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 the traditional Chinese medicine Chansu, has been reported to have potent antitumor activity. In this study, in vitro metabolism of CB among species was compared with respect to metabolic profiles, enzymes involved, and catalytic efficiency by using liver microsomes from human (HLM), mouse (MLM), rat (RLM), dog (DLM), minipig (PLM), and monkey (CyLM). Significant species differences in CB metabolism were revealed. In particular, species-specific deacetylation and epimerization combined with hydroxylation existed in RLM, whereas hydroxylation was a major pathway in HLM, MLM, DLM, PLM, and CyLM. Two monohydroxylated metabolites of CB in human and animal species were identified as 1α-hydroxycinobufagin and 5β-hydroxybufalinobufagin by using liquid chromatography-mass spectrometry and two-dimensional NMR techniques. CYP3A4 was identified as the main isoform involved in CB hydroxylation in HLM on the basis of the chemical inhibition studies and screen assays with recombinant human cytochrome P450s. Furthermore, ketoconazole, a specific inhibitor of CYP3A, strongly inhibited CB hydroxylation in MLM, DLM, PLM, and CyLM, indicating that CYP3A was responsible for CB hydroxylation in these animal species. The 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 Km and total intrinsic clearance value (Vmax/Km) similar to those for 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 gargarizans Gantar). Chansu has long been recognized in China and other Asian countries as an important constituent of some famous traditional Chinese medicine (TCM) formulas, such as Chansu (Ye et al., 2006; Zhao et al., 2006), Huang Xiao Yan Wan (Zhou et al., 1992), Cinobufagin (CB) (Hong et al., 1992), Shexiang Baoxin Wan (Song et al., 2000), and Niu (1992). A number of studies have demonstrated that several TCM formulas, such as Liu Shen Wan, have antitumor activity. In this study, we focused on its biological activity and toxicity, but little attention was paid to its metabolism, which may influence its efficacy and toxicity in vivo. CB and its analogs in Chansu have several documented toxicities: these digoxin-like components are extremely cardiotoxic to local anesthetic, 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 were focused on its biological activity and toxicity, but little attention was paid to its metabolism, which may influence its efficacy and toxicity in vitro. CB and its analogs in Chansu have several documented toxicities: these digoxin-like components are extremely cardiotoxic (Gowda et al., 2003) and act rapidly to alter intracellular calcium stores from cardiomyocytes even in small doses (Bick et al., 2002). In addition, as potent Na⁺-K⁺-ATPase inhibitors, CB and its analogs have been shown to be associated with a high mortality rate (Ko et al., 1996; Brubacher et al., 1999), and some of them are even more potent one of the most intensively investigated bufadienolides because of its natural abundance (approximately 4–6% dry weight) and potent biological activities, such as its cardiotonic, blood pressure-stimulating, local anesthetic, 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).

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ABBREVIATIONS: TCM, traditional Chinese medicine; CB, cinobufagin; HLM, human liver microsomes; MS, mass spectrometry; ABT, 1-amino-benzotriazole; DAD, diode array detector; LC, liquid chromatography; P450, cytochrome P450; UFLC, ultrafast liquid chromatography; ESI, electrospray ionization; MLM, Swiss-Hauschka mouse liver microsome(s); DLM, beagle dog liver microsome(s); PLM, minipig liver microsome(s); CyLM, cynomolgus monkey liver microsome(s); DSB, 16-deacetylcinobufagin; RLM, Sprague-Dawley rat liver microsomes; 1-HCB, 1α-hydroxylicinobufagin; 5-HCB, 5β-hydroxylicinobufagin.
than the well know poisonous cardiac glycoside ouabain (Bick et al., 2002). Therefore, a study on the metabolic/clearance pathway(s) of CB in humans is necessary for clinical risk assessment of this toxic compound and CB-containing TCMs.

To date, the metabolism of CB has been studied mostly in rats. Previous studies revealed that most of the in vivo metabolites isolated from rat bile are mono- or dihydroxylated derivatives of 3-epi-deacteylbufagin (Ma et al., 2007; Ning et al., 2010), suggesting that deacteylation and epimerization are major metabolic pathways in rats (Toma et al., 1987; Zhang et al., 1992). It should be noted that the biotransformation of CB, including epimerization of 3β-hydroxyl, deacteylation of the C-16 ester, and introduction of hydroxyl, could reduce its bioactivity significantly. On the other hand, significant differences in metabolic pathways among different species may exist, and the various metabolites and their concentrations can potentially affect in vivo bioactivities and toxicities. All these reasons encouraged 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 humans and different experimental animals including mouse, rat, dog, minipig, and monkey to compare the similarities and differences in the enzymes involved 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 two-dimensional NMR.

