Characterization of Phase I Metabolism of Resibufogenin and Evaluation of the Metabolic Effects on Its Antitumor Activity and Toxicity

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ABSTRACT

Resibufogenin (RB), one of the major active compounds of the traditional Chinese medicine Chansu, has displayed great potential as a chemotherapeutic agent in oncology. However, it is a digoxin-like compound that also exhibits extremely cardiotoxic effects. The present study aimed to characterize the metabolic behaviors of RB in humans as well as to evaluate the metabolic effects on its bioactivity and toxicity. The phase I metabolic profile in human liver microsomes was characterized systemically, and the major metabolite was identified as marinobufagenin (5β-hydroxyresibufogenin, 5-HRB) by liquid chromatography–mass spectrometry and nuclear magnetic imaging techniques. Both cytochrome P450 (P450) reaction phenotyping and inhibition assays using P450-selective chemical inhibitors demonstrated that CYP3A4 was mainly involved in RB 5β-hydroxylation with much higher selectivity than CYP3A5. Kinetic characterization demonstrated that RB 5β-hydroxylation in both human liver microsomes and human recombinant CYP3A4 obeyed biphasic kinetics and displayed similar apparent kinetic parameters. Furthermore, 5-HRB could significantly induce cell growth inhibition and apoptosis in A549 and H1299 by facilitating apoptosome assembly and caspase activation. Meanwhile, 5-HRB displayed very weak cytotoxicity of human embryonic lung fibroblasts, and in mice there was a greater tolerance to acute toxicity. In summary, CYP3A4 dominantly mediated 5β-hydroxylation and was found to be a major metabolic pathway of RB in the human liver, whereas its major metabolite (5-HRB) displayed better druglikeness than its parent compound RB. Our findings lay a solid foundation for RB metabolism studies in humans and encourage further research on the bioactive metabolite of RB.

Introduction

Chansu, the dried skin secretions of the giant toad (containing Bufo bufo gargarizans Cantor and Bufo melanostictus Schneider), is an important traditional Chinese medicine (TCM) and is widely used in China and other Asian countries for treating a number of ailments in the clinic, including swelling, pain, heart failure, and cancer (Nogawa et al., 2001; Li et al., 2010). It is also used as a raw medicinal material in many famous complicated TCM formulas distributed as over-the-counter drugs, namely Liushen Pill, Shexiang Baoxin Pill, and Kyushin (Shimizu and Morishita, 1996; Ma et al., 2009). Resibufogenin (RB) is one of the major active compounds in Chansu with a content of 4–10%, and it is regarded as a vital marked compound for the quality control of Chansu and its related TCM preparations (Ma et al., 2009). Many studies in recent years indicate that resibufogenin possesses significant pharmacological and toxicological effects, including cardiotonic, anesthetic, antitumor, and cardiotoxic effects. Furthermore, both preclinical and clinical studies show that RB and its analogs possess the skeletons of bufadienolide are potent anticancer agents (Mijatovic et al., 2007; Newman et al., 2008). It has been reported that RB exhibits strong cytotoxic activities against human myeloid leukemia, prostate cancer, and human hepatoma cells, with IC50 values of approximately 10–50 nM (Kamano et al., 1998). The

ABBREVIATIONS: ABT, 1-aminobenzotriazole; CYP3cide, PF-4981517; DAD, diode array detector; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FBS, fetal bovine serum; HLF, human embryonic lung fibroblasts; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; 5-HRB, 5β-hydroxyresibufogenin; LC, liquid chromatography; MS, mass spectrometry; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; NSCLC, non–small cell lung cancer; P450, cytochrome P450; PBS, phosphate-buffered saline; PF-4981517, 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1H-pyrazol-4-yl]-4-[3S]-3-piperidin-1-yl-pyrrolidin-1-yl]-1H-pyrazolo[3,4-d] pyrimidine; RB, resibufogenin; TCM, traditional Chinese medicine; TEPA, triethlenetriphosphoramide; UFLC, ultra-fast liquid chromatography.

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cytotoxic activities of RB against human hepatoma Bel-7402, human gastric cancer BGC-823, and human cervical carcinoma HeLa were comparable to or stronger than taxol (Ye et al., 2005). With the intensive investigation of the underlying molecular mechanisms, it was shown that RB and its analog-mediated Na⁺/K⁺-ATPase targeting could circumvent various chemoresistance pathways as a novel way of attacking resistant cancer cells. Moreover, the various molecular pathways targeted by Na-K can be more deleterious to biologically aggressive cancer cells than to normal cells (Mijatovic and Kiss, 2013). This collective information demonstrated that RB is a chemotherapeutic candidate that has great potential to serve as a chemotherapeutic agent in oncology (Kamano et al., 1998; Qi et al., 2011).

