In Vitro Studies on the Oxidative Metabolism of 20(S)-Ginsenoside Rh2 in Human, Monkey, Dog, Rat, and Mouse Liver Microsomes, and Human Liver S9

Liang Li, Xiaoyan Chen, Jialan Zhou, and Dafang Zhong

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

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ABSTRACT:

20(S)-Ginsenoside Rh2 (Rh2)-containing products are widely used in Asia, Europe, and North America. However, extremely limited metabolism information greatly impedes the complete understanding of its clinical safety and effectiveness. The present study aims to systematically investigate the oxidative metabolism of Rh2 using a complementary set of in vitro models. Twenty-five oxidative metabolites were found using liquid chromatography-electrospray ionization ion-trap mass spectrometry. Six metabolites and a metabolite intermediate were synthesized. The metabolites were structurally identified as 26-hydroxy Rh2 (M1-1), (20(S,24R)-epoxydammarane-12,25-diol-3β-0-glucopyranoside (M1-3), (20(S,24R)-epoxydammarane-12,25-diol-3β-0-glucopyranoside (M1-5), 26,27-dihydroxy Rh2 (M3-6), (20(S,24S)-epoxydammarane-12,25,26-triol-3β-0-glucopyranoside (M3-10), (20(S,24R)-epoxydammarane-12,25,26-triol-3β-0-glucopyranoside (M3-11), and 26-aldehyde Rh2 on the basis of detailed mass spectrometry and nuclear magnetic resonance data analysis. Double-bond epoxidation followed by rearrangement and vinyl-methyl group hydroxylation represent the initial metabolic pathways generating monooxygenated metabolites M1-1 to M1-5. Further sequential metabolites (M2–M5) from the dehydrogenation and/or oxygenation of M1 were also detected. CYP3A4 was the predominant enzyme involved in the oxidative metabolism of Rh2, whereas alcohol dehydrogenase and aldehyde dehydrogenase mainly catalyzed the metabolic conversion of alcohol to the corresponding carboxylic acid. No significant differences were observed in the phase I metabolite profiles of Rh2 among the five species tested. Reactive epoxide metabolite formation in both humans and animals was evident. However, GSH conjugate M6 was detected only in cynomolgus monkey liver microsomal incubations. In conclusion, Rh2 is a good substrate for CYP3A4 and could undergo extensive oxidative metabolism under the catalysis of CYP3A4.

Introduction

20(S)-Ginsenoside Rh2 (Rh2) (Fig. 1), a trace constituent of red ginseng, was first reported by Kitagawa et al. (1983). Structurally, Rh2 is a dammarane-type triterpenoid saponin formed by a glucose sugar moiety glycosidically bonded to the hydroxy group at the C-3 position of 20(S)-protopanaxadiol (PPD). A number of studies have shown that Rh2 exhibits excellent cancer prevention effects, especially the induction of apoptosis (Park et al., 1997; Ham et al., 2003; Cheng et al., 2005). In addition, Rh2 has been reported to exert synergetic effects on some chemotherapeutic agents even at a nonfatal dose (Kikuchi et al., 1991; Jia et al., 2004; Wang et al., 2006; Xie et al., 2006). The synergistic mechanisms of Rh2 have yet to be fully characterized. The modulation of efflux transporter activity related to multidrug resistance [e.g., P-glycoprotein (P-gp) and breast cancer resistance protein] has been considered a possible factor (Jin et al., 2006; Zhang et al., 2010). However, metabolism information on Rh2 is scant and mostly limited to its degradation in the gastrointestinal tract to form an aglycone. Various Rh2-containing products are sold over-the-counter in Asian, European, and North American markets.