Materials and Methods

Chemicals. CB was isolated from Chansu by one of the authors (J.N.) and subsequently identified by NMR and MS techniques. The purity was greater than 98% as determined by high-performance liquid chromatography-DAD. 1-Aminobenzotriazole (ABT), sulfaphenazole, quinidine, clomethiazole, furafylline, 8-methoxysoralen, omeprazole, glucose-6-phosphate dehydrogenase, NADP⁺, and D-glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Ketoconazole was obtained from MP Biomedicals (Salon, OH). Montelukast was from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylthiophosphoramide was purchased from Acorus 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, 18–20 g) and Sprague-Dawley rats (n = 10, male, 180–220 g) were purchased from Dalian Medical University (Dalian, China). The animals had free access to tap water and a pellet diet. All of the mice and rats were euthanized by decapitation, and livers were rapidly excised and pooled for microsome preparation. Cynomolgus monkeys (n = 3, male, 2.7–3.0 kg, 4 years old) were provided by the Animal Center of the Chinese Academy of Medical Sciences (Beijing, China). Colony-bred Chinese Bama minipigs (n = 3, male, 10–12 kg, 6 months old) and beagle dogs (n = 3, male, approximately 10 kg, 12 months old) were obtained from the Department of Animal Science, Third Military Medical University (Chongqing, China). These animals were euthanized by intravenous injection of pentobarbital sodium (150 mg/kg i.v.); 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 et al., 2009), and the method of Lowry et al. (1951) was adopted to determine the concentration of microsomal protein by using bovine serum albumin as a standard.

Pooled human liver microsomes (prepared from five male and five female human liver microsomal samples) were obtained from BD Gentest (Woburn, MA). cDNA-expressed recombinant human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 derived from baculovirus-infected insect cells coexpressing NADPH-P450 reductase were also obtained from BD Gentest. cDNA-expressed CYP2C19 in Escherichia coli coexpressing NADPH-P450 reductase was purchased from New England Biolabs (Beijing) Ltd. (Beijing, China). All microsomal samples and recombinant human P450 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), an NADPH-generating system (1 mM NADP⁺, 10 mM glucose 6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂) and liver microsomes. In all experiments, CB (25 mM dissolved in acetone previously) was serially diluted to the required concentrations, and the final concentration of acetone did not exceed 1% (v/v) in the mixture. After preincubation at 37°C for 3 min, the reaction was initiated by addition of the NADPH-generating system and further incubated at 37°C in a shaking water bath. The reaction was terminated by the addition of ice-cold acetone (100 μl). The mixture was kept on ice until it was centrifuged at 20,000g for 10 min at 4°C. Aliquots of supernatants were stored at −20°C until analysis. Control incubations without the NADPH-generating system or without substrate or without microsomes were performed to ensure that metabolite formation was microsome- and NADPH-dependent. All incubations throughout the study were performed in three experiments performed in duplicate with S.D. values generally less than 10%.

UFLC-DAD and UFLC-ESI-MS Analysis. The UFLC system was equipped with a CBM-20A communications bus module, an SIL-20AHT 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 × 2.0 mm, 2.2 μm; Shimadzu, Kyoto, Japan) 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 to 2 min, 96 to 76% B; 2 to 5 min, 76 to 64% B; 5 to 10 min, 64 to 49% B; 10 to 13 min, 49 to 5% B; and 13 to 16 min, balanced to 96% B. The flow rate was 0.4 ml/min, and the column temperature was kept at 40°C. CB and its metabolites were detected at 294 nm and quantified according to the calibration curves of authentic standards.

A Shimadzu LC-MS-2010EV 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⁻) spectra from m/z 100 to 800. The detector voltage was set at +1.75 and −1.55 kV for positive and negative ion detection, respectively. The curved desolvation line temperature and the block heater temperature were both set at 250°C. Other MS detection conditions were as follows: interface voltage, 4 kV; curved desolvation line voltage, 18 V; nebulizing gas (N₂) flow, 1.5 l/min; and drying gas (N₂) pressure, 0.06 MPa. Data processing was performed using LC-MS Solution software (version 3.41).

