Comparative Metabolism of Cinobufagin in Liver Microsomes from Mouse, Rat, Dog, Minipig, Monkey and Human

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Abstract

Cinobufagin (CB), a major bioactive component of Traditional Chinese Medicine Chansu, has been reported with potent antitumor activity. In this study, a comparative in vitro metabolism of CB was investigated by using liver microsomes from human (HLM), mouse (MLM), rat (RLM), dog (DLM), minipig (PLM) and monkey (CyLM), with respect to the metabolic profiles, involved enzymes and catalytic efficiency. Significant species differences in CB metabolism were revealed. In detail, the species-specific deacetylation and epimerization combined hydroxylation existed in RLM, while hydroxylation was a major pathway in HLM, MLM, DLM, PLM and CyLM. Two mono-hydroxylated metabolites of CB in human and animal species were identified as 1α-hydroxycinobufagin and 5β-hydroxycinobufagin by using LC–MS and 2D-NMR techniques. CYP3A4 was assigned as the main isoform involved in CB hydroxylation in HLM based on the chemical inhibition studies and screen assays with recombinant human CYPs. Furthermore, ketoconazole, a specific inhibitor of CYP3A, could strongly inhibit CB hydroxylation in MLM, DLM, PLM and CyLM, indicating that CYP3A was responsible for CB hydroxylation in these animal species. Apparent substrate affinity and catalytic efficiency for 1α and 5β hydroxylation of CB in liver microsomes from various species were also determined. PLM appears to have similar K_m values and total intrinsic clearance value (V_max/K_m) compared with HLM, and the total microsomal intrinsic clearance values for CB obeyed the following order, Mouse > Dog > Monkey > Human > Minipig. These findings provide vital information to better understand the metabolic behaviors of CB among various species.
Introduction

Chansu, also called toad venom or toad poison, is prepared from the dried white secretion of the auricular glands and the skin glands of Chinese toads (*Bufo melanostictus Schneider* and *Bufo bufo gargarzinus Gantor*). Chansu has long been recognized in China and other Asian countries as an important constituting material in some famous traditional Chinese medicine (TCM) formulas, such as Liushenwan (Hong et al., 1992), Shexiangbaoxinwan (Song et al., 2000) and Niuhuangxiaoyanwan (Zhou et al., 1992). Cinobufagin (CB), the principal constituent from Chansu (Ye et al., 2006; Zhao et al., 2006), is one of the most intensively investigated bufadienolides due to its natural abundance (ca. 4–6% dry weight) and potent biological activities, such as the cardiotonic, blood pressure-stimulating, local anaesthetic, antimicrobial and anticancer activities (Hong et al., 1992; Kamano et al. 1998; Ma et al., 2009). In recent years, more attention has been paid to the antitumor activity of CB. It has been well documented that CB could not only induce apoptosis against prostate cancer cell lines (Yeh et al., 2003), but also inhibit the migration and proliferation of human hepatic cells *in vitro* (Kamano et al., 2002; Su et al., 2003).

During the past two decades, most studies relevant to CB have been focused on its biological activity and toxicity, but little attention was paid to its metabolism which may influence on its efficacy and toxicity *in vivo*. CB and its analogous in Chansu have several documented toxicities, these digoxin-like components are extremely cardiotoxic (Gowda et al., 2003) and act rapidly to alter intracellular calciumstores from cardiomyocytes even in small doses (Bick et al., 2002). In addition, as potent Na\(^+\)-K\(^+\)-ATPase inhibitors, CB and its analogous have been shown to be able to cause high mortality rate (Ko et al., 1996;
Brubacher et al., 1999), and part of them are even more potent than a famous poisonous cardiac glycoside ouabain (Bick et al., 2002). Thereafter, a study on metabolic/clearance pathway(s) of CB in human is necessary for clinical risk assessment of this toxic compound and CB-containing Traditional Chinese Medicine (TCMs).

To date, the metabolism of CB has been studied mostly in rats. Previous studies revealed that most of in vivo metabolites isolated from rat bile are mono- or di-hydroxylated derivatives of 3-epi-deacetylcinobufagin (Ma et al., 2007; Ning et al., 2010), suggesting that deacetylation and epimerization are major metabolic pathways in rat (Tóma et al., 1987; Zhang et al., 1992). It should be noted that the biotransformation of CB including epimerization of 3β-hydroxy, deacetylation of C-16 ester, and introduction of hydroxyl could reduce its bioactivity significantly. On the other hand, significant difference in metabolic pathway among different species may exist, and the varied metabolites and their concentrations can affect the in vivo bioactivities and toxicities potentially. All these reasons encourage us to investigate the metabolic behaviors of CB among different species.

