Life Sciences 218 (2019) 314–323

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Time-dependent inhibition of carbamazepine metabolism by piperine in anti-epileptic treatment

Tianjing Ren[a], Mengbi Yang[a], Min Xiao[a], Junjie Zhu[b], Wen Xie[b], Zhong Zuo[a,c,*]

[a] School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region
[b] Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA, USA
[c] State Key Laboratory of Research on Bioactivities and Clinical Applications of Medicinal Plants, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region

ARTICLE INFO

Keywords:
Piperine
Carbamazepine
Drug interactions
Drug metabolisms
Time-dependent inhibition

ABSTRACT

Aims: The first-line anti-epileptic agent carbamazepine has narrow therapeutic index and can potentially interact with piperine, the major component from black pepper. The present study aimed to delineate the mechanism of such interaction for safe usage of carbamazepine during epilepsy control.

Materials and methods: The effect of piperine on carbamazepine hepatic metabolism was examined using rat or human liver microsomes. Mechanistic static model was applied to predict the extent of interaction. In addition, liver microsomal activities, mRNAs and protein expressions of genes regulating carbamazepine metabolism were evaluated after two weeks oral administrations of 3.5 and 35 mg/kg piperine in rats. Moreover, the effect of piperine on the xenobiotic receptor constitutive androstane receptor (CAR) was further accessed.

Key findings: Time-dependent inhibitory effect of piperine on carbamazepine metabolism was observed, with k_{inact} and K_I of 0.0153 min^{-1} and 18.34 μM for rat, and 0.0093 min^{-1} and 9.45 μM for human. Based on such in-vitro metabolic parameters, further estimation using mechanistic static model indicated that piperine could increase the AUC of CBZ by 7% and 11% in rat and human, respectively. Significant inhibition on rat liver microsomal activity, Cyp3a2 mRNA and protein expression, CAR mRNA were demonstrated with piperine at 35 mg/kg. Yet, no direct effect on the activity of CAR for piperine was found.

Significance: We have demonstrated the time-dependent inhibition by piperine on carbamazepine metabolism as the interaction mechanism. Prolonged use of piperine at high dose could increase carbamazepine concentrations through inhibiting metabolic enzyme activities and their related genes expressions.

1. Introduction

The major bioactive component from black pepper, known as piperine, is associated with great variety of pharmacological actions, including anti-inflammation, anti-oxidation and anti-cancer [1–3]. It also demonstrated anti-epileptic effect in mice through TNF-α reduction and GABAergic system regulation [4]. A clinical report showed the effect of piperine in epileptic seizure control after 2 weeks treatment [5]. Among 150 cases in this report, 113 cases were able to receive lower dose of anti-epileptic drugs after combined treatment with piperine. Thus, there is high possibility for combined use of piperine with epileptic drugs on epilepsy control. Notably, piperine has a unique role in influencing the bioavailability of other therapeutic agents. Oral administration of piperine could significantly increase the systematic exposure of fexofenadine and domperidone in rats [6,7], and increase AUC, C_{max} and decrease the clearance of drugs such as propranolol, theophylline and midazolam in human [8,9]. Mechanisms of these interactions may be related to the influence of piperine on drug metabolic enzyme. Piperine could inhibit the CYP3A4-catalyzed formation of the verapamil metabolites in human liver microsome [10]. Such inhibition is relatively selective as it is more potent against CYP3A4 with IC_{50} of 2.12 μM, compared to CYP1A2 and CYP2C9 with IC_{50} of 14.19 and 89.62 μM, respectively [11]. Decreased hepatic CYP-450 content and benzphetamine *N*-demethylase activity mediated by rCyp3a (rat CYP3A) also found after administrated with 100 mg/kg piperine in rats [12,13]. Therefore, the interactions between piperine and therapeutic agents, especially for CYP3A substrates, may be attributed to the effect on metabolism.

Carbamazepine (CBZ) is the first-line treatment for partial seizures, generalized tonic-clonic seizures and mixed seizures. Optimal concentration for seizure control is 4–12 μg/ml in human plasma and higher concentration may cause side effects such as diplopia, nystagmus

* Corresponding author at: School of Pharmacy, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region.
E-mail address: joanzuo@cuhk.edu.hk (Z. Zuo).

https://doi.org/10.1016/j.lfs.2018.12.060
Received 26 November 2018; Received in revised form 31 December 2018; Accepted 31 December 2018
Available online 03 January 2019

and aplastic anemia [14]. Due to such narrow therapeutic index, therapeutic drug monitoring is often required to ensure the effectiveness and prevent the toxicity. CBZ can be metabolized into its major metabolite, carbamazepine-10,11-epoxide (CBZE) in liver primarily by CYP3A4 with minor involvement of CYP2C8 [15]. This major metabolite contributes to both seizure control and toxicity [16]. Besides, nuclear xenobiotic receptors such as pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) also contribute to the metabolism of CBZ by regulate genes involved in drug metabolism including CYP3A4 [17]. The potential interactions between piperine and CBZ have been evaluated in only few reports. It was found 20 mg piperine could significantly increase the AUC and half-life of CBZ in epileptic patients receiving long-term treatment of CBZ, [18]. Another study was carried out in healthy volunteer receiving single dose of CBZ with pretreatment of 20 mg piperine for 10 days and decreased elimination of CBZ and the ratio of $AUC_{0\text{-inf,CBZE}}/AUC_{0\text{-inf,CBZ}}$ and $C_{max,CBZE}/C_{max,CBZ}$ were found [19]. Since the dose of piperine used in previous studies was only close to its daily consumption level, further studies with higher dose piperine are needed to evaluate its influence on patients with CBZ for epilepsy control. Moreover, lack of mechanism of pharmacokinetics interaction between CBZ and piperine would further prevent the prediction of their interactions at different dose regimens.

Based on the clinical pharmacokinetics interaction and previous metabolic enzyme alteration findings, the present study is proposed aiming to investigate the mechanism of interaction between piperine and CBZ by determining the inhibition type and obtaining related kinetic parameters used for prediction of pharmacokinetics interaction in rat and human. In the meantime, effect of piperine at different dose levels on CBZ metabolic enzyme activity and related genes expression will also be evaluated.

2. Materials and methods

As summarized in Table 1, to comprehensively understand the pharmacokinetics interaction between piperine and CBZ, we have conducted a) in-vitro studies including liver microsome and hCAR transfected cells to determine inhibition kinetics and study the effect of piperine on CAR activity, respectively, b) in-silico mechanistic static model to estimate the extent of such interaction and c) in-vivo rats study to evaluate the extent of change in liver microsome metabolic activity, related metabolic gene mRNA and protein expression.

2.1. Materials

Piperine (purity > 97%) was purchased from Aldrich Chem. Co. (Milwaukee, WI). CBZ and it metabolite CBZE were bought from Sigma-Aldrich Co. (St. Louis, MO). For in-vitro metabolism study, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH) was from Roche (Mannheim, Germany) while the pooled male Sprague–Dawley (SD) rat liver microsome, pooled human liver microsome and $MgCl_2$ were from Sigma-Aldrich Co. Berberine was used as internal standard (IS) and was bought from Sichuan Weikeqi Biological. HPLC grade acetonitrile (ACN) from RCI Labosan Ltd. (Bangkok, Thailand) and methanol (MeOH) from Merck (Darmstadt, Germany) were used without further purification. Dimethyl sulfoxide (DMSO) from Duksan Pure Chemical Co. (Ansan, Korea) and formic acid from BDH Laboratory Supplied Ltd. (Kampala, Ukraine) were used. Kollisolv® PEG-400 was bought from Sigma-Aldrich Co. For rat liver microsome extraction, ethylene diamine tetra acetic acid (EDTA) from Sigma-Aldrich, tris hydrochloride (Tris-HCl) from Bio-Rad Laboratories, Inc. (CA, USA) and glycerol was from Alfa Aesar Co., Inc. (Lancashire, UK) were used. QIAGEN RNeasy Mini Kit from QIAGEN (CA, USA), Thermo Scientific RevertAid First Strand cDNA synthesis kit from Thermo Fisher Scientific Inc. (MA, USA) and SYBR Green PCR Premix HS Taq (Real Time) kit from Gen-View Scientific Inc. (FL, USA) were used in mRNA quantification. Primary antibodies including rabbit polyclonal against CYP 3A1, 3A2, CAR and GAPDH, secondary antibody horseradish peroxidase labeled anti-rabbit IgG were all purchased from Abcam (Cambridge, UK).