However, the digoxin-like bufadienolides are cardiotoxic (Brubacher et al., 1999; Gowda et al., 2003; Kostakis and Byard, 2009) and have been shown to be associated with a high mortality rate (Xie et al., 2001). It is well known that the metabolic clearance of a given drug and the formation of new drug metabolites can significantly influence the efficacy and toxicity in vivo (Guengerich, 2006), but there is still limited attention given to the metabolic effects of RB in humans. Recently, the metabolic characteristic of RB in rats was illustrated by isolation and identification of metabolites excreted in bile (Zhu et al., 2013). Consequently, the metabolic behavior of RB in humans as well as the metabolic effects on its bioactivities and toxicity have not been well characterized.

Therefore, the objectives of this study were: 1) to elucidate the phase I metabolic pathway(s) and kinetic characterization of RB in human liver microsomes, 2) to identify the main P450 enzymes responsible for phase I metabolism of RB, and 3) to reveal metabolic effects on its bioactivity and toxicity using in vitro and in vivo methods.

Materials and Methods

Reagents

Resbufogenin (Fig. 1) was isolated from Chansu by one of the authors (J.N.) and unambiguously identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques. Its purity was above 98% as determined by ultra-fast liquid chromatography–diode array detector (UFLC-DAD), 1-Aminobenzotriazole (ABT), clonethiazole, N-glucose-6-phosphate, furafylline, glucose-6-phosphate dehydrogenase, PF-4981517 (CYP3cide), 8-methoxypsoralen, NADP+, omeprazole, quinidine, and sulfaphenazole were purchased from Sigma-Aldrich (St. Louis, MO). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, Ohio). Montelukast was from Beijing Aleznova Pharmaceutical (Beijing, China). Triethylenthiophosphoramide (TEPA) was purchased from Acros Organics (Geel, Belgium). All other reagents were either of liquid chromatography (LC) grade or the highest grade commercially available.

A mixed pool of human liver microsomes (HLM) from twenty-five donors, human individual microsomal samples, as well as liver microsomes of male ICR/CD-1 mice, male Sprague-Dawley rats, male Dunkin-Hartley guinea pigs, male Beagle dogs, and male Cynomolgus monkeys were purchased from Research Institute for Liver Diseases (RILD, Shanghai, China); cDNA-expressed recombinant human cytochrome P450 isoforms (rhCYP), including rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6, rhCYP2E1, rhCYP3A4, rhCYP3A5, and rhCYP3A7, which were derived from baculovirus-infected insect cells coexpressing NADPH-P450 reductase, were from Cypex (Dundee, UK). All microsomal samples and rhCYP isoforms were stored at −80°C until use. Human NSCLC A549 and H1299 cell lines and the embryonic human lung fibroblasts (HLF) cell line were obtained from ATCC (Manassas, VA). The cleaved caspase-9 antibody was purchased from Cell Signaling Technology (Danvers, MA). GAPDH was from ProteinTech (Chicago, IL), and cytochrome c was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Animals

All animals were maintained and used in accordance with the animal protocol approved by the local research ethics review board of the Animal Ethics Committee of Dalian Medical University, and all experimental procedures described were carried out in accordance with the Declaration of Helsinki. ICR/CD-1 mice weighing between 18 and 21 g were obtained from the laboratory animal center of Dalian Medical University. The mice were fasted overnight with free access to water before the date of the experiment.

In Vitro Metabolism of Resbufogenin

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, 4 mM MgCl₂), and liver microsomes. In all experiments, RB (25 mM previously dissolved in acetonitrile) 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 minutes, the reaction was initiated by adding NADPH-generating system and the mixture 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 20,000g for 10 minutes at 4°C. Aliquots of supernatants were stored at −20°C until analysis. Control incubations without NADPH-generating system or without substrate or without microsomes were carried out to ensure that metabolite formation was microsome- and NADPH-dependent. All incubations throughout the study were carried out in three experiments performed in duplicate with S.D. values generally below 10%.

Assays with Recombinant Human Cytochrome P450 Isoforms. Eleven cDNA-expressed human cytochrome P450 isoforms coexpressing NADPH-P450 reductase (rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6, rhCYP2E1, rhCYP3A4, rhCYP3A5, and rhCYP3A7) were used to screen the involved isofrom(s) for the hydroxylation of RB in HLM. The incubations with isoforms were carried out under the standard assay procedure described in the text. To generate adequate metabolites for detection, a relatively high substrate concentration (25 and 250 μM) was selected and incubated with each of the recombinant P450s (40–160 nM) at 37°C for 30 minutes.

Fig. 1. The 5β-hydroxylation of RB in HLM.

Resbufogenin (RB)  5β-Hydroxy-resbufagenin (5-HRB)
Metabolism of RB and Its Effects on Anticancer Activity

UFLC equipped with DAD detector was used to quantify the metabolite(s) of RB.