The specific aims of the current study were to reveal the metabolic pathway(s) of CB in human and different experimental animals including mouse, rat, dog, minipig and monkey, as well as comparing the similarities and differences in involved enzymes and their biotransformation activities on CB in liver microsomes from various species. Moreover, the anticancer activities of two hydroxylated metabolites of CB in human liver microsomes (HLM) were measured after structure elucidation by 2D-NMR.
Materials and methods

Chemicals

CB was isolated from Chansu by the author (J. Ning) and unambiguously identified by NMR and MS techniques. The purity is above 98% determined by HPLC-DAD. 1-Aminobenzotriazole (ABT), sulfaphenazole, quinidine, clomethiazole, furafylline, 8-methoxypsoralen, omeprazole, glucose-6-phosphate dehydrogenase, NADP⁺, D-glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio, USA). Montelukast was from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylenethiophosphoramide (TEPA) was purchased from Acros Organics (Geel, Belgium). All other reagents were either of LC grade or of the highest grade commercially available.

Enzyme Sources

Swiss–Hauschka (ICR) mice (n=20, male, 18 to 20 g) and Sprague–Dawley (SD) rats (n=10, male, 180–220 g) were purchased from Dalian Medical University (Dalian, China). The animals had free access to tap water and pellet diet. All the mice and rats were euthanized by decapitation, and the livers were rapidly excised and pooled for microsome preparation, respectively. Cynomolgus monkeys (n=3, male, 2.7 to 3.0 kg, 4 years old) were provided by Animal Center of Chinese Academy of Military Medical Sciences (Beijing, China). Colony-bred Chinese Bama minipigs (n=3, male, 10 to 12 kg, 6 months old) and beagle dogs (n=3, male, about 10 kg, 12 months old) were obtained from Department of Animal Science, Third Military Medical University (Chongqing, China). These animals were euthanized by i.v. injection of pentobarbital sodium (150 mg/kg b.wt.); tissue samples were
harvested within 5 min after death. All liver specimens were stored in liquid nitrogen until microsome preparation. All procedures involving animals complied with the Laboratory Animal Management Principles of China. Microsomes were prepared from liver tissue by differential ultracentrifugation as described previously (Liu HX et al. 2009), and the Lowry method was adopted to determine the concentration of microsomal protein by using bovine serum albumin as a standard (Lowry et al. 1951).

Pooled human liver microsomes (prepared from 5 male and 5 female human liver microsomal samples) were obtained from BD Gentest Corp. (Woburn, MA, USA). cDNA-expressed recombinant human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 derived from baculovirus-infected insect cells coexpressing NADPH-CYP reductase were also obtained from BD Gentest Corp. (Woburn, MA, USA). cDNA-expressed CYP2C19 in *Escherichia coli* coexpressing NADPH-CYP450 reductase was purchased from New England Biolabs (Beijing) Ltd. (Beijing, China). All microsomal samples and recombinant human CYP isoforms were stored at -80°C until use.

**Incubation Conditions**

The incubation mixture, with a total volume of 200 µl, consisted of 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM NADP+, 10 mM glucose-6-phosphate, 1 unit/ml of glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂), and liver microsomes. In all experiments, CB (25 mM dissolved in acetonitrile previously) was serially diluted to the required concentrations and the final concentration of acetonitrile did not exceed 1% (v/v) in the mixture. After preincubation at 37°C for 3 min, the reaction was initiated by adding NADPH-generating system and further incubated at 37°C in a
shaking water bath. The reaction was terminated by the addition of ice-cold acetonitrile (100 µl). The mixture was kept on ice until it was centrifuged at 20,000 × g for 10 min at 4ºC. Aliquots of supernatants were stored at -20ºC until analysis. Control incubations without NADPH-generating system or without substrate or without microsomes were carried out to ensure that metabolites formation was microsomes and NADPH dependent. All incubations throughout the study were carried out in three experiments performed in duplicate with S.D. values generally below 10%.

**UFLC-DAD and UFLC-ESI-MS Analysis**

The UFLC system equipped with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, a CTO-20AC column oven and an SPD-M 20A diode array detector. A Shim-pack XR-ODS (750 mm × 2.0 mm, 2.2 µm, Shimadzu) analytical column with an ODS guard column (5 mm × 2.0 mm, 2.2 µm, Shimadzu) was used to separate CB and its metabolites. The mobile phase consisted of CH₃CN (A) and water containing 0.5% (v/v) formic acid (B), with the following gradient profile: 0–2 min, 96%–76% B; 2-5 min, 76%–64% B; 5–10 min, 64%–49% B; 10-13 min, 49%–5% B; 13-16 min, balanced to 96% B. The flow rate was 0.4 ml/min and the column temperature was kept at 40ºC. CB and its metabolites were detected at 294 nm and quantified according to the calibration curves of authentic standards.

Shimadzu LC-MS-2010EV (Kyoto, Japan) instrument with an ESI interface was used for identification of CB and its metabolites. Mass detection was performed in both positive-ion mode (ESI⁺) and negative ion mode (ESI⁻) from m/z 100 to 800. The detector voltage was set at +1.75 kV, and –1.55 kV for positive and negative ion detection, respectively. The curved
desolvation line temperature (CDL) and the block heater temperature were both set at 250°C. Other MS detection conditions were as follows: interface voltage, 4 kV; CDL voltage, 40 V; nebulizing gas (N₂) flow was 1.5 L/min and the drying gas (N₂) pressure was set at 0.06 MPa. Data processing was performed using the LC-MS Solution version 3.41 software.