2.2. Effect of piperine on CBZ metabolism by in-vitro rat and human liver microsome systems

2.2.1. IC_{50} determination

To understand the effect of piperine on CBZ metabolism, different concentrations of piperine were first incubated with CBZ in the in-vitro microsomal metabolic system. The incubation was carried out by pre-incubating CBZ at 50 μM, its maximum human therapeutic concentration [14], with 1 mg/ml pooled rat or human liver microsome and 6 mM $MgCl_2$ in 50 mM phosphate buffer (pH = 7.4) at 37 °C for 2 min. In the meantime, different concentrations of piperine (0, 0.1, 1, 5, 10, 50, 100 μM) were added into the system. MeOH was used to dissolve both CBZ and piperine and was present at final concentration (*v/v*) of 1% in 200 μl incubation system. The reaction was initiated by addition of 5 mM NADPH and terminated 1 h after by adding 600 μl ice-cold IS solution (200 ng/ml berberine (IS) in ACN and MeOH (1:1, *v/v*)). The samples were then mixed and centrifuged at 14000 rpm for 10 min at 4 °C. The supernatants were subjected to LC-MS/MS for analyzing the formation of CBZE. The percentage of control was calculated by the ratio CBZE formed in presence of piperine to that in absence of piperine. The sigmoidal dose–response curves were then constructed by plotting percentage of control versus log of piperine concentration to obtain IC_{50} values were further calculated using GraphPad Prism (version 3.03 GraphPad Software, La Jolla, CA).

Table 1
Summary of the type, model, design and indication of various studies involved in the current research.

Study type	Subject/model	Study design		Indications
In-vitro	Rat/human liver microsome	50 μM CBZ + 0–100 μM piperine		IC_{50}
		50–400 μM CBZ + 0–20 μM piperine		Reversible inhibition potential
		50–400 μM CBZ + 0–50 μM piperine		Time-dependent inhibition potential
	Transiently hCAR transfected HEK293T cells	0, 1, 40 μM piperine		CAR transcription activity
In-silico	Mechanistic static model	**Piperine concentration source**		Extent of metabolic interaction
		Rat plasma concentration after 3.5 mg/kg p.o.		
		Rat plasma concentration after 35 mg/kg p.o.		
		Human plasma concentration after 20 mg/person p.o.		
In-vivo	Male SD rats	**Groups**	**Dose (once daily for 14 days)**	Extent of change in rat liver microsome metabolic activity, related metabolic gene mRNA and protein expression
		Control	Vehicle	
		LD PIP	3.5 mg/kg piperine	
		HD PIP	35 mg/kg piperine	

2.2.2. Analytical procedure for CBZE quantification

The CBZE concentration analysis was performed on Agilent 6430 triple quadrupole mass spectrometer equipped with and electrospray ionization source (Agilent Technologies, Inc., Santa Clara, CA). Separation of CBZE and IS was achieved by a Waters ACQUITY UPLC® BEH C18 column (2.1 × 50 mm, 1.7 mm) equipped with VanGuard™ pre-column (2.1 × 5 mm, 1.7 mm, Waters Corporation, Milford, MA). The mobile phase consisted of 0.2% formic acid in water (A) and ACN (B) with gradient setting as 30 → 40% B (0–3 min), 40 → 70% B (3–4 min), 70% B (4–8 min), 70 → 30% B (8–8.1 min) till 11 min. The flow rate was 0.15 ml/min at ambient temperature and the auto-sampler was kept in 4 °C with injection volume of 2 μl. MS/MS detection was performed in positive mode using multiple reaction monitoring (MRM) at *m/z* 253.1 → 180 for CBZE and *m/z* 336.1 → 320.1 for IS. All the data was analyzed by Agilent MassHunter Quantitative Analysis version B.03.01 software (Agilent Technologies, Santa Clara, CA).

2.2.3. Reversible inhibition potential of CBZ metabolism by piperine

Piperine was then tested as reversible inhibitor on the CBZ metabolism. CBZ at different concentration levels (50, 100, 200, 400 μM) were pre-incubated with 1 mg/ml pooled rat or human liver microsome and 6 mM $MgCl_2$ in 50 mM phosphate buffer (pH = 7.4) across four concentration levels of piperine around its IC_{50} level (0, 5, 10, 20 μM). The reaction was initiated by addition of 5 mM NADPH and terminated 1 h after by adding 600 μl ice-cold IS solution. The concentrations of CBZE in the samples were analyzed using previously described sample treatment and analytical method. The metabolite formation rates (v) were calculated and plotted with CBZ (s) and piperine (i) concentrations to construct graphical plots for determination of inhibition mode, including Lineweaver–Burk plot (1/v versus 1/s), Dixon plot (1/v versus i) and Cornish-Bowden plot (s/v versus i) [20,21]. *K*i values, the intrinsic inhibition parameter that is independent of substrate concentration and incubation condition, were further derived from the replots of slope from Lineweaver–Burk plot versus i.

2.2.4. Time-dependent inhibition potential of CBZ metabolism by piperine

To further test the time-dependent inhibition potential of piperine, primary incubation was carried out by pre-incubation of various concentration of piperine around its IC_{50} level (0, 1.25, 5, 10, 20, 50 μM) with 2 mg/ml rat or human liver microsome, 5 mM NADPH and 6 mM $MgCl_2$ for 0, 15, 30, 45, 60 min at 37 °C. After different pre-incubation time, 10 μl aliquots of the primary incubation was transferred to 90 μl secondary incubation containing 400 μM CBZ with 5 mM NADPH and 6 mM $MgCl_2$ to yield a 10-fold dilution. The secondary incubation was further incubation for 20 min at 37 °C followed by adding 300 μl ice-cold IS solution to stop the reaction. The samples were treated with previously described method and analyzed by LC-MS/MS.

To obtain the kinetic parameters for time-dependent inhibition, enzyme activities were first calculated by metabolite formed under different piperine concentration levels versus that without piperine after various pre-incubation times. The natural logarithmic enzyme activities were then plotted against pre-incubation time at each concentration level of piperine. The negative slopes of each line were determined as apparent inactivation rate constants (k_{obs}) and were plotted against piperine concentrations. The plots were fitted into non-linear least squares regression to determine the limit maximum inactivation rate constant (k_{inact}) and the inhibitor concentration yielding half of maximum inactivation (K_I) base on the following equation [22]:

$$k_{obs} = \frac{k_{inact} \cdot I}{K_I + I} \quad (1)$$

2.2.5. Estimating the extent of metabolic interaction base on mechanistic static model

The potential change of the drug exposure caused by the time-dependent inactivator could be predicted by a mechanistic static model developed by Mayhew et al. The model required kinetic constants of inactivation (k_{inact} and K_I) and in-vivo inactivator concentration. The AUC ratio of drug in presence of inactivator to that in absence of inactivator (AUC_i/AUC) could be calculated by the following equation:

$$\frac{AUC_i}{AUC} = \frac{1}{\left(\frac{f_m}{1+\left(\frac{k_{inact}\cdot[I]_{in-vivo}}{K_I \cdot k_{deg}}\right)}\right) + (1 - f_m)} \quad (2)$$

where k_{deg} is the first-order rate constant of in-vivo degradation of P450 enzyme with an initial estimate of 0.0008 min^{-1} for rat and 0.00032 min^{-1} for human [23,24]. The f_m should be the fraction of total clearance that affect P450 enzyme involved, since we monitored the inactivation on formation of CBZE by piperine, f_m was considered as the fraction of CBZ converted to CBZE to the total CBZ clearance. Based on the literature data, f_m was approximate to 0.5 in rat and 0.6 in human [25,26].