Jinxing capsule, a healthcare product, was approved by the Chinese State Food and Drug Administration for use in neoadjuvant therapy in 2006. A recommended oral dose of 81 mg/day for Rh2 (40.5 mg/time b.i.d.), the main active ingredient, is indicated in the package insert (Asia Pharmaceutical Group, Haikou, China). Jinxing capsule is claimed to improve immune function and increase disease resistance for patients undergoing treatment and rehabilitation for many kinds of cancer. During the preclinical evaluation (D. Zhong, unpublished data), we found that Rh2 could be absorbed moderately with an oral bioavailability of 28% (25 mg/kg) in Wistar rats and 55% (8 mg/kg) in beagles. After an oral administration of 50 mg/kg, the peak Rh2
concentration (60 μM) in rat liver was approximately 20 times higher than that in plasma. No Rh2-related material was detected in rat urine, and minimal Rh2 (less than 10%) was detected in rat feces and bile, which imply that extensive metabolism followed by secretion into bile is the major route of Rh2 excretion.

Rh2 metabolism in rats after a single oral administration (50 mg/kg) was subsequently studied in our laboratory (Deng et al., 2009). Major metabolic pathways observed included deglycosylation, oxygenation, and both sulfate and glucuronate conjugation. Small amounts of PPD were detected in rat plasma, whereas monooxygenated, dioxygenated, and carboxylated derivatives of both Rh2 and PPD represented the major circulating metabolites. A GSH conjugate derived from monooxygenated Rh2 was determined as one of the major metabolites in rat bile. Cysteine adducts of both Rh2 and PPD were also detected in rat feces. Thus, some chemically reactive intermediates were formed in vivo. To the best of our knowledge, the metabolites of Rh2 in humans have not been reported to date.

Chemically reactive metabolite formation is an unwanted feature of any drug or drug candidate during discovery and development. Therefore, substantially decreasing or eliminating the metabolic activation liability of drug candidates has become the ultimate goal of researchers in the field of drug metabolism and toxicology (Kumar et al., 2008). Detecting and characterizing reactive metabolites with appropriate trapping agents in both experimental animals and humans are highly important initial steps so that informed evaluation of hidden risks can be made in a timely manner (Evans and Baillie, 2005).

A range of rodent (mice and rats) and nonrodent (dogs and monkeys) animal species are routinely used for preclinical metabolism and toxicology tests in many pharmaceutical companies to evaluate the absorption, distribution, metabolism, excretion, and toxicology profiles of new candidate drugs. Nevertheless, given the differences in isoform composition, expression, and catalytic activities of drug-metabolizing enzymes across species, extensive differences in drug metabolism and toxicity between animals and humans have been found. In this context, choosing the most relevant animal species on which to conduct studies and extrapolating the results to humans are huge challenges for researchers (Martignoni et al., 2006; Baillie and Rettie, 2011).

The objectives of the present study were as follows: 1) to characterize the structures of oxidative metabolites, including reactive intermediates of Rh2 and 2) to identify the cytochrome P450 (P450) and non-P450 enzymes responsible for Rh2 biotransformation.
Materials and Methods

Materials. 20(S)-Ginsenoside Rh2 (purity >98%) was purchased from Shanghai Huaiy Bio Technology Co., Ltd. (Shanghai, China). Pooled human liver microsomes (HLM), S9, and cytosol (mixed-gender pool of 50 donors), male cynomolgus monkey liver microsomes (CyLM), male beagles [dog liver microsomes (DLM)], male Sprague-Dawley rats [rat liver microsomes (RLM)], and male CD-1 mice [mouse liver microsomes (MLM)], as well as recombinant P450 enzymes CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11 were purchased from BD Gentex (Woburn, MA). NADPH, NAD⁺, GSH, GSH ethyl ester (GEE), N-acetyl-cysteine (NAC), 4-methylpyrazole hydroxylide, xilazine, and disulfiram were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade (Merck, Darmstadt, Germany). 3-Chloroperoxybenzoic acid (m-CPBA) was purchased from Alfa Aesar China (Tianjin) Co., Ltd. (Beijing, China). Analytical-grade dichloromethane and methanol, selenium dioxide (SeO₂), tert-butyl hydroperoxide, and salicylic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Purified water was generated with a Milli-Q Gradient System (Millipore Corporation, Molsheim, France). C18 reversed-phase silica gel (150–200 mesh; Merck) was used for column chromatography. Deuterium methanol and pyridine were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Microsomal and S9 Fraction Incubations of Rh2 and Isolated Metabolites. Stock solutions of Rh2 (3, 10, and 50 mM) were prepared in methanol. The final methanol concentration in the incubation was 0.1% (v/v). The liver microsomes and S9 fraction were carefully thawed on ice before the experiment. NADPH (2 mM) was added into open-to-air polyethylene tubes heated in a water-bath shaker at 37°C containing 10 μM Rh2 solution in 100 mM potassium phosphate buffer (pH 7.4) with or without GSH, GEE, or NAC at a final concentration of 2 mM. The total incubation volume was 200 μl. After 3 min preincubation at 37°C, the incubation reactions were initiated with the addition of microsome proteins (1 mg/ml). After undergoing incubation for 60 min, the reactions were terminated with an equal volume of ice-cold acetone trile. Control samples without NADPH were included. Each incubation was performed in duplicate. Individual incubations of Rh2 (3 and 50 μM) and six isolated metabolites (M1-1, M1-3, M1-4, M3-3, M3-10, and M3-11; 10 μM Rh2) were performed in the same incubation conditions described above.