Preparation and Purification of Hydroxylated Metabolites by Biotransformation. Mucor polymorphus AS 3.3443 was purchased from the China General Microbiological Culture Collection Center (Beijing, China). All culture and biotransformation experiments using filamentous fungi were performed in potato medium, and each liter of potato medium contained 200 g of potato and 20 g of glucose. Preparative scale biotransformation of CB by M. polymorphus 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 to 30°C. The substrate (10 mg) was added...
to 350 ml of precultured medium for 36 h. In total, 300 mg of substrate were used. The incubation was continued for 4 additional days under the fermentation conditions. Culture conditions 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 were incubated under the above conditions.

The culture was filtered, and then the filtrate was extracted five times with the same volume of ethyl acetate. The organic phase was collected and concentrated to dryness in vacuo. The residues were applied to a 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 to VII. Fraction V was separated by preparative high-performance liquid chromatography with an YMC ODS-A column (10 × 250 mm, 5 μm) and eluted with methanol-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 are given on the δ scale and were referenced to tetramethylsilane at δ = 0 ppm for 1H and 13C.

**Chemical Inhibition Study.** The hydroxylation of CB in HLM in the absence or presence of selective inhibitors for different P450 isoforms were measured to explore the enzyme(s) involved in this biotransformation. In brief, CB (20 μM, relevant to the K_m values) was incubated in HLM (0.125 mg of protein/ml) with an NADPH-generating system in the absence (control) or presence of known P450 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, and ketoconazole (1 μM) for CYP3A4. Inhibition by furafylline (10 μM), 8-methoxypsoralen (0.1 μM), and sulfaphenazole (10 μM), and ketoconazole (1 μM) were used to explore the inhibitory effect toward CB (20 μM) hydroxylation in MLM, DLM, PLM, and CyLM.

**Assay with Recombinant P450s.** Nine cDNA-expressed human P450 isoforms coexpressing 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 performed under the above conditions with HLM. To generate adequate metabolites for detection, a relatively high substrate concentration (50 μM) was selected and incubated with each of the recombinant P450s (40–80 nM) at 37°C for 30 min. UFLC with a DAD 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 that 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, and 250 μM) was incubated with the pooled HLM (0.125 mg protein/ml), MLM (0.1 mg protein/ml), DLM (0.12 mg protein/ml), PLM (0.125 mg protein/ml), and CyLM (0.1 mg protein/ml) for 10 min or incubated with recombinant CYP3A4 (4 nM) for 20 min. All incubations were performed in three independent experiments in duplicate. The apparent k_cat 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-Hofsee plots. Kinetic constants were reported as the value ± S.E. of the parameter estimate.

**Cytotoxicity Study of Cinobufagin Metabolites.** Human hepatoma cell line Bel-7402 was maintained in GIBCO RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. For 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 with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method (Mizutani et al., 1995). The activity is shown as the IC_50 value, a parameter that stands for the concentration (micromolar) 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 the monohydroxylated metabolites of CB by UFLC-ESI-MS, and the formation of monohydroxycinobufagin was time-, NADPH-, and microsome-dependent (data not shown). An extra dihydroxycinobufagin (M-3) can be detected in MLM under the same incubation conditions, and this minor metabolite can also be found in other species by increasing the microsome concentration or extending the incubation time. In contrast, 16-deacetylcinobufagin (DCB) was the major metabolite of CB in RLM without an NADPH-generating system (Fig. 3A), whereas at least eight metabolites could be detected in RLM with an NADPH-generating system (Fig. 3B), indicating that complex metabolic pathways are involved in CB metabolism in rat.

The metabolites of CB in MLM with an NADPH-generating system were characterized by UFLC-DAD-ESI-MS. With positive ion ESI, CB and its metabolites can produce intact molecular ions [M + H]^+...
and significant acetonitrile adducts \([M + \text{CH}_3\text{CN} + \text{H}]^+\) that 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), whereas peaks 1 (5.28 min) and 3 (5.61 min) were tentatively assigned as monohydroxylated deacetylcinobufagin and peak 2 (5.40 min) was tentatively assigned as 3-ketohydroxylated deacetylcinobufagin by UFLC-ESI-MS. It is evident from Fig. 3 and Table 1 that DCB was the most abundant metabolite of CB in RLM, whereas hydroxylated and epimerized derivatives of cinobufagin and DCB were also detected. Of note, two monohydroxylated derivatives (peaks 5 and 6) in RLM corresponded well to the monohydroxylated metabolites of CB in HLM (M-1 and M-2, respectively).