**Preparation and Purification of Hydroxylated Metabolites by Biotransformation**

*Mucor polymorphosporus* AS 3.3443 was purchased from China General Microbiological Culture Collection Center (Beijing, China). All culture and biotransformation experiments using filamentous fungi were performed in potato medium, (L): 200 g potato and 20 g glucose. Preparative scale biotransformation of CB by *M. polymorphosporus* AS 3.3443 was carried out in a 1000 ml Erlenmeyer flask containing 400 ml of the medium. The flasks were placed on the rotary shakers, operating at 180 rpm at 28-30°C. The substrate (10 mg) was added to 350 ml pre-cultured medium for 36 h. In total, 300 mg of substrate were used. The incubation was continued for four additional days under the fermentation conditions. Culture controls consisted of fermentation blanks in which microorganisms grew without substrate but with the same amount of acetone. Substrate controls contained the sterile medium with the same amount of substrate and incubated under the above conditions.

The culture was filtered, and then the filtration was extracted with the same volume of EtOAc for five times. The organic phase was collected and concentrated to dryness *in vacuo*. The residues were applied to silica gel column (45 g, 200-300 mesh, Ø 2.5×35 cm) and eluted with petroleum ether (60-90°C)-ethyl acetate (in a gradient manner from 100:1 to 1:1, at a flow rate of 1.5 ml/min) to afford Fractions (I-VII). The Fraction V was separated by preparative HPLC with an YMC ODS-A column (10×250 mm, 5 u) and eluted with
MeOH–H₂O (55:45, v/v) to give compounds M-1 (6 mg) and M-2 (5 mg).

NMR Spectroscopy

All NMR experiments were performed on a Varian INOVA-500 NMR spectrometer. ¹H and ¹³C NMR spectra (at 500 and 125 MHz, respectively) were measured at room temperature (22°C). Chemical shifts were given on the δ scale and were referenced to tetramethylsilane (TMS) at δ = 0 ppm for ¹H and ¹³C.

Chemical Inhibition Study

The hydroxylation of CB in HLM in the absence or presences of selective inhibitors for different CYP isoforms were measured to explore the involved enzyme(s) for this biotransformation. In brief, CB (20 μM, relevant to the Kₘ values) was incubated in HLM (0.125 mg protein/ml) with an NADPH-generating system in the absence (control) or presence of known CYP isoform-specific inhibitors/substrates. The selective inhibitors and their concentrations were as follows (Bjornsson et al. 2003): montelukast (2 μM) for CYP2C8 (Walsky et al. 2005), sulfaphenazole (10 μM) for CYP2C9, omeprazole (20 μM) for CYP2C19, quinidine (10 μM) for CYP2D6, clomethiazole (50 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A4. Inhibition by furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, TEPA (50 μM) for CYP2B6 (Rae et al. 2002) and ABT (500 μM) for broad CYPs (Emoto et al. 2003) were examined by adding CB after pre-incubation with NADPH-generating system at 37°C for 20 min. In addition, furafylline (10 μM), sulfaphenazole (10 μM), and ketoconazole (1 μM) were used to explore the inhibitory effect towards CB (20 μM) hydroxylation in MLM, DLM, PLM and CyLM.

Assay with Recombinant CYPs
Nine cDNA-expressed human CYP isoforms co-expressing NADPH-P450 reductase (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) were used to screen the involved isoform(s) for hydroxylation of CB in HLM. The incubations were carried out under the above conditions with HLM. To generate adequate metabolites for detection, a relative high substrate concentration (50 µM) was selected and incubated with each of the recombinant CYPs (40-80 nM) at 37ºC for 30 min. UFLC with DAD detector was used to quantify the metabolites of CB.

**Kinetic Study**

To estimate kinetic parameters of hydroxylation of CB in liver microsomes from human and other species, as well as recombinant CYP3A4, the incubation conditions were optimized to ensure the formation rates of 1-HCB and 5-HCB were in the linear range in relation to incubation time and protein concentration. CB (5, 10, 25, 50, 75, 100, 150, 200, 250 µM) was incubated with the pooled human liver microsomes (HLM, 0.125 mg protein/ml), mouse liver microsomes (MLM, 0.1 mg protein/ml), dog liver microsomes (DLM, 0.12 mg protein/ml), minipig liver microsomes (PLM, 0.125 mg protein/ml) and monkey liver microsomes (CyLM, 0.1 mg protein/ml) for 10 min or incubated with recombinant CYP3A4 (4 nM) for 20 min. All incubations were carried out in three independent experiments in duplicate. The apparent $K_m$ and $V_{max}$ values were calculated from nonlinear regression analysis of experimental data according to the Michaelis-Menten equation, and the results were graphically represented by Eadie-Hofstee plots. Kinetic constants were reported as the value ± standard error (S.E.) of the parameters estimate.