Chemical Inhibition Assays. The hydroxylation of RB in HLM with the absence or presence of selective inhibitors for different P450 isoforms was measured to explore the involved enzyme(s) for this metabolism. In brief, RB (10 μM) was incubated in HLM (0.05 mg protein/ml) with an NADPH-generating system in the absence (control) or presence of known P450 isoform-specific inhibitors. 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 CYP3A. Inhibition by furafylline (10 μM) for CYP1A2, 8-methoxypsoralen (2.5 μM) for CYP2A6, TEPA (50 μM) for CYP2B6 (Rae et al., 2002), CYP3A (2 μM) for CYP3A4 (Walsky et al., 2012) and ABT (500 μM) for broad P450s (Emoto et al., 2003) were examined by adding RB after preincubation with NADPH-generating system at 37°C for 40 minutes.

Kinetic Characterization. To estimate kinetic parameters of RB hydroxylation in human liver microsomes as well as recombinant CYP3A4, the incubation conditions were optimized to ensure that formation rates of 5-HRB were in relation to incubation time and protein concentration in the linear range. RB (1, 2, 5, 10, 25, 50, 75, 100, 150, 200, and 250 μM) was incubated with HLM (0.05 mg protein/ml) or incubated with rCYP3A4 (5 nM) for 20 minutes. All incubations were carried out in three independent experiments in duplicate. The apparent Km and Vmax 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 plus or minus standard error of the parameters estimates.

Correlation Studies. The formation rates of the metabolites described for RB (5 μM, near Km value) were determined in a panel of HLM prepared from 12 individual donors. RB was incubated with HLM (0.05 mg protein/ml) for 15–60 minutes to ensure that the formation rate of 5-HRB was in relation to incubation time and protein concentration in the linear range. These values were compared with the levels of CYP3A4 or CYP3A5 in HLM. The concentrations of CYP3A4 and CYP3A5 in HLM were determined by liquid chromatography–tandem mass spectrometry, using multiple reaction monitoring mode and isotope-labeled peptide as the internal standards. Specific peptides for ETVNFLR (for CYP3A4) and SLGPGVGMK (for CYP3A5) were selected for their quantification by using transition ions of m/z 439.7/549.3 and m/z 468.3/678.5, respectively. The correlation parameter was expressed by the linear regression coefficient (r). P < 0.05 was considered statistically significant.

UFLC-DAD and UFLC-ESI-MS Analysis. The UFLC system was equipped with a CMB-20A communications bus module, an SIL-20AHT autosampler, two LC-25AD pumps, a DGU-20A vacuum degasser, a CTO-20AC column oven and an SPD-M 20A diode array detector. A Shim-pack XR-ODS (75 mm × 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 RB and its metabolites. The mobile phase consisted of CH3CN (A) and water containing 0.2% (v/v) formic acid (B) with the following gradient profile: 0–2 minutes, 96–76%; 2–5 minutes, 76–64%; 5–10 minutes, 64–49%; 10–13 minutes, 49–5%; and 13–16 minutes, balanced to 96%. The flow rate was 0.4 ml/min and the column temperature was kept at 40°C. RB and its metabolites were detected at 299 nm and quantified according to the calibration curves of authentic standards.

A Shimadzu LC-MS-2010EV instrument with an electrospray ionization (ESI) interface was used for identification of RB and its metabolite(s). Mass spectrometry 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, 1.5 l/min; and drying gas (N2) pressure, 0.06 MPa. Data processing was performed using the LC-MS Solution version 3.4.1 software (Shimadzu).

Metabolite Purification and Identification

In Vitro Biosynthesis and the Isolation of Major Metabolite. The predominant metabolite was biosynthesized by using the mixture of cyanomolops monkey liver microsomes (CyLM) (90%) and HLM (10%). Above all, the major biotransformed product by CyLM was characterized by UFLC-DAD-ESI-MS and it was found to have retention time, UV spectra, and mass spectra identical to the metabolite in HLM. The incubation system was scaled up to 250 ml. RB (200 μM) was incubated with the liver microsomes (final protein concentration, 1.0 mg/ml) and the NADPH-generating system (1 mM NADPH, 10 mM glucose-6-phosphate, 1 unit/ml of glucose-6-phosphate dehydrogenase, and 4 mM MgCl2) for 4 hours at 37°C. Under these conditions, approximately 35% of RB was converted to 5-HRB. Acetonitrile (250 ml) was added to the reaction mixture to precipitate the protein. After centrifugation at 20,000g for 15 minutes at 4°C, the supernatant was separated and extracted with ethyl acetate (250 ml × 3). The organic layer was combined and dried in vacuo, and the residue was redissolved in acetonitrile (1.5 ml) and injected into a high-performance liquid chromatography (HPLC) instrument. The HPLC system (Shimadzu) consisted of a SCL–10A system controller, two LC–10AT pumps, an SIL–10A auto injector, and an SPD–10AVP UV detector. A C18 column (4.6 mm × 150 mm, 5 μm) was used to separate RB and its metabolite. The mobile phase was 65% methanol in water. The eluent was monitored at 299 nm with a flow rate of 1.2 ml/min, and the fractions containing 5-HRB were collected and dried in vacuo. The purity of 5β-hydroxyresibufogenin was above 98% by HPLC–UV analysis.

