolic pathways to humans were selected as experimental animals.[17] Species-specific differences exist in the function and expression of drug-metabolizing enzymes between humans and animals.[18] In addition, metabolic studies *in vitro* are often used to screen animal species quickly and conveniently.[19]

The metabolic transformation of most drugs *in vivo* is mainly carried out in the liver, which can be divided into the phase I metabolic reaction and the phase II metabolic reaction. In this study, the species-specific differences in NADPH-and UGDPH-dependent metabolism of asarinin were identified first using different LMs. Asarinin had undergone phase I and phase II metabolism, and the metabolite peak was obviously observed. The phase I metabolic characteristics of asarinin revealed that there were metabolites in LMs of five species, including humans, dogs, mini pigs, monkeys, and rats. Rats and monkeys exhibited stronger metabolic capacities compared with other species, humans and rats showed the weaker metabolic capacity for asarinin compared with other species in phase I (CYPs) reaction systems. Monkeys showed stronger metabolic capacities in phase II reaction systems, and one different peak (M2) was detected in HLMs.

Asarinin was incubated with thirteen recombinant human CYP isoforms, when asarinin was incubated with CYP3A5, CYP2C8, CYP1A1, and CYP4F2, metabolites were observed by HPLC, which proved that

Table 2: Kinetic parameter values

Species	K_m (μM)	V_{max} (pmol/min/mg protein)	CLint (μL/min/mg protein)(V_{max}/K_m)
Humans	4.68	37.78	8.17
Dogs	3.47	22.73	6.67
Mini pigs	22.23	111.4	5.01
Monkeys	9.83	52.52	5.34
Rats	36.9	483.9	13.11

V_{max}: The estimated maximum velocity; K_m: The affinity constant of substrate

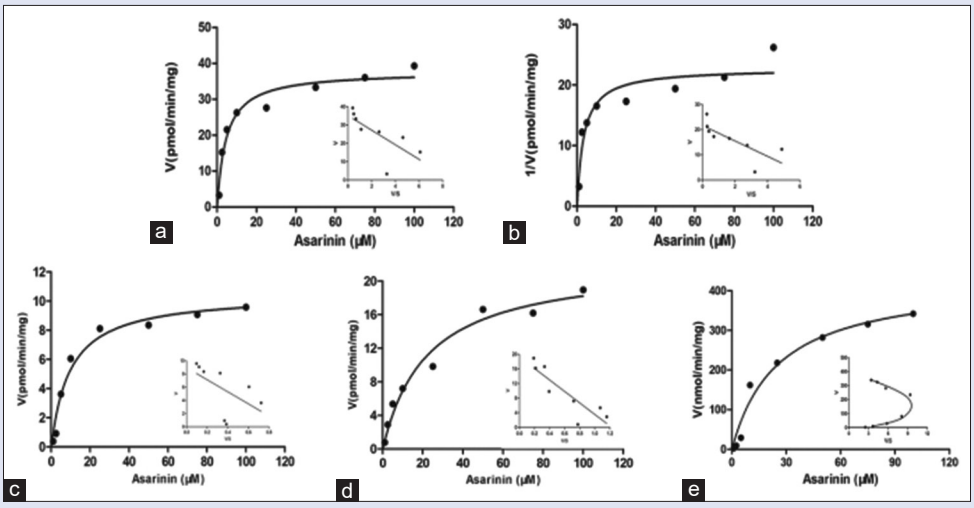

Figure 5: Kinetic of asarinin metabolism in human liver microsomes (a), dog microsomes (b), mini pig microsomes (c), monkeys microsomes (d), and rabbit microsomes (e)