**Cytotoxicity Study of Cinobufagin Metabolites**
Human hepatoma cell line (Bel-7402) was maintained in RPMI1640 medium (GIBCO/BRL, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin. For the cytotoxicity detection, the cells were harvested by 0.25% trypsin and culture-seeded in 96-well cell culture plates. Appropriate dilutions of the compounds tested were added into the cultures. After incubation at 37°C for 72 h, the survival rates of the cancer cells were evaluated by MTT method (Mizutani et al., 1995). The activity was shown as IC50 value, a parameter that stands for the concentration (μM) of the compound tested to give 50% inhibition of cell growth. The data are expressed as the mean ± S.D (n=3).
Results

Biotransformation of Cinobufagin by Liver Microsomes from Different Species

Two product peaks were observed when CB (100 μM) was incubated with the hepatic microsomes from mouse, dog, minipig, monkey, or human (0.3 mg protein/ml) along with the NADPH-generating system for 30 min (Fig. 2). These two peaks (M-1 and M-2) were identified as mono-hydroxylated metabolites of CB by UFLC-ESI-MS, and the formation of mono-hydroxylcinobufagin was time-, NADPH- and microsomes- dependent (data not shown). An extra dihydroxylcinobufagin (M-3) can be detected in MLM under the same incubation conditions, and this minor metabolite can also be found in other species with increasing microsomes concentration or extending incubation time. In contrast, 16-deacetylcinobufagin (DCB) was the major metabolite of CB in RLM without NADPH-generating system (Fig. 3A), while at least eight metabolites could be detected in RLM with a NADPH-generating system (Fig. 3B), indicating complex metabolic pathways involved in CB metabolism in rat.

The metabolites of CB in RLM with a NADPH-generating system were characterized by UFLC-DAD-ESI-MS. With the positive-ion ESI, CB and its metabolites can produce intact molecular ions [M+H]⁺ and significant acetonitrile adducts [M+CH₃CN+H]⁺ which can be used for molecular mass determination (Table 1). Most metabolites can be assigned by comparison of retention times, UV spectra and mass spectra with authentic standards (Ye et al., 2006; Ma et al., 2007), while peak 1 (5.28 min) and 3 (5.61 min) were tentatively assigned as mono-hydroxylated deacetylcinobufagin, peak 2 (5.40 min) was tentatively assigned as 3-keto-hydroxylated deacetylcinobufagin by UFLC-ESI-MS, respectively. It is
evident from Fig. 3 and Table 1 that DCB was the most abundant metabolite of CB in RLM, while hydroxylated and epimerized derivatives of cinobufagin and DCB were also detected. Notably, two mono-hydroxylated derivatives (peak 5 and 6) in RLM corresponded well to the mono-hydroxylated metabolites of CB in HLM (M-1 and M-2, respectively).

**Preparation and Identification of Mono-hydroxylated Metabolites**

To elucidate their absolute structures and metabolic labile sites of CB in HLM, two mono-hydroxylated metabolites of CB were biosynthesized by microorganism (Srisilam and V, 2003) and characterized by 2D-NMR. Eighteen strains of filamentous fungi (from 7 genera) were initially screened by LC-MS for their biotransformation capability (from CB to mono-hydroxycinobufagin). Among the cultures screened, *Mucor polymorphosporus* AS 3.3443 was found to be able to convert cinobufagin into several mono-hydroxylated metabolites. More importantly, two major biotransformed products by this strain were characterized by UFLC-DAD-ESI-MS and found that they have identical retention times, UV spectra and mass spectra to M-1 and M-2 of CB in HLM, respectively (Supplemental File).

These two biotransformed products by microorganism were also isolated and characterized by $^1$H-NMR and $^{13}$C-NMR. The $^1$H-NMR and $^{13}$C-NMR spectral data of two mono-hydroxylated metabolites were listed in Table 2. Compared with NMR data of cinobufagin, $^{13}$C-NMR spectrum of M-1 displayed that the carbon signal at δ 29.3 (C-1) shifted downfield to δ 71.8 (CH). The other carbon signals of M-1 were similar to that of cinobufagin. In HMBC spectrum, the carbon signal at δ 71.8 correlated with the proton signals of δ 0.99 (H-19) and δ 1.93 (H-2). In $^1$H-$^1$H COSY spectrum, the proton signal at δ 1.93 (H-2) had correlations with the proton signals at δ 3.62 (H-1) and δ 4.00 (H-3), these
data suggested that the hydroxyl group should be at C-1 site. In NOESY spectrum, the NOE enhancements between the proton signal of $\delta$ 3.62 (H-1) and the signals at $\delta$ 0.99 (19-Me) and $\delta$ 1.61 (5-H), suggested the $\alpha$-configuration of 1-OH. Thus M-1 can be assigned as 1$\alpha$-hydroxylcinobufagin (1-HCB) which is a novel compound.

The $^1$H-NMR spectrum of M-2 revealed that the proton signal of H-5 at $\delta$1.69 disappeared by comparison of NMR data of cinobufagin. The $^{13}$C-NMR spectrum of M-2 showed that the carbon signal of C-5 shifted downfield to $\delta$73.5. Furthermore, the NMR data of M-2 agreed well with the spectral data of cinobufotalin reported in previous studies (Ye et al., 2006; Ma et al., 2008). Taking together, these evidences indicated that M-2 was 5$\beta$-hydroxylcinobufagin (5-HCB).