2.3. Impact of long-term use of piperine on enzymes mediating CBZ metabolism in rat

2.3.1. Animal treatments

To further study the influence of piperine on CBZ hepatic metabolism after long-term use, the rat liver microsomal activity was evaluated after oral administration of piperine for consecutive 14 days. Male SD rats with body weight of 180–200 g were obtained from the Laboratory Animal Services Center at the Chinese University of Hong Kong and the experimental procedures were conducted under the approval of Animal Ethics Committee of the Chinese University of Hong Kong. The rats were divided into 3 groups with 6 rats in each group. The low dose piperine treatment group (LD PIP) received daily oral administration of 3.5 mg/kg piperine prepared by diluting piperine stock solution in DMSO with PEG-400 and H_2O (DMSO: PEG-400: H_2O = 1:5:4, *v/v/v*). The high dose treatment group (HD PIP) received 35 mg/kg piperine while the control group received vehicle for 14 days. The two studied doses of piperine were determined based on its daily or clinical doses and scaling factor between rat and human recommend by FDA [27]. Piperine dose of 3.5 mg/kg was in the range of its equivalent content in black pepper at human daily consumption level (14–54 mg piperine/person/day) [28], while its dose of 35 mg/kg was in the range of pepper extract clinical recommended dose for epilepsy control (280–560 mg piperine/person/day) [5]. After 14 days, rats were sacrificed and perfused with ice-cold saline to collect liver. Half of liver was stored at −80 °C for further analysis and the rest was immediately used to prepare microsome.

2.3.2. Rat liver microsome preparation

The livers were cut into small pieces for microsome extraction by differential centrifugation method [29]. Briefly, chopped liver samples were homogenized with around 2 times volume (*v/w*) of homogenizing buffer (10 mM EDTA, 0.1 M Tris–HCl, 150 mM KCl, pH 7.4) on ice. The homogenates were then centrifuged at 12,500*g* at 4 °C for 15 min. The supernatants were transferred into centrifuge tubes for high-speed centrifuge at 102,000*g* under 4 °C for 60 min using L8-70 Beckman ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The resultant microsomal pellets were re-suspended with microsomal buffer (10 mM EDTA 0.05 M Tris–HCl, 20% glycerol, pH 7.5) and stored at −80 °C until use. Protein concentrations of the prepared microsomes were determined by protein assay kit using micro-plate reader (Benchmark, Japan).

2.3.3. Effect of piperine on hepatic CBZ metabolism activity in rat liver microsome

To determine the effect of piperine on hepatic CBZ metabolism activity, the rat liver microsomes from different treatment groups were

incubated with 50 μM CBZ following the previous incubation condition. After pre-incubation of CBZ and 1 mg/ml the prepared rat liver microsomes with 6 mM $MgCl_2$, 5 mM NADPH were added to start the reaction. Ice-cold IS solutions were added after 1 h of incubation and CBZE formations were analyzed by LC-MS/MS method. The CBZE formations with rat liver microsomes after piperine treatments were compared with that in control groups and their ratios were calculated as percentage of control.

2.3.4. Effect of piperine on mRNA expression of genes involved in CBZ metabolism in rat liver

The hepatic mRNA expression levels of genes involved in CBZ metabolism were compared between piperine treatment groups and control group to evaluate the effect of piperine on mRNA expression following previously described method [30]. Liver samples from the 2 rats in same dosing group were pooled and weighed for around 20 mg. The samples were homogenized and purified for RNA extraction by QIAGEN RNeasy Mini Kit. The UV absorbance ratios of 260/280 (> 1.9) were measured to validate the quality of extracted RNA. The validated RNA was used for further reversed transcription with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit in a thermal cycler. Following cDNA preparation, PCR was initiated by mixing cDNA with multiscribe reverse transcriptase, RT buffer, random hexamer primers, 0.5 mM each of dATP, dGTP, dCTP, dTTP and primer with sequence listed in Table 2 [31–33]. After 3 min of pre-heating at 94 °C, 40 cycles were run with 10 s at 94 °C, 20 s at 63 °C and 30 s at 72 °C followed with 95 °C for 5 s, 65 °C for 1 min then reached 97 °C before cooling back to 40 °C. The expression levels were detected by Roche LightCyclers® 480 Real-time PCR System (Roche applied science, Switzerland) using SYBR Green PCR Premix HS Taq (Real Time) kit. The fold of changes for each gene from different treatment groups were corrected with its GAPDH and compared by following method: $\Delta Ct = Ct(target) - Ct(GAPDH)$, $\Delta(\Delta Ct) = \Delta Ct$ (treatment group) $- \Delta Ct$ (control group), Fold of change $= 2^{-\Delta(\Delta Ct)}$, where Ct (cycle threshold) is the number of amplification cycles for fluorescent signal to reach a detectable level.

2.3.5. Effect of piperine on protein expression of genes regulating CBZ metabolism in rat liver

The protein expression levels of rCyp3a1, rCyp3a2, rCAR and GAPDH in rat livers after treatment with piperine were further analyzed by Western blotting and compared with control group. The liver samples were pooled from 2 rats in same treatment groups and denatured for loading on 10% SDS–polyacrylamide gel. After separation by a Mini-Protean II system (Bio-Rad), the gels were transblotted onto a PVDF membrane (Immobilon transfer membrane, Millipore, Billerica, MA, USA). The membranes were blocked with 5% of BSA followed by an over-night incubation with primary antibody (rCyp3a1, rCyp3a2, rCAR and GAPDH), and 2-hour incubation of secondary antibody. The immunoblot bands were visualized by immersing the enhanced chemiluminescence solution (Millipore, Billerica, MA, USA) for 60 s before capturing the digital chemiluminescence images with FluorChem Q Imaging System (Alpha Innotech Corporation, Santa Clara, CA). The intensities of bands were quantified by Image Lab™ (Bio-Rad Laboratories, Inc., CA, USA) and corrected by GAPDH for comparison between different groups.

Table 2
PCR primer for genes involved in CBZ metabolism and reference gene in rat.

Gene	Direction	Sequence
rCyp3a1	Sense	CCGCCTGGATTCTGTGCAGA
	Antisense	TGGGAGGTGCCTTATTGGGC
rCyp3a2	Sense	GCTACTACAAGGGCTTAGGGAG
	Antisense	CTTGCCTGTCTCCGCCTCTT
rCyp2c13	Sense	CTGGCAATCATGGTGACTGA
	Antisense	GAAACTCCTTGCTGTCATGC
rPXR	Sense	TGTGACGGCAAGGGCTTTTTC
	Antisense	CTTGAGCAAGGAGATCTGGTCCTC
rCAR	Sense	CAGCCTGCAGGTTGCAGAAG
	Antisense	TTCCACAGCCGCTCCCTTGA
GAPDH	Sense	GTGGACCTCATGGCCTACAT
	Antisense	TGTGAGGGAGATGCTCAGTG

2.3.6. Transient transfection and luciferase reporter gene assay

The CAR-responsive tk-PBRE-Luc reporter was constructed by inserting the phenobarbital response element (PBRE) derived from the mouse Cyp2b10 gene into the tk-Luc as described previously [34]. The human embryonic kidney HEK293T cells were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and streptomycin. The cells were transfected with empty vector or the human CAR (hCAR) expression vector, β-galactosidase vector, and tk-PBRE-Luc reporter for 6 h. After transfection, the cells were treated with vehicle (0.1% DMSO), or piperine at 1 and 40 μM for 24 h. Piperine concentration at 1 μM was used as it was close to the average human plasma concentration level at daily piperine consumption level, while 40 μM piperine was used to represent the plasma concentration level that may be achieved after dosing piperine at its therapeutic level [35]. The luciferase reporter activities in cell lysates were measured and normalized against co-transfected β-galactosidase activity.

2.4. Statistical analyses

Experimental data from both in-vitro and in-vivo studies were expressed as mean plus standard deviation (± SD). Statistical differences between multiple groups in microsome activity, mRNA and protein expression tests were evaluated by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. Two-way ANOVA were used to analyze CAR activity data. Statistical significance was considered when the p value was < 0.05 during the test.

3. Results

3.1. Time-dependent inhibitory effect of piperine on CBZ hepatic metabolism in rat and human

3.1.1. IC_{50} determination

The effect of piperine on CBZ metabolism was tested by incubating 50 μM CBZ with piperine at various concentration levels in rat or human liver microsome incubation system. The metabolic rate of CBZ was determined by the formation rate of CBZE. It was found that CBZE formations were decreased after adding piperine in both rat and human incubation system, indicating that piperine could inhibit CBZ metabolism in both rat and human. As shown in Fig. 1, the calculated IC_{50} of piperine inhibition on CBZ metabolism in liver microsomes were 9.20 ± 1.36 μM and 10.00 ± 1.22 μM for rat and human, respectively. The similar IC_{50} levels of piperine between rat and human liver microsomes also suggest that rat liver microsome could be used to estimate such inhibitory effect of piperine on CBZ metabolism in human.