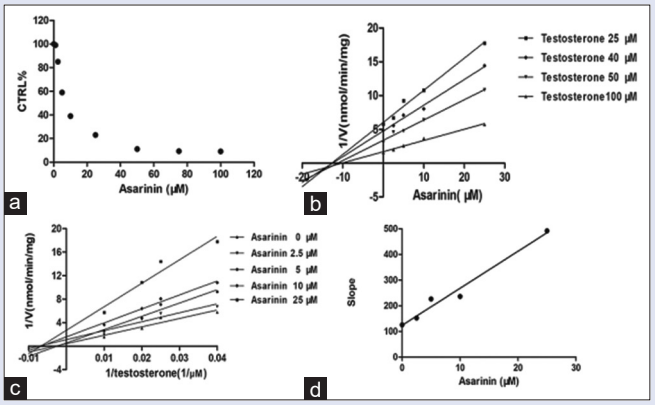

Figure 6: Inhibition of asarinin on CYP3A4 (a). Dixon plot of inhibition of asarinin at different concentrations on testosterone 6 β - hydroxylation (b). Lineweaver-Burk plot of inhibitory effect of asarinin on testosterone 6 β - hydroxylation (c). The slopes from Lineweaver-Burk plot versus the concentrations of asarinin (d)

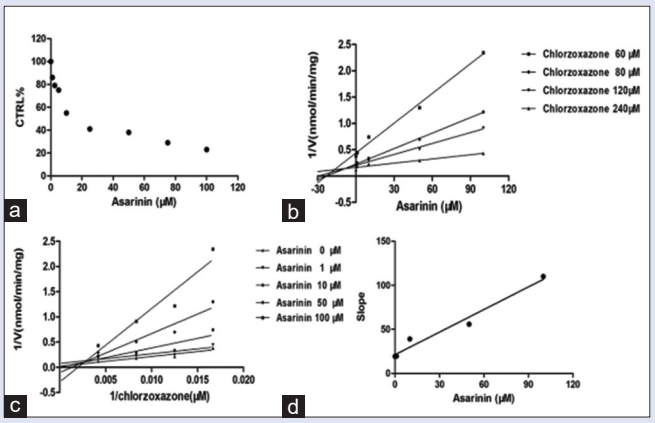

Figure 8: Inhibition of asarinin on CYP2E1 (a). Dixon plot of inhibition of asarinin at different concentrations on chlorzoxazone 6-hydroxylation (b). Lineweaver-Burk plot of inhibitory effect of asarinin on chlorzoxazone 6-hydroxylation (c). The slopes from Lineweaver-Burk plot versus the concentrations of asarinin (d)

CYP3A5, CYP2C8, CYP1A1, and CYP4F2 were CYP isoforms catalyzing the metabolism of asarinin.

In the past few years, rats have been widely applied in the study of toxicology and pharmacokinetics of asarinin.[20] However, the differences between human and common experimental animals in the metabolic pathway and characteristics of asarinin have not been revealed. The enzyme kinetics of asarinin in the phase I reaction system was studied, the kinetic parameter values were calculated. When the reaction was performed by more than one enzyme with largely different kinetic properties, Biphasic kinetics can be observed. Enzymatic kinetic analyses showed that phase I metabolism profiles of asarinin for humans, dogs, monkeys, and pigs followed the classic Michaelis–Menten kinetics, while such biotransformation in rats accorded with biphasic kinetics. The comparable K_m values of the various species studied reflected similarities.[21] Here, the K_m, V_{max}, and CL_{int} values in rats were much higher than others, the K_m value of dogs was similar to that of human beings. The similar Km and CL_{int} values in DLMs and HLMs showed similar catalytic and affinity efficiency of CYPs for asarinin in the two mammals. HLMs and DLMs metabolized asarinin more efficiently than