**Chemical Inhibition Studies**

Selective inhibitors of nine major CYP isoforms were used to screen the CYP isoform(s) responsible for the formation of two mono-hydroxylated metabolites in HLM (Fig. 4). ABT, a broad specificity CYP inactivator, inhibited the formation of 1-HCB and 5-HCB completely, suggesting that CYPs were responsible for the CB hydroxylation in HLM. Among nine CYP isoforms selective inhibitors tested, ketoconazole inhibited the formation of 1-HCB and 5-HCB completely, while inhibitors of other CYP isoforms did not exhibit a significant inhibition (less than 30% inhibition, $p>0.05$) towards the formation of these two metabolites. These findings suggested that the formation of 1-HCB and 5-HCB was mainly catalyzed by CYP3A.

In order to explore whether the metabolic enzyme(s) responsible for CB hydroxylation in mouse, dog, mini-pig, and monkey were also CYP3A isoenzymes, furafylline and
sulfaphenazole, the selective inhibitors of CYP1A and CYP2C, respectively, as well as ketoconazole, a potent selective inhibitor for CYP3A, were used. The results were illustrated in Fig. 5. It was evident that furafylline (10 μM) and sulfaphenazole (10 μM) slightly inhibited the formation of 1-HCB and 5-HCB in all species above mentioned, while ketoconazole (1 μM) could inhibit the formation of 1-HCB and 5-HCB near completely, implying that CYP3A played an important role in the hydroxylation of CB among these species.

**Assays by Recombinant Human CYP Isoforms**

In order to further verify the CYP isoform(s) involved in the metabolism of CB in human, the activity of CB oxidation was determined using nine cDNA-expressed CYP isoforms. After incubation at 37ºC for 30 min, two hydroxylated metabolites were formed exclusively by CYP3A4, none of the metabolites were observed in the incubation with CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 (less than 0.01 pmol/min/pmol CYP). The formation rates of CYP3A4 for 1-HCB and 5-HCB were 14.01 ± 0.90 pmol/min/pmol CYP and 26.02 ± 1.70 pmol/min/pmol CYP, respectively.

**Kinetic Study**

Over the whole concentration range tested, hydroxylation of CB in liver microsomes from human and four common animal species, as well as in recombinant CYP3A4, obeyed the Michaelis-Menten kinetics, as evidenced by Eadie-Hofstee plot (Fig. 6). The kinetic parameters including $K_m$, $V_{max}$ and the intrinsic clearance ($V_{max}/K_m$) for CB hydroxylation were determined and listed in the Table 3. In human liver microsomes, the $K_m$ values for the formation of 1-HCB and 5-HCB were 12.5 and 20.4 μM, respectively, while the $V_{max}$ values
for the formation of 1-HCB and 5-HCB were 0.59 and 1.17 nmol/min/mg, respectively. The 
$K_m$ values for the formation of 1-HCB and 5-HCB in recombinant CYP3A4 were 16.0 and 
32.6 µM, respectively, and $V_{\text{max}}$ values for the formation of 1-HCB and 5-HCB were 19.22 
and 45.81 pmol/min/pmol CYP, respectively. In liver microsomes from four experimental 
animals, the $K_m$ values for 1-HCB formation ranged from 3.3 to 34.2 µM, while the $K_m$
values for 5-HCB formation ranged from 3.4 to 20.4 µM. The intrinsic clearance ($C_{\text{Lint}}$) for 
the formation of 1-HCB and 5-HCB diversified greatly among different species, with the 
range of 14.5-595.0 and 54.6-644.6 µl/min/mg proteins, respectively.

**Cytotoxicity Studies of Cinobufagin Metabolites**

Taking into account that 1$\alpha$ and 5$\beta$-hydroxylation of CB by CYP3A are major metabolic 
pathways in human liver, it is necessary to evaluate the bioactivities of these two 
mono-hydroxylated metabolites (1-HCB and 5-HCB) compared with CB. The *in vitro* 
cytotoxicity tests of 1-HCB, 5-HCB and CB were evaluated by using Bel-7402 cell line. The 
result showed that IC$_{50}$ values were 0.37 ± 0.05 (M-1), 8.62 ± 0.42 (M-2) and $4.9 \times 10^{-2}$ ± 
$0.16 \times 10^{-2}$ µM (CB), respectively. This finding showed that 1$\alpha$ or 5$\beta$-hydroxylation of CB 
were capable to reduce the cytotoxic activities (increasing the IC$_{50}$ values about 7.5-fold and 
175-fold for 1-HCB and 5-HCB, respectively) against Bel-7402 cells, suggesting that these 
hydroxylation pathways might serve as the deactivation routs.
Discussion