Preparation and Identification of Monohydroxylated Metabolites. To elucidate their absolute structures and the metabolic labile sites of CB in HLM, two monohydroxylated metabolites of CB were biosynthesized by microorganisms and characterized by 2D NMR. Eighteen strains of filamentous fungi (from seven genera) were initially screened by LC-MS for their biotransformation capability (from CB to monohydroxycinobufagin). Among the cultures screened, \(M.\) polymorphosporus AS 3.3443 was found to be able to convert cinobufagin into several monohydroxylated metabolites. Of more importance, two major biotransformed products by this strain were characterized by UFLC-DAD-ESI-MS and they were found to have retention times, UV spectra, and mass spectra identical to those of M-1 and M-2 of CB in HLM (M-1 and M-2, respectively).

These two products biotransformed by microorganism were also isolated and characterized by \(^1\text{H}\) NMR and \(^{13}\text{C}\)-NMR. The \(^1\text{H}\) NMR and \(^{13}\text{C}\)-NMR spectral data of two monohydroxylated metabolites are listed in Table 2. Compared with NMR data of cinobufagin, the \(^{13}\text{C}\) NMR spectrum of M-1 displayed a carbon signal at \(\delta 29.3\) (C-1) shifted downfield to \(\delta 71.8\) (CH). The other carbon signals of M-1 were similar to that of cinobufagin. In the heteronuclear multiple-bond correlation spectroscopy spectrum, the carbon signal at \(\delta 71.8\) correlated with the proton signals of \(\delta 0.99\) (H-19) and \(\delta 1.93\) (H-2). In the \(^1\text{H}-^1\text{H}\) correlation spectroscopy spectrum, the proton signal at \(\delta 1.93\) correlated with the proton signals at \(\delta 3.62\) (H-1) and \(\delta 4.00\) (H-3); these data suggested that the hydroxyl group should be at the C-1 site. In the nuclear Overhauser effect spectroscopy spectrum, the nuclear Overhauser 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 \(-\text{O}^\circ\)-configuration of 1-OH. Thus, M-1 can be assigned as 1-\(-\text{O}^\circ\)-hydroxylcinobufagin (1-HCB), which is a novel compound.

The \(^3\text{H}\) NMR spectrum of M-2 revealed that the proton signal of H-5 at \(\delta 1.69\) disappeared by comparison with NMR data for cinobufagin. The \(^{13}\text{C}\) 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). Taken together, these data indicated that M-2 was 5-\(-\text{O}^\circ\)-hydroxylcinobufagin (5-HCB).

Chemical Inhibition Studies. Selective inhibitors of nine major P450 isoforms were used to screen the P450 isoform(s) responsible for the formation of two monohydroxylated metabolites in HLM (Fig. 4). ABT, a broad-specificity P450 inactivator, inhibited the formation of 1-HCB and 5-HCB completely, suggesting that P450s were responsible for the CB hydroxylation in HLM. Among the nine P450 isoform selective inhibitors tested, ketoconazole inhibited the formation of 1-HCB and 5-HCB completely, whereas inhibitors of other P450 isoforms did not exhibit significant inhibition (less than 30% inhibition, \(p > 0.05\)) toward the formation of these two metabolites.
findings suggested that the formation of 1-HCB and 5-HCB was mainly catalyzed by CYP3A.

To explore whether the metabolic enzymes responsible for CB hydroxylation in mouse, dog, minipig, 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 are 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 mentioned above, whereas ketoconazole (1 μM) could inhibit the formation of 1-HCB and 5-HCB nearly completely, implying that CYP3A played an important role in the hydroxylation of CB among these species.