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 spectra.

Cytotoxicity Assays of Resibufogenin and Its Metabolite

Cell Culture. Human NSCLC A549 and H1299 cell lines and HLF cell line were maintained in either RPMI 1640 medium or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Cell Viability Assay. Cell viability was determined by a Cell Proliferation Kit I (MTT) (Roche Diagnostics, Indianapolis, IN). Briefly, lung cancer cell lines (6 × 103 cells/well) were seeded into 96-well plates. Cells were allowed to adhere overnight, and then the cells were changed to fresh medium containing various concentrations of 5-HRB or RB dissolved in dimethyl sulfoxide (DMSO); final concentration, 0.1%). After 48 hours incubation, the growth of cells was measured. The effect on cell viability was assessed as the percent cell viability compared with untreated control group, which were arbitrarily assigned 100% viability. The compound concentrations required to cause 50% cell growth inhibition (IC50) were determined by interpolation from dose-response curve. Experiments were performed in triplicate and at least three independent experiments were carried out.

In Vitro Migration Assay. A scratch assay (wound-healing assay) was performed to detect cell migration. The cells were grown to full confluence in six-well plates and wounded with a sterile 100-μl pipette tip after 6 hours of serum starvation and then washed to remove detached cells from the plates. Cells were treated with indicated doses of 5-HRB in full medium and kept in a CO2 incubator. After 48 hours, medium was replaced with phosphate-buffered saline (PBS) buffer, the wound gap was observed, and cells were photographed using a Leica DM 14000B microscope fitted with digital camera.

Colon Formation Assay. To analyze the cell sensitivity to 5-HRB, we used a colony formation assay in vitro. Briefly, A549 cells (1 × 105 per well) were seeded in six-well plates containing 2 ml growth medium with 10% FBS and cultured for 24 hours. Then, removed the medium, and cells were exposed to various concentrations of 5-HRB. After 18 hours, cells were washed with PBS and supplemented with fresh medium containing 10% FBS. The cultures were maintained at 37°C, 5% CO2 incubator for 14 days, allowing viable cells to grow into macroscopic colonies. The medium was removed, and the colonies were counted after staining with 0.1% crystal violet.

Confocal Immunofluorescence. For confocal microscopy analysis, cells grown on chamber slides were washed in PBS and fixed for 30 minutes at room temperature with 4% paraformaldehyde. The cells were then permeabilized with 0.2% TritonX-100 for 3 minutes. The samples were blocked with 10% bovine serum albumin (BSA) in PBS for 30 minutes. Antibodies against cytochrome c in the 1% blocking solution were added to the sample and incubated overnight at 4°C. Non-immune rabbit IgG and mouse IgG were included as controls. After five 10-minute washes with PBS, fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies were added in blocking solutions and incubated for 1 hour in a darkroom at room temperature.
After five additional 10-minute washes, samples were examined with a Leica confocal microscope, and images were processed with Image-Pro Plus 5.1 software. More than 100 cells were inspected per experiment, and cells with typical morphology were presented.

**Western Blot Analysis.** Cell lysate proteins were separated by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gels and then electrophoretically transferred to a polyvinylidene fluoride membrane. Western blots were probed with the specific cleaved caspase-9 antibody. GAPDH was used as control for sample loading. The protein bands were detected by enhanced chemiluminescence. Similar experiments were performed at least three times. The total protein concentration was determined using a bichinchoninic acid protein assay kit.

**Acute Toxicity Assay.** Acute toxicities of RB and 5-HRB were tested using a variation of the method described by MTT methods. Six different doses of each drug were used for the LD50 index determination, with each experimental group being composed of five mice for this purpose. The compounds RB and 5-HRB were administered by intraperitoneal injection. The animals were observed at 10 minutes, 30 minutes, and 60 minutes after administration to access the numbers of deaths and possible toxicological symptoms.

**Data Analysis and Statistics.**

Kinetic constants for RB hydroxylation by HLM or rhCYP3A4 were obtained by fitting experimental data to the biphasic Michaelis-Menten kinetics using Origin (OriginLab Corporation, Northampton, MA). The biphasic Michaelis-Menten equation is

\[
K_m = \frac{(V_{\text{max}} + [S])}{(K_m1 + [S])} + \frac{(V_{\text{max}2} + [S])}{(K_m2 + [S])},
\]

where \(v\) is the rate of reaction, \(V_{\text{max}}\) is the maximum velocity, \(K_m\) is the Michaelis constant, and \([S]\) is the substrate concentration.

The IC50, representing the inhibitor concentration that inhibits 50% of control activity, is determined by nonlinear curve fitting with Origin. Kinetic constants and IC50 values are reported as the value ± S.E. of the parameter estimate.