In the past thirty years, rat has been widely used in pharmacokinetic and toxicological studies of Chansu and cinobufagin (Tóma et al., 1987; Zhang et al., 1992; Gowda et al., 2003; Xu et al., 2007; Liang et al. 2008; Jiang et al., 2009). However, until now, the differences in metabolic pathway and metabolic behavior of CB between human and common experimental animals have not been revealed yet. In this study, a comparison of metabolic profiles, involved enzymes and their catalytic efficiency for CB metabolism in liver microsomes from different species were performed. Our results revealed that complex pathways including deacetylation, epimerization and hydroxylation were involved in metabolism of CB in RLM, while the species-specific deacetylation of CB was an important metabolic pathway which was well characterized previously (Zhang et al., 1992). In sharp contrast, the CYP-mediated hydroxylation was thought be the major pathway responsible for the elimination of CB in human liver, because none of hydrolyzed, epimerized and glucuronidated metabolites were formed and CB concentration was not decreased in HLM incubation (data not shown). Except of rat, comparable metabolic profiles were observed when CB was incubated with liver microsomes from human, monkey, minipig, dog and mouse along with the NADPH-generating system. These results revealed that the large difference in metabolic profiles between rat and other species. This could also be used to explain why the major metabolites of CB in rat, namely deacetylcinobufagin and 3-epi-deacetylcinobufagin, can not be observed in serum of the dog via oral or intravenous administration of CB (Tóma et al., 1987).

For further exploration of the similarity on the enzymes responsible for hydroxylation of
CB in liver microsome from various species, chemical inhibition studies by using selective inhibitors were used. It is evident from chemical inhibition studies and assay with recombinant CYPs that both 1α and 5β hydroxylation of CB in HLM were catalyzed predominantly by CYP3A4, while the 1α and 5β hydroxylation of CB in liver microsomes from monkey, minipig, dog and mouse were also CYP3A-mediated. These findings suggested the potential similarity in the metabolic enzymes for CB hydroxylation in liver microsome from species mentioned above. In contrast, a specific B-type esterase(s) localized in the microsomal fraction of rat liver was responsible for the hydrolysis of CB (Zhang et al., 1992). The large difference in metabolic profiles and involved metabolic enzymes between rat and human suggested that rat was not a suitable surrogate animal model for pharmacokinetic, toxicological and pharmacological studies of CB, and the in vivo data obtained from rat should be cautiously utilized for interspecies extrapolation.

Although animal model(s) has been commonly used in preclinical studies to evaluate the pharmacokinetics and toxicity in human, it should be pointed out that a suitable animal model(s) for in vivo DMPK studies should have the relevant metabolism behaviors compared with human, including identical metabolic profiles, equivalent or similar metabolic enzymes and closed catalytic efficiency (Martignoni et al., 2006). CYP3A is the most important isoform involved in the metabolism of CB in human and above mention animal species, but various CYP3A isoforms expressed in various species with different substrate specificities and catalytic efficiency. In this study, a comparative study on CB metabolism in human and four experimental animals with similar profiles was carried out to determine the catalytic efficiency of CB hydroxylation by liver microsomes from various species. None of the
animal species are completely similar to human with respect to the enzyme activities and the catalytic efficiency for the formation of 1-HCB and 5-HCB are diversified. HLM, PLM and MLM have the similar $K_m$ values for the formation of 1-HCB (M-1), but none of the experimental animal species have the closed $K_m$ values for the formation of 5-HCB (M-2). The intrinsic clearance values ($V_{max}/K_m$) for 1-HCB and 5-HCB in dog were much higher than that in human, due to the high affinity of DLM (low $K_m$ values) for the formation of these two metabolites. Both CyLM and MLM displayed the high catalytic activity for the formation of 1-HCB and 5-HCB, which could be attributed to the higher total CYP levels/mg liver microsomal protein in monkeys and mice compared with human (Martignoni et al., 2006). Comparatively, PLM exhibited similar $K_m$ values and total intrinsic clearance value compared with HLM. These results indicated that minipig might be a good choice to serve as a surrogate model for DMPK studies of CB.

The structure-activity relationship studies on bufadienolides revealed that biotransformation of CB including epimerization, deacetylation, and hydroxylation could reduce its bioactivities including anticancer activity and cardiotonic activity (Kamano, 1970; Kamano et al., 1998). This study also demonstrated that 1$\alpha$ and 5$\beta$-hydroxylation of CB by CYP3A would reduce its cytotoxicity activity. In this case, the effects on CYP3A may lead to large variations on exposure of CB which can influence the in vivo potency or bring severe adverse reactions. As a substrate of CYP3A, more adverse drug-drug reactions or undesirable effects may occur when CB co-administrated with those drugs which are substrate, inducer or inhibitor of CYP3A. It has been reported that many compounds including xenobiotics and endogenous steroids could cause marked modifications on CYP3A-mediated metabolism.
(Nakamura et al., 2002; Usmani et al., 2003; Zhou et al., 2007; Zhou, 2008). Their effects on CYP3A including alternating the kinetic behaviors, activating or inhibiting the biotransformation of other substrates, would make the pharmacokinetic behaviors more complicated. It also should be noted that the metabolism of CB could be affected by other bufadienolides coexisted in Chansu, due to their similar structures and their potential inhibitory effect on CYP3A4 (Shimada et al., 2006; Li et al., 2009). The inhibition of CYP3A by other bufadienolides in Chansu can lead to accumulation of CB at a relative high level in vivo, which might cause some serious adverse reactions. Therefore, more attention should be paid on the interactions of CB with its coadminstrated drugs and other bufadienolides in clinic.