Assays with Recombinant Human P450 Isosorns. To further verify the P450 isoform(s) involved in the metabolism of CB in humans, the oxidation activity of CB was determined using nine cDNA-expressed P450 isoforms. After incubation at 37°C for 30 min, two hydroxylated metabolites were formed exclusively by CYP3A4;

TABLE 1
Retention times, molecular weights, and MS data for metabolites of CB in RLM

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>t_R (min)</th>
<th>Mol. Wt.</th>
<th>Identification</th>
<th>Molecular Ions (ESI+, m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.28</td>
<td>416</td>
<td>Monohydroxyl-deacetylcinobufagin</td>
<td>417 [M + H]^+</td>
</tr>
<tr>
<td>2</td>
<td>5.40</td>
<td>414</td>
<td>3-Ketohydroxyl-deacetylcinobufagin</td>
<td>455 [M + CH3CN + H]^+</td>
</tr>
<tr>
<td>3</td>
<td>5.61</td>
<td>416</td>
<td>Monohydroxyl-deacetylcinobufagin</td>
<td>456 [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>5</td>
<td>7.35</td>
<td>458</td>
<td>1α-Hydroxyacinobufagin</td>
<td>442 [M + CH3CN + H]^+</td>
</tr>
<tr>
<td>6</td>
<td>7.60</td>
<td>458</td>
<td>5β-Hydroxycinobufagin</td>
<td>459 [M + H]^+</td>
</tr>
<tr>
<td>7</td>
<td>8.32</td>
<td>400</td>
<td>3-epi-Deacetylcinobufagin</td>
<td>401 [M + H]^+</td>
</tr>
<tr>
<td>8</td>
<td>10.59</td>
<td>442</td>
<td>3-epi-Cinobufagin</td>
<td>443 [M + CH3CN + H]^+</td>
</tr>
</tbody>
</table>

t_R, retention time.

TABLE 2
1H NMR (500 MHz, DMSO) and 13C NMR (125 MHz, DMSO) spectral data for M-1 and M-2