3.1.2. Reversible inhibition and Ki determination

To further investigate the inhibition type of piperine on CBZ metabolism, piperine was incubated with CBZ at different concentrations to construct representative plots for reversible inhibition. As it was shown in Lineweaver–Burk plot, Dixon plot and Cornish-Bowden plot in Fig. 2 for Rat, all lines intersect at the same point on x-axis, thus piperine was identified as non-competitive inhibitor on CBZ metabolism in rat liver microsome incubation system. The representative non-competitive inhibition features were also demonstrated in human liver microsome incubation system (Fig. 2 for Human), indicating the similar inhibition type of piperine in rat and human. The Ki determined by the x-axis intersection in the replots of slope from Lineweaver–Burk plot were found to be 22.24 μM and 11.81 μM for rat and human,

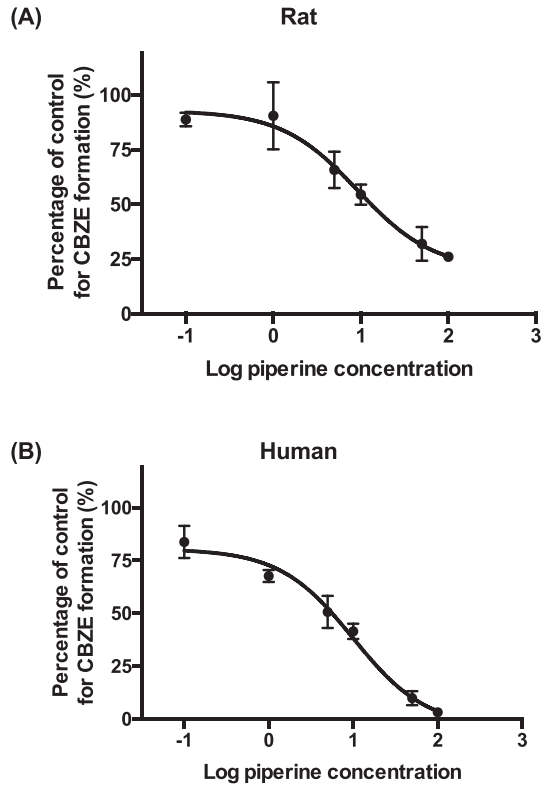

Fig. 1. Sigmoidal dose–response curves of piperine on CBZ metabolism in rat (A) and human (B) liver microsome incubation system. Each point represents mean ± S.D. in triplicate experiments.

repetitively.

3.1.3. Time-dependent inhibition

Since non-competitive inhibition and time-dependent inhibition demonstrated similar kinetic patterns in the representative plots, the time-dependent inhibition potential was further evaluated by pre-incubation of liver microsome with piperine for different time and then tested the remaining metabolic activity on CBZ metabolism. As it was shown in Fig. 3, longer pre-incubation of rat or human liver microsome with piperine could result in further inhibition on CBZ metabolism at each piperine concentration level, indicating the time-dependent inhibition on CBZ metabolism by piperine in rat and human. When comparing the remaining metabolic activity with different piperine concentrations at same pre-incubation period, lower metabolic activity was found with higher piperine concentrations, indicating the inhibition is concentration-dependent as well. In addition, the inhibition is NADPH-dependent as no further inhibition was found after pre-incubation in the absence of NADPH. Therefore, the inhibition of CBZ metabolism by piperine is time-, concentration- and NADPH-dependent, suggesting the mechanism-based inhibition by piperine. The kinetic parameters of mechanism-based inhibition (k_{inact} and K_I) could be calculated from the double-reciprocal plot of k_{obs} and piperine concentrations (Fig. 3B for both Rat and Human). The k_{inact} and K_I were found to be 0.0153 min^{-1} and 18.34 μM for rat, and 0.0093 min^{-1} and 9.45 μM for human.

3.1.4. Static modeling of metabolism-based DDI on systematic exposure of CBZ

The in-vitro kinetic parameters of mechanism-based inhibition (k_{inact} and K_I) were incorporated into the mechanistic static model (Eq. (2)) to predict the potential magnitude of change in CBZ exposure in rat and human. Since it have been demonstrated that the maximum plasma concentrations in rat after oral administration of piperine at 3.5 mg/kg and 35 mg/kg were 0.45 μM and 6.92 μM, and their respective unbound plasma fractions were 0.019 and 0.024 in our previous studies, the in-vivo piperine concentration available to inactivate CBZ metabolism in rat could be estimated [36,37]. The result showed no AUC change of CBZ at the 3.5 mg/kg of piperine while an AUC fold change of 1.07 at 35 mg/kg of piperine. The concentration in human used for predication was obtained from the maximum plasma concentration at steady state after 7 days consecutive administration of 20 mg piperine, which was 2.09 μM [35]. Given unbound fraction of 0.03 in human plasma, the estimated AUC fold change of CBZ was 1.11 by piperine in human [38].

3.2. Long-term piperine treatment led to significant inhibition on activity and gene expression of enzymes mediating CBZ metabolism in rat

3.2.1. Decreased microsomal activity

The effect of piperine on microsomal activity was further investigated using the liver microsome from rats treated with 3.5 mg/kg and 35 mg/kg piperine for 14 days. The metabolic activities were evaluated by CBZE formation using CBZ as probe substrate. Compared with control group, a significant decrease of CBZE formation was found in high dose piperine treatment group with relative CBZE formation of 67.0 ± 18.8% compared to control [$F(2,15) = 7.42$, $p < 0.05$], suggesting the in-vivo inhibition of CBZ metabolic activity by long-term administration of high dose piperine. The relative CBZE formation in low dose piperine treatment group was found to be 81.7 ± 16.5% of that from control group, yet no significant difference was found between the two groups.

3.2.2. Reduced mRNA and protein expression of gens regulating CBZ metabolism

The influence of piperine on mRNA and protein expression of genes regulating CBZ metabolism was further assessed using liver samples of rats treated with multiple dose of piperine. As demonstrated in Fig. 4, in comparison to control group, significant decreases were found on the rCyp3a2 and rCAR mRNA expression level in high dose piperine treatment group [$F(2,6) = 6.78$, $p < 0.05$ for rCyp3a2 and $F(2,6) = 7.64$, $p < 0.05$ for rCAR]. The rCyp3a2 mRNA expression level was decreased to 38% of control while the rCAR was decreased to 59% of control. The mRNA expression levels of other genes showed no significant difference. In the meantime, multiple dose of piperine at low dose level showed no significant influence on any of the genes regulating CBZ metabolism. Therefore, piperine at high dose level could decrease the rCyp3a2 and rCAR mRNA expression to inhibit CBZ metabolism after multiple dose of administration.

Since the mRNA of rCyp3a2 and rCAR were downregulated after high dose treatment of piperine, the protein expression levels of these genes in together with rCyp3a1 were further evaluated. As it was shown in Fig. 5, a significant decrease in rCyp3a2 in high dose treatment group while with no significant influence on rCAR or rCyp3a1. The low dose treatment group did not influence any of the protein expression levels. Compared with mRNA expression change, only rCyp3a2 was significantly inhibited at high dose concentration level [$F(2,6) = 8.21$, $p < 0.05$], which is consistent with our findings on microsome metabolic activity and mRNA expression. Thus, it is suggested that multiple dose of 35 mg/kg piperine could significantly inhibit the CBZ metabolism through inactivating microsomal activity and inhibiting rCyp3a2 mRNA and protein expression level in rat liver.