In summary, the metabolic pathways of CB in human and five various experimental animals were elucidated in the present study. The species-specific deacetylation and epimerization together with hydroxylation are biotransformation pathways in RLM, while hydroxylation of CB at C-1 and C-5 sites by CYP3A are major metabolic pathways in liver microsomes from human, monkey, dog, minipig and mouse. The metabolic behaviors of CB among different species have been elucidated with respect to the similarities on metabolic profiles, involved enzymes and their catalytic efficacy, which would contribute to the animal selection in the toxicological and pharmacokinetic studies of CB or several TCMs containing CB.
Acknowledgment

The authors would like to thank Prof. Hong Wei (Third Military Medical University) for providing the liver of monkey, minipig and dog.
Authorship Contributions

Participated in research design: Ma, Ge, Liang, and Yang.

Conducted experiments: Ning, and Ge.

Performed data analysis: Ma, Ning, and Ge.

Wrote or contributed to the writing of the manuscript: Ning, Ge, Liang, Wang, and Yang.

Other: Zhang, Huang, and Li contributed to the preparation of authentic standards.
References


constituents in rat plasma after oral administration of Shexiang Baoxin pill by HPLC-ESI-MS/MS. *Biomed Chromatogr* **23**: 1333-1343.


Herbal Medicine 23: 490.
Footnotes

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Figure legends

Figure 1. Structure of cinobufagin.

Figure 2. Representative UFLC profiles of CB and its metabolites in liver microsomes from human, minipig, dog, monkey and mouse. CB (100 µM) was incubated with liver microsomes (0.3 mg/ml) from different species with NADPH-generating system at 37°C for 30 min.

Figure 3. Representative UFLC profiles of CB and its metabolites (peak 1 to 8) in rat. CB (100 µM) was incubated with RLM (0.3 mg/ml) at 37°C for 30 min without NADPH-generating system (A) and with NADPH-generating system (B).

Figure 4. Effects of selective CYP inhibitors on the formation of two mono-hydroxylated metabolites (M-1 and M-2) in HLM. Results are the mean ± S.D. from three experiments carried out in duplicate.

Figure 5. Effects of furafylline, sulfaphenazole and ketoconazole on the formation of two mono-hydroxylated metabolites (M-1 and M-2) in CyLM, PLM, DLM and MLM. Results are the mean ± S.D. from three experiments carried out in duplicate.

Figure 6. Michaelis-Menten plots of CB metabolism in human liver microsomes. CB (2.5-250 µM) was incubated with HLM (0.125 mg/ml) at 37°C for 10 min with NADPH-generating system. An Eadie-Hofstee plot was shown as an inset to illustrate monophase kinetics. Data points represent the mean of triplicate determinations.

Figure 7. The metabolic pathways of CB in liver microsomes from human, monkey, dog, minipig and mouse.
Table 1. Retention times (tR), molecular weights (M.W.) and MS data for metabolites of CB in RLM.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>tR (min)</th>
<th>M.W.</th>
<th>Identification</th>
<th>Molecular ions</th>
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<tbody>
<tr>
<td>1</td>
<td>5.28</td>
<td>416</td>
<td>Mono-hydroxyl-deacetylcinobufagin</td>
<td>417 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>458 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>2</td>
<td>5.40</td>
<td>414</td>
<td>3-Keto-hydroxyl-deacetylcinobufagin</td>
<td>415 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>456 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>3</td>
<td>5.61</td>
<td>416</td>
<td>Mono-hydroxyl-deacetylcinobufagin</td>
<td>417 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>458 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>4</td>
<td>6.95</td>
<td>400</td>
<td>16-Deacetylcinobufagin</td>
<td>401 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>442 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>5</td>
<td>7.35</td>
<td>458</td>
<td>1a-Hydroxylcinobufagin</td>
<td>459 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>6</td>
<td>7.60</td>
<td>458</td>
<td>5β-Hydroxylcinobufagin</td>
<td>459 [M+H]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>7</td>
<td>8.32</td>
<td>400</td>
<td>3-Epi-deacetylcinobufagin</td>
<td>401 [M+H]+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>442 [M+CH₃CN+H]+</td>
</tr>
<tr>
<td>8</td>
<td>10.59</td>
<td>442</td>
<td>3-Epi-cinobufagin</td>
<td>484 [M+CH₃CN+H]+</td>
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</tbody>
</table>

a: tR, retention time (min)
Table 2. $^1$H- (500 MHz, DMSO) and $^{13}$C-NMR (125 MHz, DMSO) spectral data for M-1 and M-2.