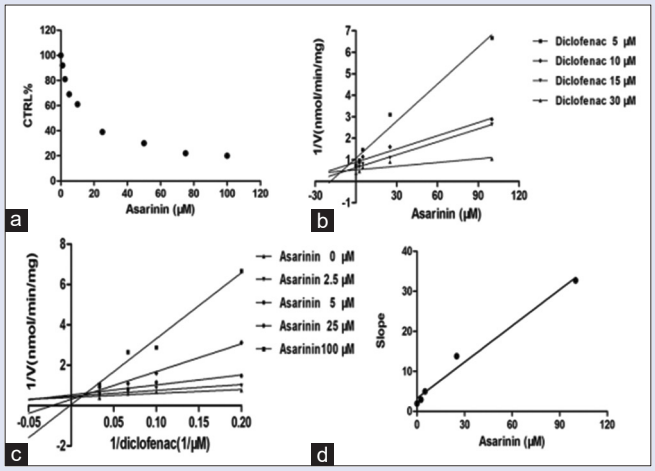

Figure 7: Inhibition of asarinin on CYP2C9 (a). Dixon plot of inhibition of asarinin at different concentrations on diclofenac 4 '- hydroxylation (b). Lineweaver-Burk plot of inhibitory effect of asarinin on diclofenac 4 '- hydroxylation (c). The slopes from Lineweaver-Burk plot versus the concentrations of asarinin (d)

those in PLMs and MLMs, which might be due to the much higher affinity. On the contrary, the increased V_{max} in MLMs and PLMs indicated that these liver samples transformed asarinin relatively efficiently than those in HLMs and DLMs. These results indicated that these were significant species differences. Furthermore, dog might be the ideal animal model for studying the metabolism of asarinin.

The hepatic clearance was predicted *in vivo* and the metabolic stability with different LMs was assessed. The CL_H (%) versus hepatic blood flow (QH) was 29.9%, 33.1%, and 35.0% for RLMs, HLMs, and DLMs, respectively. In general, drugs with CL_H more than 70% QH were defined as high clearance drugs, and drugs <30% QH were defined as low clearance drugs. According to the values predicted, asarinin was classified as a medium-clearance drug in humans, dogs, and rats. The result was also confirmed using metabolic stability assay.

During the drug development process, it is important to investigate how a target drug inhibits or induces the enzymes involved in the biotransformation of the drug. The effects could cause the molecular basis of interactions with other concomitantly administered drugs and could confirm some toxic effects.[22]

For the metabolism of most drugs, cytochrome P450 (CYP) 3A is the most important subfamily of metabolic enzymes, and CYP3A4 is the most member in the CYP3A family. CYP3A4 is known to play a regulatory role in the metabolism of >40% of the market drugs, and has a wide range of substrate specificity and can be inhibited (induced) by multiple drugs and regulated by some protein receptors.[23] The main reason for potential DDI is inhibiting the metabolism of its specific substrates (e.g., Puerarin, oridonin, warfarin).[24] In this study, asarinin showed strong inhibitory effects on the activity of CYP3A4, 2C9, and 2E1 in HLMs. Asarinin noncompetitively inhibited the activity of CYP3A4, which suggested the potential DDI between asarinin, and some drugs metabolized by CYP3A4. CYP2C9 is a major subtype which plays a crucial role in the pharmacokinetics of a variety of drugs.[25] Asarinin inhibited the activity of CYP2E1 and 2C9 in a competitive manner, which indicated that there were potential DDIs between asarinin and some drugs metabolized by CYP2C9 and 2E1.

CONCLUSION

In vitro metabolism experiments, HPLC profiles showed substantial differences among the species in the biotransformation of asarinin.

Meanwhile, dog might be an appropriate model to study the metabolism of asarinin. CYP3A5, CYP2C8, CYP1A1, and CYP4F2 were identified as the main CYPs involved in asarinin metabolism, which could be used to predict the metabolic characteristics of asarinin among the species due to different levels of expression and activities of CYPs in different species. In inhibition experiments, asarinin exhibited strong inhibitory effects on CYP2C9, CYP2E1, and CYP3A4. The inhibitory types of asarinin can be seen from the Dixon and Lineweaver-Burk plots, asarinin competitively inhibited the activity of CYP2C19 and 2E1, and noncompetitively inhibited the activity of CYP3A4. For these reasons, when asarinin-containing herbs are used with these drugs, the potential DDIs should be monitored. *In vitro* experiments showed that there were potential DDIs between asarinin and some drugs, but further studies are warranted to assess the risk of clinically significant DDIs.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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