**Results.**

**Biotransformation of RB by Human Liver Microsomes.** The in vitro metabolic profiles of RB were characterized after incubation with human hepatic microsomes (0.3 mg protein/ml) along with the NADPH-generating system for 30 minutes (Fig. 2). The metabolites were characterized by UPLC-DAD-ESI-MS. The positive-ion mode was adopted for liquid chromatography mass spectrometry analysis, owing to the fact that it is more sensitive than the negative-ion mode for analysis of target analytes. Mass spectra were dominated by [M+H]+ and [M+CH3CN+H]+. The m/z for the [M+H]+ and [M+CH3CN+H]+ of the metabolite in human hepatic microsomes were 401.2 and 442.2, respectively. Accordingly, the molecular weight (MW) of metabolite was calculated to be 400 (increasing m/z 16 compared with RB), indicating this metabolite was a monohydroxylated form.

**Identification of the Main Monohydroxylated Metabolite.** To elucidate the main metabolic site of RB, this metabolite was purified and further identified by 1H-NMR and 13C-NMR. The spectral data of the monohydroxylated metabolite is listed in Table 1. Compared with NMR data of RB, the 13C-NMR spectrum of this metabolite showed the disappearance of tertiary carbon at δ 35.5 (C-5); meanwhile, the additional oxygenated quaternary carbon at δ 74.3 (CH) was also observed. The carbon signals of C-4, C-6, and C-10 shifted down to δ 36.8 (δ +8.6), and δ 40.6 (δ +5.6), respectively. The upfield shifts of C-1 (δ +4.4) and C-19 (δ +6.8) were observed because of the γ-gauche effect, which suggested that an additional hydroxyl group was located at C-5 position. Furthermore, the NMR data of metabolite agreed well with the spectral data of marinobufagenin reported previously (Ma et al., 2008). On the basis of above analysis, the hydroxylated metabolite was identified as 5β-hydroxylresibufogenin (5-HRB, Fig. 1).

**Chemical Inhibition Assays.** A broad specificity P450 inactivator and nine selective inhibitors of major P450 isoforms were used to screen the P450 isoform(s) responsible for the formation of 5-HRB in HLM (Fig. 3A). ABT, a broad specificity P450 inactivator, completely inhibited the formation of 5-HRB, suggesting that P450s were responsible for RB hydroxylation in HLM. Among the selective inhibitors tested, ketoconazole (CYP3A) inhibitor strongly inhibited the catalytic activity of HLM, further implying the prominent role of CYP3A in RB hydroxylation. Furthermore, CYP3Cide, a potent and specific time-dependent inhibitor of CYP3A4 (Walsky et al., 2012), exhibited similar inhibitory effects on RB 5β-hydroxylation in HLM and rhCYP3A4 (Supplemental Fig. 1). Meanwhile, inhibitors of other P450 isoforms did not exhibit a significant inhibition (less than 20% inhibition, \(P > 0.05\)) toward the formation of 5-HRB. These findings suggest that the formation of 5-HRB was selectively catalyzed by CYP3A4.

**Assays by Recombinant Human P450 Isosforms.** To further verify the P450 isoform(s) involved in RB metabolism in humans, the formation of 5-HRB was determined using 11 rhCYP isoforms. After incubation at 37°C for 30 minutes, one hydroxylated metabolite was formed exclusively by CYP3A family (including CYP3A4, CYP3A5, and CYP3A7); no metabolite was observed in the incubation with rhCYP1A2, rhCYP2A6, rhCYP2B6, rhCYP2C8, rhCYP2C9, rhCYP2C19, rhCYP2D6, and rhCYP2E1 (less than 0.01 nmol/min per nanomole P450) (Fig. 3B). The formation rates of rhCYP3A4 for 5-HRB were 16.4 ± 0.7 and 53.8 ± 3.6 nmol/min per nanomole P450 at substrate concentrations of 25 and 250 μM, respectively. However, RB 5β-hydroxylation rates of rhCYP3A5 and rhCYP3A7 were very

<table>
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<tr>
<th>No.</th>
<th>1H-NMR (500 MHz, DMSO)</th>
<th>13C-NMR (125 MHz, DMSO)</th>
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**TABLE 1**
displayed the similar apparent kinetic parameters. In HLM, kinetic characterization of RB 5-hydroxylation in HLM and rhCYP3A4 showed 83% identity in amino acid sequence. Although CYP3A5 has generally been considered less important than CYP3A4, there are many controversial issues in the contribution of CYP3A5 to the total clearance of CYP3A, which may be caused by the polymorphism and interindividual and intracellular variability in expression (Daly, 2006). Therefore, in the present study, the correlations of CYP3A4 or CYP3A5 expression levels with RB hydroxylation were investigated to reveal which one of these two P450s is the predominant enzyme that was responsible for metabolism of RB in the human livers. The formation rates of 5-HRB were determined in a panel of HLMs prepared from 12 individual donors. These values were compared with the protein levels of CYP3A4 or CYP3A5 in 12 individual HLMs. As shown in Fig. 5, the linear regression coefficient (r) for 5-HRB formation rate and CYP3A4 concentration was 0.857 (P < 0.001), and for 5-HRB formation rate and CYP3A5 concentration was 0.083 (P = 0.799). There was a high correlation between 5-HRB formation rate and CYP3A4 expression levels, whereas there was a poor correlation of 5-HRB formation rate with CYP3A5 protein levels, which fully indicates the prominent contribution of CYP3A4 toward RB hydroxylation in HLM.