<table>
<thead>
<tr>
<th>No.</th>
<th>1H NMR (500 MHz, DMSO)</th>
<th>13C NMR (125 MHz, DMSO)</th>
<th>HMBC (H → C)</th>
<th>NOESY</th>
<th>1H NMR (500 MHz, DMSO)</th>
<th>13C NMR (125 MHz, DMSO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.62 brs</td>
<td>71.8 d</td>
<td>C-2, C-10</td>
<td>H-5, Me-19</td>
<td>1.25</td>
<td>24.8 t</td>
</tr>
<tr>
<td>2</td>
<td>1.93</td>
<td>32.0 t</td>
<td>C-1, C-3, C-4</td>
<td>H-3</td>
<td>1.48</td>
<td>27.2 t</td>
</tr>
<tr>
<td>3</td>
<td>4.00 (brs)</td>
<td>66.7 d</td>
<td>C-2, C-4, C-5</td>
<td>H-5</td>
<td>1.52</td>
<td>30.5 t</td>
</tr>
<tr>
<td>4</td>
<td>1.17</td>
<td>32.8 t</td>
<td>C-2, C-3, C-4, C-10</td>
<td>H-5</td>
<td>2.10 brd</td>
<td>66.5 d</td>
</tr>
<tr>
<td>5</td>
<td>1.63</td>
<td>30.1 d</td>
<td>C-4, C-6, C-10</td>
<td>Me-19</td>
<td>—</td>
<td>75.5 s</td>
</tr>
<tr>
<td>6</td>
<td>1.32</td>
<td>25.0 t</td>
<td>C-5, C-8, C-10</td>
<td>H-5</td>
<td>1.15</td>
<td>33.8 t</td>
</tr>
<tr>
<td>7</td>
<td>1.20</td>
<td>19.8 t</td>
<td>C-8, C-9</td>
<td>H-8</td>
<td>1.52</td>
<td>22.2 t</td>
</tr>
<tr>
<td>8</td>
<td>1.33</td>
<td>19.8 t</td>
<td>C-8, C-9</td>
<td>H-8</td>
<td>1.39</td>
<td>22.2 t</td>
</tr>
<tr>
<td>9</td>
<td>1.87</td>
<td>32.8 d</td>
<td>C-9,C-11, C-14</td>
<td>Me-19</td>
<td>1.98</td>
<td>31.5 d</td>
</tr>
<tr>
<td>10</td>
<td>1.73</td>
<td>39.9 d</td>
<td>C-10, C-11, C-12, C-19</td>
<td>H-11,</td>
<td>1.80</td>
<td>41.1 d</td>
</tr>
<tr>
<td>11</td>
<td>1.17</td>
<td>20.6 t</td>
<td>C-8, C-9, C-12, C-13</td>
<td>H-9</td>
<td>1.24</td>
<td>21.0 t</td>
</tr>
<tr>
<td>12</td>
<td>1.48</td>
<td>38.7 t</td>
<td>C-9, C-11, C-13, C-18</td>
<td>H-18</td>
<td>1.41</td>
<td>38.8 t</td>
</tr>
<tr>
<td>13</td>
<td>1.65</td>
<td>44.6 s</td>
<td>C-14, C-16, C-17</td>
<td>H-16</td>
<td>1.65</td>
<td>44.3 s</td>
</tr>
<tr>
<td>14</td>
<td>1.48</td>
<td>44.6 s</td>
<td>C-14, C-16, C-17</td>
<td>H-16</td>
<td>1.65</td>
<td>44.3 s</td>
</tr>
<tr>
<td>15</td>
<td>3.75 (s)</td>
<td>59.3 d</td>
<td>C-14, C-16, C-17</td>
<td>H-16</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>
<td>C-13, C15, C-25</td>
<td>H-15, H-17</td>
<td>5.42 (d, 9.5)</td>
<td>74.5 d</td>
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<tr>
<td>17</td>
<td>2.85 (9.5)</td>
<td>48.9 d</td>
<td>C-13, C-14, C-16, C-20</td>
<td>H-16</td>
<td>3.35 (d, 9.5)</td>
<td>48.8 d</td>
</tr>
<tr>
<td>18</td>
<td>0.71 (s)</td>
<td>16.9 q</td>
<td>C-12,C-13,C-14</td>
<td>H-8, H-21</td>
<td>0.62 (s)</td>
<td>16.5 q</td>
</tr>
<tr>
<td>19</td>
<td>0.99 (s)</td>
<td>18.7 q</td>
<td>C-1,C-5,C-10</td>
<td>H-1, H-5, H-8</td>
<td>0.91 (s)</td>
<td>16.8 q</td>
</tr>
<tr>
<td>20</td>
<td>1.65</td>
<td>116.1 s</td>
<td>C-20</td>
<td>Me-18</td>
<td>—</td>
<td>152.2 d</td>
</tr>
<tr>
<td>21</td>
<td>7.48 (s)</td>
<td>152.2 d</td>
<td>C-20</td>
<td>Me-18</td>
<td>7.35 (s)</td>
<td>152.2 d</td>
</tr>
<tr>
<td>22</td>
<td>7.81 (d, 9.5)</td>
<td>148.6 d</td>
<td>C-20, C-23</td>
<td>H-23, H-26</td>
<td>7.80 (d, 9.5)</td>
<td>148.4 d</td>
</tr>
<tr>
<td>23</td>
<td>6.24 (d, 9.5)</td>
<td>112.9 d</td>
<td>C-22, C-24</td>
<td>H-22</td>
<td>6.23 (d, 9.5)</td>
<td>112.9 d</td>
</tr>
<tr>
<td>24</td>
<td>1.65</td>
<td>169.4 s</td>
<td>C-25</td>
<td>Me-18</td>
<td>1.81 (s)</td>
<td>20.2 q</td>
</tr>
<tr>
<td>25</td>
<td>1.82 (s)</td>
<td>20.2 q</td>
<td>C-25</td>
<td>Me-18</td>
<td>1.81 (s)</td>
<td>20.2 q</td>
</tr>
</tbody>
</table>

DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple-bond correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.
no metabolites were observed in the incubation with CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (less than 0.01 pmol per min/pmol P450). The formation rates of CYP3A4 for 1-HCB and 5-HCB were 14.01 ± 0.90 and 26.02 ± 1.70 pmol per min/pmol P450, respectively.

**Kinetic Study.** Over the whole concentration range tested, hydroxylation of CB in liver microsomes from humans and four common animal species, as well as in recombinant CYP3A4, obeyed Michaelis-Menten kinetics, as evidenced by the 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 are 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, whereas the $V_{max}$ values for the formation of 1-HCB and 5-HCB were 0.59 and 1.17 nmol per 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 $V_{max}$ values for the formation of 1-HCB and 5-HCB were 19.22 and 45.81 pmol per min/pmol P450, respectively. In liver microsomes from four experimental animals, the $K_m$ values for 1-HCB formation ranged from 3.3 to 34.2 μM, whereas the $K_m$ values for 5-HCB formation ranged from 3.4 to 20.4 μM. The intrinsic clearance ($CL_{int}$) for the formation of 1-HCB and 5-HCB differed greatly among different species, with a range of 14.5 to 595.0 and 54.6 to 644.6 μl per min/mg protein, respectively.