3.2.3. Effect of piperine on the transcriptional activity of CAR

We used the transient transfection and report gene assay to determine the effect of piperine on the transcriptional activity of CAR. Since the mRNA and protein expression of rCyp3a2 was found downregulated after treatment of high doses of piperine, the effect of piperine on the transcriptional activity of CAR was determined by CAR responsive tk-PBRE-Luc reporter in HEK293T cells transiently transfected with the CAR expression vector. As it was found in Fig. 6,

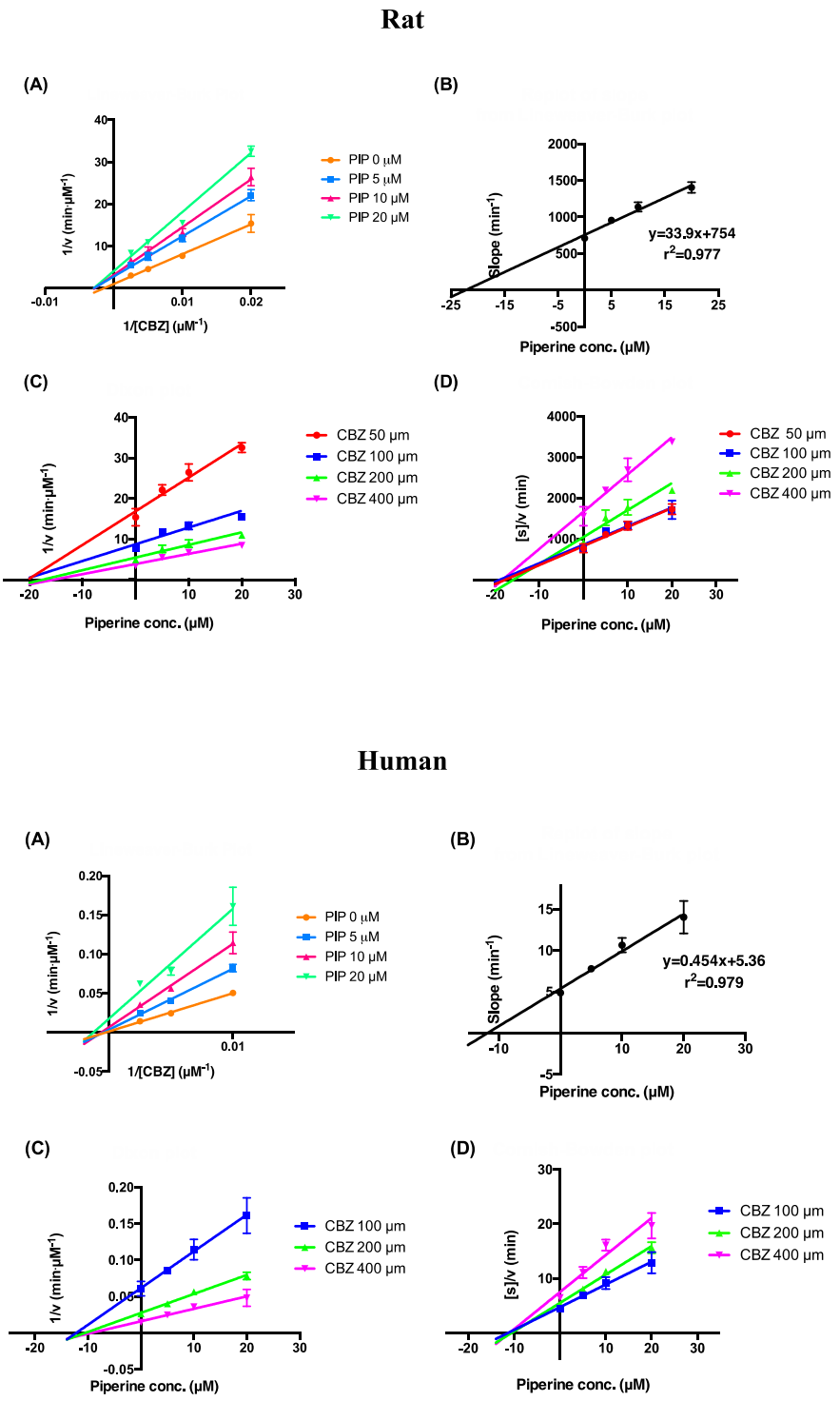

Fig. 2. Representative plots for reversible inhibition of CBZ metabolism by piperine in rat (Upper) and human (Bottom) liver microsome incubation system. (A) Lineweaver–Burk plot. (B) Replot of slope from Lineweaver–Burk plot versus piperine concentration. (C) Dixon plot. (D) Cornish-Bowden plot. Each point represents mean ± S.D. in triplicate experiments.

factorial two-way ANOVA revealed a significant effect of vectors [$F(1,12) = 1873$, $p < 0.001$] on the CAR activity, indicating that the hCAR transfection could successfully increase the reporter activity, which is consistent with the known constitutive activity of CAR. However, there exists no significant effect [$F(2,12) = 1.09$, $p = 0.37$] on CAR activity by piperine treatments in the two-way ANOVA, suggesting that piperine might not have a direct effect on the transcriptional activity of CAR. In addition, there was also no significant interaction between the effect of treatments and vectors [$F(2, 12) = 0.77$, $p = 0.48$].

4. Discussion

The present study for the first time demonstrated the mechanism of the inhibitory effect of piperine on CBZ metabolism. Piperine has been characterized as a mechanism-based inhibitor on CBZ metabolism in in-vitro studies with rat and human liver microsomes. Such inhibition could result from the decreased microsomal activity and rCyp3a2

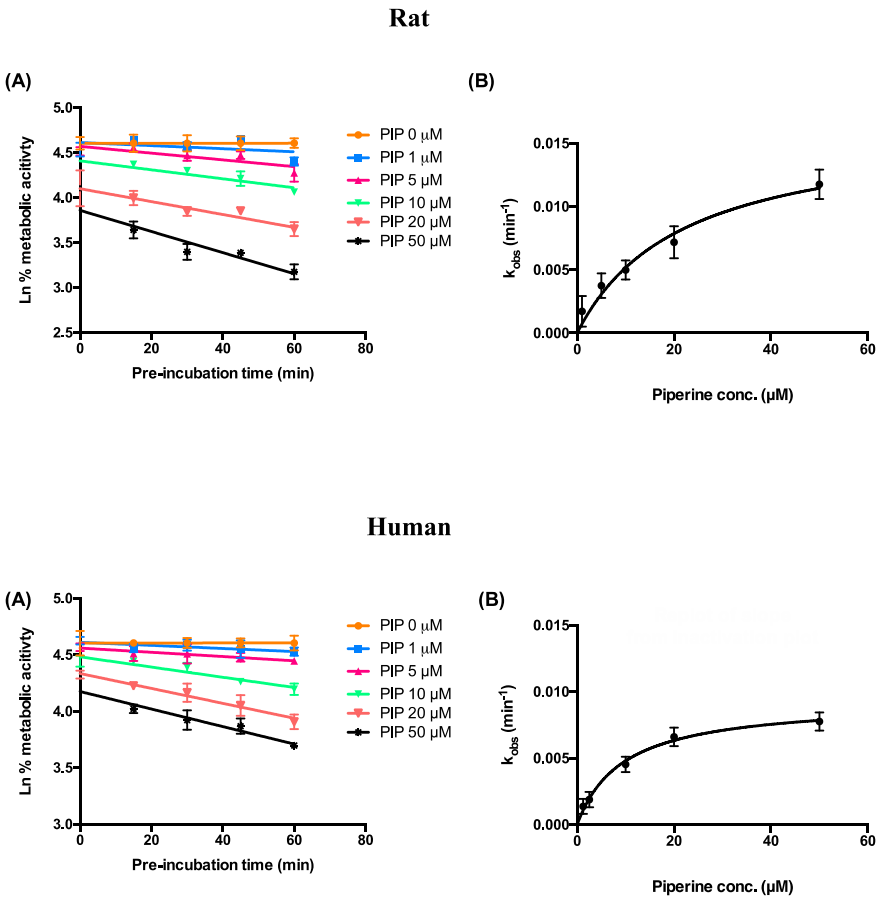

Fig. 3. Representative plots for irreversible inhibition of CBZ metabolism by piperine in rat (Upper) and human (Bottom) microsome incubation system. (A) CBZ metabolic activity remained after pre-incubation with different concentration of piperine vs. pre-incubation time plot. (B) Observed inactivation rates (k_{obs}) vs. piperine concentration plot for inactivation kinetic parameter calculation (k_{inact} and K_I). Each point represents mean ± S.D. in triplicate experiments.

mRNA and protein expression level after long-term consumption of high dose piperine.