<table>
<thead>
<tr>
<th>No.</th>
<th>1$\alpha$-hydroxyl cinobufagin (M-1)</th>
<th>5$\beta$-hydroxyl cinobufagin (M-2)</th>
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<tr>
<td></td>
<td>$^1$H-NMR</td>
<td>$^{13}$C-NMR</td>
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<tr>
<td>1</td>
<td>3.62 brs</td>
<td>71.8 d</td>
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<tr>
<td>2H$_a$</td>
<td>1.93</td>
<td>32.0 t</td>
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<tr>
<td>H$_b$</td>
<td>1.68</td>
<td>H-1</td>
</tr>
<tr>
<td>3</td>
<td>4.00 (brs)</td>
<td>66.7 d</td>
</tr>
<tr>
<td>4H$_a$</td>
<td>1.78</td>
<td>32.8 t</td>
</tr>
<tr>
<td>H$_b$</td>
<td>1.82</td>
<td>H-5</td>
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<tr>
<td>5</td>
<td>1.63</td>
<td>30.1 d</td>
</tr>
<tr>
<td>6H$_a$</td>
<td>1.32</td>
<td>25.0 t</td>
</tr>
<tr>
<td>H$_b$</td>
<td>1.73</td>
<td>H-5</td>
</tr>
<tr>
<td>7H$_a$</td>
<td>1.20</td>
<td>19.8 t</td>
</tr>
<tr>
<td>H$_b$</td>
<td>1.33</td>
<td>H-8</td>
</tr>
<tr>
<td>8</td>
<td>1.87</td>
<td>32.8 d</td>
</tr>
<tr>
<td>9</td>
<td>1.73</td>
<td>39.9 d</td>
</tr>
<tr>
<td>10</td>
<td>---</td>
<td>40.5 s</td>
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<tr>
<td>11H$_a$</td>
<td>1.17</td>
<td>20.6 t</td>
</tr>
<tr>
<td>H$_b$</td>
<td>1.40</td>
<td>C-13</td>
</tr>
<tr>
<td>12H$_a$</td>
<td>1.48</td>
<td>38.7 t</td>
</tr>
<tr>
<td>H$_b$</td>
<td>1.65</td>
<td>C-18</td>
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<tr>
<td>13</td>
<td>---</td>
<td>44.6 s</td>
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<tr>
<td>14</td>
<td>---</td>
<td>71.9 s</td>
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<tr>
<td>15</td>
<td>3.75 (s)</td>
<td>59.3 d</td>
</tr>
<tr>
<td>16</td>
<td>5.45(d, 9.5)</td>
<td>74.6 d</td>
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<tr>
<td>17</td>
<td>2.85 (9.5)</td>
<td>48.9 d</td>
</tr>
<tr>
<td>18</td>
<td>0.71 (s)</td>
<td>16.9 q</td>
</tr>
<tr>
<td>19</td>
<td>0.99 (s)</td>
<td>18.7 q</td>
</tr>
<tr>
<td>20</td>
<td>---</td>
<td>116.1 s</td>
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<tr>
<td>21</td>
<td>7.48 (s)</td>
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<td>22</td>
<td>7.81 (brs)</td>
<td>148.6 d</td>
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<td>23</td>
<td>6.24 (d, 9.5)</td>
<td>112.9 d</td>
</tr>
<tr>
<td>24</td>
<td>---</td>
<td>160.9 s</td>
</tr>
<tr>
<td>25</td>
<td>---</td>
<td>169.4 s</td>
</tr>
<tr>
<td>26</td>
<td>1.82(s)</td>
<td>20.2 q</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>M-1 (1-HCB)</th>
<th>M-2 (5-HCB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td>Human</td>
<td>0.59 ± 0.01</td>
<td>12.5 ± 0.8</td>
</tr>
<tr>
<td>Monkey</td>
<td>2.12 ± 0.04</td>
<td>34.2 ± 2.4</td>
</tr>
<tr>
<td>Dog</td>
<td>1.28 ± 0.03</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Minipig</td>
<td>0.25 ± 0.02</td>
<td>17.2 ± 3.8</td>
</tr>
<tr>
<td>Mouse</td>
<td>8.39 ± 0.15</td>
<td>14.1 ± 1.3</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>19.22 ± 0.68</td>
<td>16.0 ± 2.6</td>
</tr>
</tbody>
</table>

K<sub>m</sub> values were in μM; V<sub>max</sub> values were in nmol/min/mg for liver microsomes, or in pmol/min/pmol CYP for CYP3A4; V<sub>max</sub>/K<sub>m</sub> values were in µl/mg/min for liver microsomes, or in µl/pmol/min for CYP3A4. The range of substrate concentrations was 5-250 μM. Each value was the mean ± S.D. of three determinations performed in duplicate.
Figure 3
Figure 4
Figure 5: Residual Activity (%) for different compounds across different species.

- Monkey
- Dog
- Mouse
- Minipig

Compounds: Furafylline, Sulfaphenazole, Ketoconazole

Species: M-1, M-2

Bars represent the residual activity, with error bars indicating variability.
Figure 6
Cinobufagin (CB) → M-1 + M-2

Figure 7