**Discussion**

In the past 30 years, the rat has been widely used in pharmacokinetic and toxicological studies of Chansu and cinobufagin (Toma 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 the metabolic pathway and metabolic behavior of CB between human and common experimental animals have not been revealed. In this study, a comparison of metabolic profiles, enzymes involved, and 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, whereas species-specific deacetylation of CB was an important metabolic pathway that was well characterized previously (Zhang et al., 1992). In sharp contrast, P450-mediated hydroxylation was thought to be the major pathway responsible for the elimination of CB in human liver, because none of the hydrolyzed, epimerized, and glucuronidated metabolites were formed, and the CB concentration was not decreased in the HLM incubation (data not shown). Except for rat, comparable metabolic profiles were observed when CB was incubated with liver microsomes from human, monkey, minipig, dog, and mouse along with an NADPH-generating system. These results revealed the large difference in metabolic profiles between rat and other species and could explain why the major metabolites of CB in rat, namely deacetylcinobufagin and 3-epi-deacetylcinobufagin, cannot be observed in the serum of the dog with oral or intravenous administration of CB (Toma et al., 1987).

For further exploration of the similarity of the enzymes responsible for hydroxylation of CB in liver microsome from various species,
chemical inhibition studies with use of selective inhibitors were used. It is evident from chemical inhibition studies and assays with recombinant P450s that both 1α- and 5β-hydroxylation of CB in HLM were catalyzed predominantly by CYP3A4, whereas 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 microsomes 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 metabolic enzymes involved 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 used cautiously for interspecies extrapolation.

Although animal models have been commonly used in preclinical studies to evaluate pharmacokinetics and toxicity in humans, it is important to point out that a suitable animal model for in vivo drug metabolism and pharmacokinetics studies should have the relevant metabolism behaviors compared with those of humans, including identical metabolic profiles, equivalent or similar metabolic enzymes, and closed catalytic efficiency (Martignoni et al., 2006). CYP3A is the most important isozyme involved in the metabolism of CB in humans and the above-mentioned animal species, but various CYP3A isoforms are expressed in various species with different substrate specificities and catalytic efficiency. In this study, a comparative study of CB metabolism in humans and four experimental animals with similar profiles was performed to determine the catalytic efficiency of CB hydroxylation by liver microsomes from various species. None of the animal species is completely similar to humans with respect to enzyme activities, and catalytic efficiencies for the formation of 1-HCB and 5-HCB are diversified. HLM, PLM, and MLM have similar enzyme activities, and catalytic efficiencies for the formation of 1-HCB and 5-HCB (M-2). Intrinsic clearance values similar to those of HLM. These results indicated that minipig might be a good choice to serve as a surrogate model for drug metabolism and pharmacokinetics studies of CB.

The structure-activity relationship studies of 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 1α- and 5β-hydroxylation of CB by CYP3A would reduce its cytotoxicity activity. In this case, the effects on CYP3A may lead to large variations in exposure of CB, which can influence the in vivo potency or cause severe adverse reactions. Because CB is a substrate of CYP3A, more adverse drug-drug reactions or undesirable effects may occur when CB is coadministered with drugs that are substrates, inducers, or inhibitors of CYP3A. It has been reported that many compounds including xenobiotics and endogenous steroids could cause marked modifications in CYP3A-mediated metabolism (Nakamura et al., 2002; Usmani et al., 2003; Zhou et al., 2007; Zhou, 2008). Their effects on CYP3A, including altering kinetic behaviors or activating or inhibiting the biotransformation of other substrates, would make pharmacokinetic behaviors more complicated. It also should be noted that the metabolism of CB could be affected by other bufadienolides coexisting in Chansu, because of 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 relatively high level in vivo, which may cause some serious adverse reactions. Therefore, more attention should be paid to the interactions of CB with its coadministered drugs and other bufadienolides in the clinic.

In summary, the metabolic pathways of CB in humans and five experimental animals were elucidated in the present study. The species-specific deacetylation and epimerization together with hydroxy-
loration are biotransformation pathways in RLM, whereas hydroxyla-
tion of CB at C-1 and C-5 sites by CYP3A is a major metabolic
pathway 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 in metabolic profiles,
enzymes involved, and catalytic efficacy, which would contribute to
animal selection in toxicological and pharmacokinetic studies of CB
or other TCMs containing CB.

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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, Ma, Ge, Liang, Wang, and Yang.

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

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