During the in-vitro study, piperine was initially tested as reversible inhibitor since it is the most common cause for CYP inhibition [39]. The reversible inhibition could be further divided into different types including competitive, non-competitive, uncompetitive, and mixed-type inhibition based on the binding site of inhibitor with enzyme [40]. From the representative plots of in-vitro inhibition studies using rat or human liver microsome, the patterns of non-competitive inhibition were demonstrated for piperine, indicating that piperine may bind to another active site of the enzyme or the enzyme-substrate complex. The result was consistent with Laurie P. Volak's study, in which piperine was found as a selective non-competitive inhibitor for human CYP3A with *Ki* of 5.4 ± 0.3 μM for triazolam 1′-hydroxylation [11]. However, since the irreversible inhibitors are noncompetitive in nature, similar kinetic pattern could be obtained from irreversible and noncompetitive inhibition if no pre-incubation of inhibitor with enzyme was taken into account [41]. For mechanism-based (irreversible) inhibition, a metabolite-intermediate complex (MI) could be formed during the CYP metabolism with capacity to tightly bind the CYP enzyme [23]. In comparison with the reversible inhibitor, mechanism-based inhibition required de novo protein synthesis to return the metabolic activity, thus would result in more profound inhibitory effect. In such case, the potential of piperine as irreversible inhibitor was further examined by testing remaining enzyme activity using CBZ as probe after pre-incubation of piperine with liver microsome. The result showed greater loss on CBZ metabolic activity with longer pre-incubation time and higher piperine concentration, suggesting time and concentration-dependent inhibition on CBZ metabolism by piperine. Stronger inhibitory effect after pre-incubation of piperine has also been mentioned in

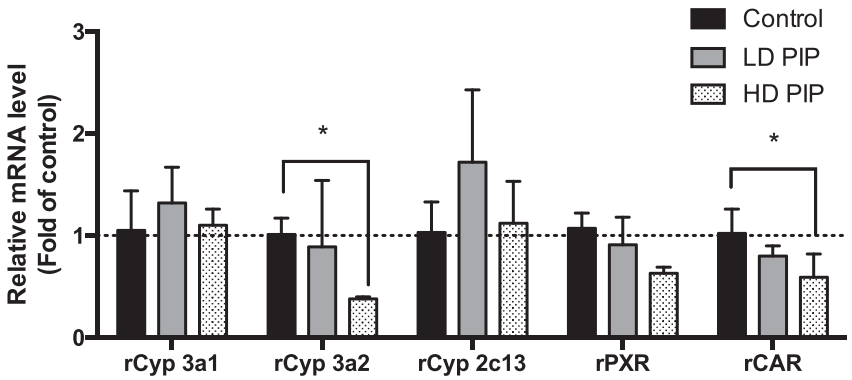

Fig. 4. Comparison of liver mRNA expression levels of genes regulating CBZ metabolism from rats after 14 days treatment of 3.5 mg/kg or 35 mg/kg piperine (LD PIP or HD PIP) or vehicle (Control). (*$p < 0.05$, compared with control group). Each point represents mean ± S.D. (n = 6).

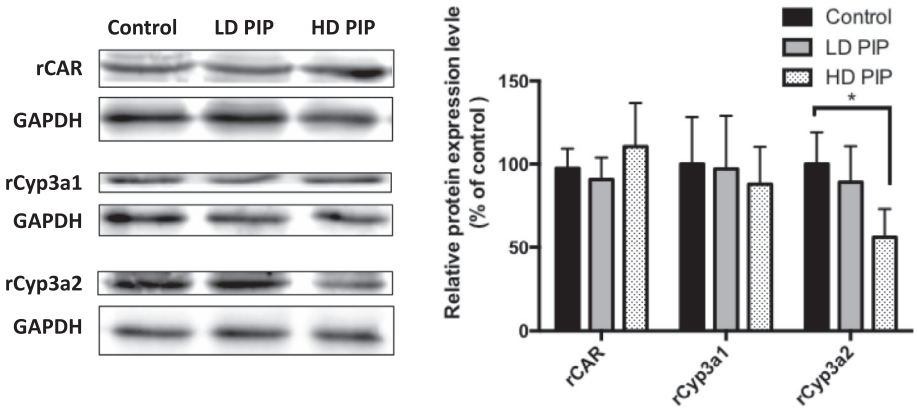

Fig. 5. Further comparison of liver rCAR, rCyp3a1 and rCyp3a2 protein expression levels from rats after 14 days treatment of 3.5 mg/kg or 35 mg/kg piperine (LD PIP or HD PIP) or vehicle (Control) by western blot (*$p < 0.05$, compared with control group). Each point represents mean ± S.D. (n = 6).

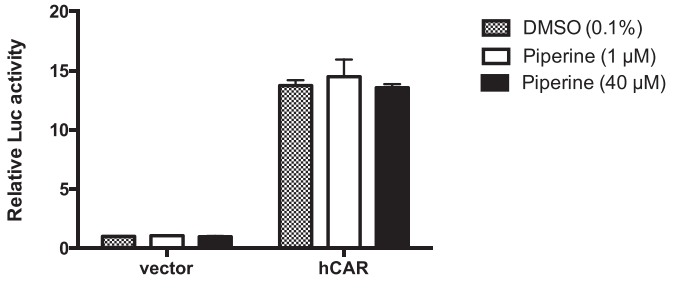

Fig. 6. Luciferase activities in HEK293T cells transfected with tk-PBRE-Luc reporter gene and empty or hCAR expression vectors after treated with piperine at their respective concentrations. The results were shown as the relative luciferase activity with respect to vehicle (0.1% DMSO) in empty vectors (*$p < 0.5$). Each point represents mean ± S.D. (n = 3).

previous study, where pre-incubation of piperine at 50 μM with human liver microsome for 10 min could lead to further 31% and 28% decrease of CYP3A4-mediated verapamil metabolites formation [10]. However, the study did not conduct further investigation with different time and incubation concentration. Although time-dependent inhibition was found, comparing to $k_{inact} > 0.3\,min^{-1}$ and $K_I < 1\,\mu M$ for potent inhibitor [42], piperine could not be considered as a highly potent inhibitor based on its k_{inact} and K_I of $0.0153\,min^{-1}$ and 18.34 μM for rat, and $0.0093\,min^{-1}$ and 9.45 μM for human. The inhibitory potency of piperine was similar to the anti-depressant drug fluoxetine, with k_{inact} and K_I of $0.017\,min^{-1}$ and 5.26 μM in human [23].

In addition, the mechanism-based inhibition effect of piperine may be related to its benzo-1,3-dioxiole (methylenedioxyphenyl) group to form inhibitory complex with enzyme. The carbene between two oxyl is highly reactive and will coordinate with haem iron in the enzyme by donating a pair of electrons [42]. The formed complex is essentially irreversible under physiological condition thus lead to greater pharmacokinetics impact than reversible inhibition. Other drugs with this structure, especially those act on central nervous system, also demonstrated time-dependent inhibition, such as Methylenedioxy-methamfetamine (ecstasy) and paroxetine (a serotonin re-uptake inhibitor) as mechanism-based inhibitor of CYP2D [43,44].

In the present study, we use CBZ as substrate for all the in-vitro microsome incubation study. Although the common methodology for obtaining inactivation parameters is by using the US FDA validated probe substrate for specific CYP isoforms, it may not accurately reflect the inhibition since the inhibition maybe substrate-dependent [42]. Such substrate-dependent interaction was mainly found in CYP3A4, owing to its large active site and multiple binding sites [45]. Base on the sigmoidal kinetics profile and molecular dynamics simulation of CYP3A4-mediated CBZ metabolism, multiple substrate-binding events were involved with CYP3A4 active sites [46,47]. Therefore, instead of using the established CYP3A probes, we use CBZ as the probe substrate to see the direct effect of piperine on CBZ metabolism.

The AUC fold changes of CBZ by piperine were predicted using mechanistic static model. The model not only considers the in-vitro kinetic parameters including k_{inact} and K_I, but also takes account into the in-vivo concentration that is available to the enzyme in liver [22]. The unbound hepatic inlet concentration of the inhibitor has been used as the surrogate for free concentration in liver before [48]. However, this free portal concentration will often over-estimate the interaction for mechanism-based inactivation. Instead, free systemic maximum plasma concentrations were used to yield a more accurate prediction. Thus, AUC fold changes by piperine were estimated to be 1.07 in rat and 1.11 in human by using unbound systematic concentrations based on the unbound plasma fraction of piperine in rat and human. However, more reliable predication could be made by physiologically-based pharmacokinetic (PBPK) models, as it will consider the dynamic change of inhibitor concentration and enzyme activity instead of single point estimation and is available to be adjusted with multiple intrinsic and extrinsic factors, such as clinical situation and genetic variations [49].