RB and Its Metabolite Both Inhibited Non–Small Cell Lung Cancer Cell Growth and Changed Cell Morphology. To evaluate the hydroxylation effect on cytotoxicity of RB, we first determined the effect of 5-HRB against cell proliferation of human lung cancer cells by Cell Proliferation Kit I (MTT) (Roche Diagnostics). As shown in Fig. 6, both 5-HRB and RB resulted in dose-dependent growth inhibition of non–small cell lung cancer (NSCLC) cells, and they could markedly reduce cell-to-cell contact and induce cell shrinkage compared with the control groups. The IC50 values for cell viability inhibition of 5-HRB and RB were, respectively, 91.5 ± 7.8 nM and 23.6 ± 5.4 nM in A549, and 718.1 ± 19.6 nM and 262.5 ± 16.7 nM in H1299. However, the IC50 values for cell viability inhibition in HLF were approximately 1000-fold more than those in NSCLC cells, which fully suggests that 5-HRB displayed highly selective cytotoxicity against tumor cells.

5-HRB Suppressed Cell Morphology Change and the Clonal Formation. The changes of cell morphology mediated by treatments of 5-HRB were detected in A549 cells. We analyzed the inhibition of clonal formation ability in A549 cells treated with 5-HRB at the appropriate concentrations. As shown in Fig. 7A, 5-HRB highly inhibited colony formation and resulted in a remarkable decrease at colony formation ratio. Further, we analyzed the inhibition of 5-HRB on cell motility in A549 cells by employing a wound-healing assay. Treatment with 5-HRB inhibited cell migration compared with the control group without any agent treatment. The wounding space between cell layers was occupied mostly by the migrating cells at 48 hours after making a scratch in control cells. By contrast, the gap of cells was not occupied by the migrating cells at 48 hours after treatment with 5-HRB at the indicated doses (Fig. 7B). These results suggest that 5-HRB has the perfect properties for inhibiting NSCLC-cell colony formation and migration.

5-HRB Induced Apoptosis by Regulating Cytochrome c/Caspase Signaling. Treatment with 5-HRB at the doses of 20 μM and 100 μM induced 26% and 50% apoptotic cells in A549, respectively (Fig. 8A). To determine the effect of 5-HRB on apoptosis, we detected the expression of the proapoptotic protein caspase-9 in A549 cells by Western blot analysis. 5-HRB markedly increased the expression levels of cleaved caspase-9, compared with the control group (Fig. 8C). We next performed immunofluorescence imaging (IF) analysis to monitor changes in the subcellular localization of cytochrome c in A549 cells to determine whether 5-HRB could induce cytochrome c release. As shown in Fig. 8B, treatment with 5-HRB (50 nM or 100 nM) markedly triggered the release of cytochrome c from the intermitochondrial space into the cytosol. These results demonstrate that 5-HRB induced cell growth inhibition and is associated with the increase of apoptosis that may be attributable to the facilitation of the downstream cytochrome c–dependent apoptosis assembly and caspase activation in the cytosol.

Acute Toxicity and Cardiotoxicity Evaluation. It is well known that mice and rats are less sensitive to bufadienolides than are dogs or humans. However, we used mice in the present study investigating in vivo safety of RB and its hydroxylated metabolite (5-HRB) for the
In addition, compared with other bufadienolides in Chansu, the intrinsic 5-HRB may significantly influence the in vivo anticancer effects of RB. The metabolic features indicated that the formation of 5-HRB has less toxicity than does RB. Additionally, the primary cardiotoxicities of RB and 5-HRB in neonatal rat primary cardiac myocytes were also evaluated in the present study. RB and 5-HRB did not influence the cell morphology and cardiotoxic action of bufadienolides varied greatly and the cardiotoxicity than did RB. The lactate dehydrogenase release rate of cardiac myocytes treated by RB was higher than that of 5-HRB (Supplemental Fig. 7), which suggests that 5-HRB exhibited less cardiotoxicity than did RB.