In addition to the mechanism-based inhibition by piperine, we have also demonstrated down-regulated CYPs mRNA and protein expression after prolonged use of high dose piperine in rat. Both mRNA and protein expression of rCyp3a2, the homology of human CYP3A4 that mainly contribute to the metabolism of CBZ, were decreased after the treatment of piperine [50]. As it is known that CAR played an important role in xenobiotic metabolism by modulate its primary target genes such as CYP3A and CYP2B genes, the decreased rCyp3a2 mRNA may also be attributed to the repressed CAR mRNA expression level [17]. To further investigate the influence of piperine on CAR activity, we did cell-based luciferase reporter gene assay in HEK293T cells transiently transfected with hCAR expression vector. As no significant difference was found in CAR-transfected cells upon piperine treatment, piperine may not have intrinsic inhibitory effect on hCAR. Consistent results were also demonstrated during our tests using monkey kidney-derived fibroblast (CV-1) cell transfected with hCAR or mouse CAR (mCAR), indicating piperine is neither a strong activator nor an inverse agonist for hCAR or mCAR. In addition, piperine has demonstrated both agonist and antagonist effect during screening by stably transfected HepG2 cells with hCAR vector and CYP2B6-luc reporter [51]. Such activation or inhibition effect, yet, has to be displayed in the presence of an inhibitor (PK11195) or activator (CITCO), which may be attributed to occupy ligand-binding pocket by piperine. Thus, the direct influence on CAR by piperine was not obvious. However, the possibility that piperine may inhibit the nuclear translocation of CAR could not be excluded at this point, since CAR activity has to be initiated after nuclear translocation from cytoplasm to nucleus and yet this step cannot be monitored in

transfected immortalized cells given the spontaneous nuclear translocation in these cells [52]. Beside CAR, there are other nuclear receptors involved in CYP3A regulations, such as PXR, hepatocyte nuclear factor 4 (HNF-4) and glucocorticoid receptor (GR) [53]. Since piperine has been reported to able to activate hPXR [54], it may act on other nuclear receptors to inhibit the CYP activity, which warrants further investigations.

As an antiepileptic agent with narrow therapeutic index, CBZ often requires therapeutic drug monitoring (TDM) in clinical practice. Therefore, its pharmacokinetic interactions with co-administrated substance should be taken into careful consideration [14]. In the present study, piperine demonstrated inhibition effect on CBZ metabolism, leading to increased CBZ concentration levels in-vivo. Since clinical antiepileptic regimens are usually used on long-term base, given the inhibition of piperine is time-dependent, higher inhibition on CBZ metabolism would be expected after their longer period of co-administration. With the help of inhibition kinetic parameters obtained from the current study and mechanistic static model estimation, co-administered piperine at daily consumption level (20 mg/person) could lead to 11% increase in AUC of CBZ. Based on static model, we have estimated that 40 μM of piperine in human plasma, equivalent to its plasma concentration after therapeutic dose of 420 mg/person with the assumption of linear kinetics of piperine, could lead to 90% increase of CBZ AUC. Therefore, if the patient is taking CBZ with 420 mg/person piperine, TDM and dose adjustment of CBZ are highly recommended to prevent the adverse effect. Since we have demonstrated that long-term use of piperine could inhibit the mRNA and protein expression of rCyp3a, it is also suggested that piperine may also increase the concentration levels of other anti-epileptic drugs that are CYP3A substrates, such as clobazam, diazepam and ethosuximide [55]. Therefore, it is also necessary to address the safety issues for piperine co-administration with these drugs during the clinical practice.

5. Conclusion

In summary, our study has evidenced time-dependent inhibition by piperine on carbamazepine metabolism as the mechanism of CBZ and piperine interaction. Prolonged use of piperine at high dose could not only inhibit the CBZ metabolic activity but also decreased rCyp3a2 mRNA and protein expression level and rCAR mRNA expression level. Thus, co-administration of CBZ with piperine especially after prolonged use at high dose level warrants further attention.

Conflict of interest statement

The authors declared no conflict of interest.

Acknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] Y. Dong, Z. Huihui, C. Li, Piperine inhibit inflammation, alveolar bone loss and collagen fibers breakdown in a rat periodontitis model, J. Periodontal Res. 50 (2015) 758–765.
[2] Y. Ma, M. Tian, P. Liu, Z. Wang, Y. Guan, Y. Liu, et al., Piperine effectively protects primary cultured atrial myocytes from oxidative damage in the infant rabbit model, Mol. Med. Rep. 10 (2014) 2627–2632.
[3] A.L. Greenshields, C.D. Doucette, K.M. Sutton, L. Madera, H. Annan, P.B. Yaffe, et al., Piperine inhibits the growth and motility of triple-negative breast cancer cells, Cancer Lett. 357 (2015) 129–140.
[4] G.M. da Cruz, C.F. Felipe, F.A. Scorza, M.A. da Costa, A.F. Tavares, M.L. Menezes, et al., Piperine decreases pilocarpine-induced convulsions by GABAergic mechanisms, Pharmacol. Biochem. Behav. 104 (2013) 144–153.
[5] Initial experience of treating epilepsy by piperine, Acad. J. Peking Univ. Health Sci. Cent. (1974) 214–222.
[6] M.J. Jin, H.K. Han, Effect of piperine, a major component of black pepper, on the intestinal absorption of fexofenadine and its implication on food-drug interaction, J. Food Sci. 75 (2010) H93–H96.
[7] M.S. Alhumayyd, I.A. Bukhari, A.A. Almotrefi, Effect of piperine, a major component of black pepper, on the pharmacokinetics of domperidone in rats, J. Physiol. Pharmacol. 65 (2014) 785–789.
[8] M.M. Rezaee, S. Kazemi, M.T. Kazemi, S. Gharooee, E. Yazdani, H. Gharooee, et al., The effect of piperine on midazolam plasma concentration in healthy volunteers, a research on the CYP3A-involving metabolism, Daru 22 (2014) 1–7.
[9] G. Bano, R.K. Raina, U. Zutshi, K.L. Bedi, R.K. Johri, S.C. Sharma, Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers, Eur. J. Clin. Pharmacol. 41 (1991) 615–617.
[10] R.K. Bhardwaj, H. Glaeser, L. Becquemont, U. Klotz, S.K. Gupta, M.F. Fromm, Piperine, a major constituent of black pepper, inhibits human *P*-glycoprotein and CYP3A4, J. Pharmacol. Exp. Ther. 302 (2002) 645–650.
[11] L.P. Volak, S. Ghirmai, J.R. Cashman, Curcuminoids inhibit multiple human cytochromes P450, UDP-glucuronosyltransferase, and sulfotransferase enzymes, whereas piperine is a relatively selective CYP3A4 inhibitor, Drug Metab. Dispos. 36 (2008) 1594–1605.
[12] T. Shimada, M. Mimura, K. Inoue, S. Nakamura, H. Oda, S. Ohmori, et al., Cytochrome P450-dependent drug oxidation activities in liver microsomes of various animal species including rats, guinea pigs, dogs, monkeys, and humans, Arch. Toxicol. 71 (1997) 401–408.
[13] R.R. Dalvi, P.S. Dalvi, Differences in the effects of piperine and piperonyl butoxide on hepatic drug-metabolizing enzyme system in rats, Drug Chem. Toxicol. 14 (1991) 219–229.
[14] M. Bialer, R.H. Levy, E. Perucca, Does carbamazepine have a narrow therapeutic plasma concentration range? Ther. Drug Monit. 20 (1998) 56–59.
[15] B.M. Kerr, K.E. Thummel, C.J. Wurden, S.M. Klein, D.L. Kroetz, F.J. Gonzalez, et al., Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation, Biochem. Pharmacol. 47 (1994) 1969–1979.
[16] T. Tomson, O. Almkvist, B.Y. Nilsson, J.O. Svensson, L. Bertilsson, Carbamazepine-10,11-epoxide in epilepsy. A pilot study, Arch. Neurol. 47 (1990) 888–892.
[17] T.M. Willson, S.A. Kliewer, PXR, CAR and drug metabolism, Nat. Rev. Drug Discov. 1 (2002) 259–266.
[18] S. Pattanaik, D. Hota, S. Prabhakar, P. Kharbanda, P. Pandhi, Pharmacokinetic interaction of single dose of piperine with steady-state carbamazepine in epilepsy patients, Phytother. Res. 23 (2009) 1281–1286.
[19] S.K. Bedada, R. Appani, P.K. Boga, Effect of piperine on the metabolism and pharmacokinetics of carbamazepine in healthy volunteers, Drug Res. 67 (2017) 46–51.
[20] R. Eisenthal, A. Cornish-Bowden, The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters, Biochem. J. 139 (1974) 715–720.
[21] T. Palmer, P.L. Bonner, Enzymes: Biochemistry, Biotechnology, Clinical Chemistry, Elsevier, 2007.
[22] R.S. Obach, R.L. Walsky, K. Venkatakrishnan, Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions, Drug Metab. Dispos. 35 (2007) 246–255.
[23] B.S. Mayhew, D.R. Jones, S.D. Hall, An in vitro model for predicting in vivo inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation, Drug Metab. Dispos. 28 (2000) 1031–1037.
[24] M.F. Fromm, D. Busse, H.K. Kroemer, M. Eichelbaum, Differential induction of prehepatic and hepatic metabolism of verapamil by rifampin, Hepatology 24 (1996) 796–801.
[25] S.-L. Chang, R. Levy, Inhibition of epoxidation of carbamazepine by valproic acid in the isolated perfused rat liver, J. Pharmacokinet. Biopharm. 13 (1985) 453–466.
[26] A.-C. Egnell, B. Houston, S. Boyer, In vivo CYP3A4 heteroactivation is a possible mechanism for the drug interaction between felbamate and carbamazepine, J. Pharmacol. Exp. Ther. 305 (2003) 1251–1262.
[27] FDA, Guidance for industry: bioanalytical method validation, http://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf, (2018).
[28] USDA, Production, supply and distribution database, http://apps.fas.usda.gov/psdonline/app/index.html-/app/home, (2018).
[29] J.R. Hill, In vitro drug metabolism using liver microsomes, Curr. Protoc. Pharmacol. 23 (2003) 7.8.1–7.8.11.
[30] Z. Zhang, B. Ge, L. Zhou, T.N. Lam, Z. Zuo, Induction of liver cytochrome P450s by Danshen-Gegen formula is the leading cause for its pharmacokinetic interactions with warfarin, J. Ethnopharmacol. 154 (2014) 672–686.
[31] B. Schilter, M.R. Andersen, C. Acharya, C.J. Omiecinski, Activation of cytochrome P450 gene expression in the rat brain by phenobarbital-like inducers, J. Pharmacol. Exp. Ther. 294 (2000) 916–922.
[32] V. Chirulli, V. Longo, S. Marini, A. Mazzaccaro, R. Fiorio, P.G. Gervasi, CAR and PXR expression and inducibility of CYP2B and CYP3A activities in rat and rabbit lungs, Life Sci. 76 (2005) 2535–2546.
[33] N. Kiyosawa, J.C. Kwekel, L.D. Burgoon, K.J. Williams, C. Tashiro, B. Chittim, et al., *o,p′*-DDT elicits PXR/CAR-, not ER-, mediated responses in the immature ovariectomized rat liver, Toxicol. Sci. 101 (2008) 350–363.
[34] W. Xie, J.L. Barwick, C.M. Simon, A.M. Pierce, S. Safe, B. Blumberg, et al., Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR, Genes Dev. 14 (2000) 3014–3023.
[35] X.M. Wang, W.X. Peng, Pharmacokinetics of piperine capsules in healthy volunteers, Central South Pharm. 8 (2010).
[36] C. Li, Q. Wang, T. Ren, Y. Zhang, C.W. Lam, M.S. Chow, et al., Non-linear pharmacokinetics of piperine and its herb-drug interactions with docetaxel in Sprague-Dawley rats, J. Pharm. Biomed. Anal. 128 (2016) 286–293.
[37] T. Ren, Q. Wang, C. Li, M. Yang, Z. Zuo, Efficient brain uptake of piperine and its pharmacokinetics characterization after oral administration, Xenobiotica

(2017) 1–9.
[38] V. Zabela, T. Hettich, G. Schlotterbeck, L. Wimmer, M.D. Mihovilovic, F. Guillet, et al., GABA A receptor activity modulating piperine analogs: in vitro metabolic stability, metabolite identification, CYP450 reaction phenotyping, and protein binding, J. Chromatogr. B 1072 (2018) 379–389.
[39] J.H. Lin, A.Y. Lu, Inhibition and induction of cytochrome P450 and the clinical implications, Clin. Pharmacokinet. 35 (1998) 361–390.
[40] B. Wang, Drug-Drug Interactions in Pharmaceutical Development, John Wiley & Sons, 2008.
[41] C. Mohan, K. Long, M. Mutneja, An Introduction to Inhibitors and Their Biological Applications, EMD Millipore Corp., 2013, pp. 3–13.
[42] S. Zhou, S. Yung Chan, B. Cher Goh, E. Chan, W. Duan, M. Huang, et al., Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs, Clin. Pharmacokinet. 44 (2005) 279–304.
[43] A. Heydari, K.R. Yeo, M.S. Lennard, S.W. Ellis, G.T. Tucker, A. Rostami-Hodjegan, Mechanism-based inactivation of CYP2D6 by methylenedioxymethamphetamine, Drug Metab. Dispos. 32 (2004) 1213–1217.
[44] V. Uttamsingh, R. Gallegos, J.F. Liu, S.L. Harbeson, G.W. Bridson, C. Cheng, et al., Altering metabolic profiles of drugs by precision deuteration: reducing mechanism-based inhibition of CYP2D6 by paroxetine, J. Pharmacol. Exp. Ther. 354 (2015) 43–54.
[45] K.E. Kenworthy, J.C. Bloomer, S.E. Clarke, J.B. Houston, CYP3A4 drug interactions: correlation of 10 in vitro probe substrates, Br. J. Clin. Pharmacol. 48 (1999) 716–727.
[46] H. Nakamura, N. Torimoto, I. Ishii, N. Ariyoshi, H. Nakasa, S. Ohmori, et al., CYP3A4 and CYP3A7-mediated carbamazepine 10,11-epoxidation are activated by differential endogenous steroids, Drug Metab. Dispos. 31 (2003) 432–438.
[47] C.S. Müller, T. Knehans, D.R. Davydov, P.L. Bounds, U. von Mandach, J.R. Halpert, et al., Concurrent cooperativity and substrate inhibition in the epoxidation of carbamazepine by cytochrome P450 3A4 active site mutants inspired by molecular dynamics simulations, Biochemistry 54 (2015) 711–721.
[48] R.S. Obach, R.L. Walsky, K. Venkatakrishnan, E.A. Gaman, J.B. Houston, L.M. Tremaine, The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions, J. Pharmacol. Exp. Ther. 316 (2006) 336–348.
[49] K.H. Grime, J. Bird, D. Ferguson, R.J. Riley, Mechanism-based inhibition of cytochrome P450 enzymes: an evaluation of early decision making in vitro approaches and drug-drug interaction prediction methods, Eur. J. Pharm. Sci. 36 (2009) 175–191.
[50] P. Soucek, I. Gut, Cytochromes P-450 in rats: structures, functions, properties and relevant human forms, Xenobiotica 22 (1992) 83–103.
[51] S.E. Kearney, G. Zahoránszky-Kőhalmi, K.R. Brimacombe, M.J. Henderson, Canvass: A Crowd-Sourced, Natural Product Screening Library for Exploring Biological Space, (2018).
[52] L. Li, T. Chen, J.D. Stanton, T. Sueyoshi, M. Negishi, H. Wang, The peripheral benzodiazepine receptor ligand 1-(2-chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide is a novel antagonist of human constitutive androstane receptor, Mol. Pharmacol. 74 (2008) 443–453.
[53] P. Honkakoski, M. Negishi, Regulation of cytochrome P450 (CYP) genes by nuclear receptors, Biochem. J. 347 (2000) 321–337.
[54] Y.M. Wang, W. Lin, S.C. Chai, J. Wu, S.S. Ong, E.G. Schuetz, et al., Piperine activates human pregnane X receptor to induce the expression of cytochrome P450 3A4 and multidrug resistance protein 1, Toxicol. Appl. Pharmacol. 272 (2013) 96–107.
[55] N.B. Fountain, Treatment of epilepsy: principles and practice, Neurology 58 (2002) 1578–1579.