Discussion

Recent advances in the investigation of bufadienolides have indicated that RB is a type of biologically active molecule with a wide variety of physiologic and pharmacological functions (Mijatovic et al., 2007; Newman et al., 2008). The previous studies reported that the cytotoxic and cardiotoxic action of bufadienolides varied greatly and the substituted groups of steroid skeletons could significantly influence these effects. This led us to inquire whether the biotransformation of RB that results in either active or inactive metabolites could significantly influence its efficacy and toxicity. To answer the question, we studied the metabolism profile of RB in HLMs, first by identifying the main metabolite and the involved metabolic enzyme. It was disclosed that 5-HRB was the main metabolite in HLM, which rapidly and specifically catalyzed by CYP3A4. Kinetic characterization demonstrated that RB 5β-hydroxylation in both HLM and recombinant CYP3A4 obeyed biphasic kinetics, with high affinity $K_m$ and a large in vitro intrinsic clearance value. The metabolic features indicated that the formation of 5-HRB may significantly influence the in vivo anticancer effects of RB. In addition, compared with other bufadienolides in Chansu, the intrinsic clearance of RB in rhCYP3A4 (5.63 $\mu$mol/min per picomole P450) was higher than those of cinobufagin (2.61 $\mu$mol/min per picomole P450) and bufalin (1.70 $\mu$mol/min per picomole P450), respectively (Ge et al., 2013). It implies that tiny changes in the substituted chemical groups of steroid skeletons (such as 16-OAC or 14β-OH groups) would significantly influence the binding and catalysis activities between CYP3A4 and bufadienolides. These findings may provide reference information for further investigation into the interaction as well as the metabolism-structure relationship between CYP3A4 and other bufadienolides.

Previous studies have revealed that RB can be rapidly metabolized in rats (with a short half-life of 7.5 minutes, i.v.), whereas 3-O-epimerization is the most important metabolic pathway of RB in rats (Zhu et al., 2013). The in vitro incubation study showed that 3-epiresibufogenin is the dominant metabolite of RB (100 $\mu$mol) in rat liver microsomes with a NADPH-generating system. In sharp contrast, 5-HRB is the major metabolite in liver microsomes from mouse, dog, guinea pig, monkey, and human (Supplemental Fig. 2). These findings agree well with our previous metabolic investigation of cinobufagin, which is a structural analog of RB (Ma et al., 2007; Ning et al., 2010; Ma et al., 2011). The significant differences in metabolic behaviors between rat and human suggested that rat could not serve as a preferred surrogate model for in vivo study of RB, and the in vivo pharmacological or toxicological data obtained in rat models previously may be misleading. For example, it has been reported that RB has the capability to antagonize marinobufagenin (5-HRB)-caused hypertension, proteinuria, intrauterine growth restriction, and weight gain in a rat model of human preeclampsia (Vu et al., 2006; Horvat et al., 2010; Puschett et al., 2010; Puschett, 2012), but these bioactivities have not been observed in other animals. Our results make it conceivable that marinobufagenin will be generated when RB is administrated in human, whereas 3-epi-RB and its derivatives will be generated in rat, and that

![Fig. 4. Michaelis-Menten plots of RB metabolism in HLM (A) and rhCYP3A4 (B). An Eadie-Hofstee plot is shown as an inset to illustrate biphasic kinetics. Data points represent the mean of triplicate determinations.](downloaded from dmd.aspetjournals.org at ASPET Journals on April 3, 2017)
these metabolites may serve as antagonists of marinobufagenin. Therefore, it is necessary to reevaluate in vivo data for RB and its analogs obtained in rat by using a suitable surrogate model for simulating their metabolism in humans.

It is particularly noted that marinobufagenin (5-HRB), a major metabolite of RB in HLM, is one of the endogenous mammalian bufadienolides (Fedorova et al., 2001; Prassas and Diamandis, 2008; Uddin et al., 2011, 2012). As an endogenous steroid, marinobufagenin participates in a variety of physiologic and pathophysiologic processes, including the regulation of Na⁺-K⁺-ATPase activity and the pathogenesis of arterial hypertension in mammals, including humans (Bagrov et al., 1998; Bagrov et al., 2005). It has been reported that the

![Fig. 5. The correlation between the CYP3A4 or 3A5 protein levels and RB hydroxylation rates. The correlation parameter was expressed by the linear regression coefficient (r). P < 0.05 was considered statistically significant.](image)

![Fig. 6. RB and 5-HRB inhibited cell viability and changed morphology. (A) Human lung cancer A549 and H1299 cells and HLF cells were treated with RB or 5-HRB under normal culture medium at the indicated doses. (B) The changes in cell morphology and spreading in A549 cells treated with RB or 5-HRB for 48 hours were observed and cells were photographed using a microscope fitted with the digital camera.](image)
plasma levels of marinobufagenin are closely associated with proteinuria and preeclampsia in pregnancy (Puschett et al., 2010; Puschett, 2012). Taking into account that marinobufagenin is the major metabolite of RB in HLMs, the administration of RB could increase the plasma concentration of marinobufagenin. It is easily conceivable that the administration of RB will make the condition more serious in pregnancies with preeclampsia, owing to the production of extra marinobufagenin by CYP3A4-mediated hydroxylation of RB. Therefore, adequate consideration should be taken regarding prescription of TCMs containing RB or marinobufagenin.

Recent investigations demonstrate that the Na+-K+-ATPase alpha subunit is a promising new target for developing anticancer agents (Mijatovic et al., 2007, 2008). It is even more noteworthy that RB and its metabolite, as a natural inhibitor of Na+-K+-ATPase, should be studied for antineoplastic activity. In the present study, the cytotoxic activities of 5-HRB and RB were evaluated in NSCLC, and 5-HRB still exhibited excellent cytotoxic activities against NSCLC, including A549 and H1299 cells. By monitoring the apoptosis-associated protein, this investigation indicated that the anticancer bioactivities of 5-HRB were partially mediated through the activation of cytochrome c/caspase–dependent apoptotic signaling pathways for A549 cells. However, attention should be paid to the discrepancy between the present findings and the reported structure-cytotoxic activity relationship of bufadienolides, which demonstrated that 5-hydroxylated reduced anticancer activity significantly (Kamano et al., 1998; Ma et al., 2011). The discrepancy makes apparent that the metabolic effect on bufadienolides may change case by case, making it meaningful to investigate the biotransformation of bufadienolides, which have great potential as a chemotherapeutic agents in oncology.

Considering the approximately 1000-fold discrepancy between the IC\textsubscript{50} values for cell viability inhibition in NSCLC and HLF cells, the present research suggests that malignant cells are more susceptible to the effects of RB and 5-HRB than are normal cells. To further clarify and compare the safety of RB and 5-HRB, we evaluated the acute toxicity of RB and 5-HRB in vivo. First, a comparative study of RB hydroxylation in humans and four common experimental animals (including monkey, dog, guinea pig, and mouse) was performed to determine a suitable animal model for the toxicity evaluation (Supplemental Figs. 2 and 3 and Supplemental Tables 1 and 2). The results suggested that 5β-hydroxylation of RB is the major pathway in four animal species, and mouse was selected as a preferred model because its metabolic profiles and enzyme and catalytic efficacies were similar to those of human. Our results indicated that 5-HRB possesses a relatively high tolerability in vivo.

From the perspective of drug metabolism and pharmacological evaluation, the CYP3A-mediated RB 5β-hydroxylation in humans generates the bioactive and more polar metabolite marinobufagenin, implying that RB serves as a prodrug and its in vivo anticancer effects can be conserved after biotransformation. Additionally, we also found that marinobufagenin displayed superior metabolic stability compared with RB, and the further biotransformation of marinobufagenin in HLM is very slow (data not shown). These findings make us believe that marinobufagenin has a good druglikeness and can be considered as a potential leading anticancer compound. Taken together, marinobufagenin can be considered a promising antitumor agent, owing to its excellent cancer therapeutics, metabolic stability, and the relatively high tolerability in the human body.

In summary, the profile of phase I metabolism of RB was characterized first. The major metabolite of RB in HLM was identified as 5β-hydroxyresibufogenin (5-HRB), and CYP3A4 was the major isoform responsible for RB 5β-hydroxylation with high selectivity. In
In addition, a significant species differences in 5β-hydroxylation was elucidated: In sharp contrast to the important involved 3-epimerization of RB in rat liver microsomes, 5β-hydroxylation played the dominant role in the metabolism of RB in humans. Furthermore, the major metabolite of RB could significantly induce cell growth inhibition and apoptosis in A549 and H1299 by facilitating apoptosome assembly and caspase activation, all of which implies that 5-HRB may play an important role in antitumor activities of RB in human. Our findings lay a solid foundation for further research into RB metabolism in humans, and our in vitro and in vivo safety revaluation regarding the bioactive metabolite of RB encourage its further investigation.

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Authorship Contributions

Participants in research design: Ma, Ge, Yang.
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Contributed new reagents or analytic tools: Wang, Deng.
Performed data analysis: Ning, Yu, Hou.
Wrote or contributed to the writing of the manuscript: Ning, Ge, Ma.

References


Fig. 8. 5-HRB induced apoptosis by modulating cytochrome c/caspase signaling. Human A549 cells were treated with 5-HRB at the indicated doses. At 24 hours after treatment, the apoptosis was determined by a fluorescence-activated cell sorting (FACS) analysis (A); the release of cytochrome c (Cyto c) in A549 cells was determined by immunofluorescence imaging (IF) analysis to monitor Cyto c release from the mitochondrial intermembrane space into the cytosol (B) and the levels of the cleaved caspase-9 protein in A549 cells were analyzed by Western blot (C). The apoptosis is represented by relative percentage of apoptotic cells versus that in DMSO-treated cells. (**P < 0.01, significant differences between 5-HRB treatment groups and DMSO vehicle control groups.)