Microsomal Incubations in the Presence of Inhibitors. The specific P450 enzymes involved in the oxidative metabolism of Rh2 were determined by testing six chemical inhibitors as follows (concentration and enzyme in parenthesis): α-naphthoflavone (0.1 μM CYP1A2), sulfaphenazole (1.0 μM CYP2C9), ticlopidine (0.4 mM CYP2B6 and 6.0 mM CYP2C19), quinidine (2.0 mM CYP2D6), chlorothiazide (60 μM CYP2E1), and ketoconazole (1.0 and 10.0 μM CYP3A). The inhibitor concentrations used were referred from the U.S. Food and Drug Administration Guide for Industry on Drug Interaction Studies (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCUM072101.pdf, 2006). Under the experimental conditions, ketoconazole shows inhibitory activities against Rh2 (10 μM). The inhibitors at various concentrations in 100 mM potassium phosphate buffer (pH 7.4). The final incubation volume was 200 μl. After 15 min of preincubation at 37°C, the reactions were initiated with the addition of 10 μM M1-1 (or 26-aldehyde-Rh2). The reactions were terminated with an equal volume of ice-cold acetone trile after 60 min of incubation. Controls without chemical inhibitors were included. Each incubation was performed in duplicate.

Sample Preparation for LC/MS® Analysis. All the in vitro incubation samples were prepared using the same methods. Methanol (200 μl) was added to a 200-μl aliquot of the in vitro incubations. This sample was vortex-mixed and centrifuged at 14,000g for 5 min. The supernatant was transferred into a glass tube, evaporated to dryness under a stream of nitrogen at 40°C, and then reconstituted in 100 μl of 0.1% formic acid in methanol with 0.1% formic acid in 5 mM ammonium acetate (70:30, v/v). A 20-μl aliquot of the reconstituted solution was injected into the LC/MS® system for analysis.

Chromatography. The Agilent 1200 HPLC system was equipped with a reversed-phase column (Eclipse XDB-C18, 4.6 × 150 mm i.d., 5 μM; Agilent Technologies, Santa Clara, CA) protected by a 4.0 × 3.0 mm i.d. Security Guard (5 μm) C18 guard column (Phenomenex, Torrance, CA). The mobile phase was a mixture of 0.1% formic acid in methanol (A) and 0.1% formic acid in 5 mM ammonium acetate (B) with gradients programmed as follows: initial 70% A maintained for 3 min, increased to 100% in 17 min, maintained for 5 min, and then finally decreased to 70% A in 0.1 min and maintained for 10 min. The flow rate was 0.5 ml/min, and the injection volume was 20 μl. The effluent from the LC column was diverted to waste for the first 1.5 min after the injection to avoid contamination with nonvolatile salts and background interferences.

Ion-Trap Mass Spectrometry. The LC/MS® experiment was performed on an Agilent 6330 LC/MSD Trap XCT ultra (Agilent Technologies, Waldbronn, Germany). The mass spectrometer (MSD) was equipped with an electrospray ionization source. The ionization mode was positive. The interface and MSD parameters were as follows: nebulizer pressure, 50 psi (N₂); dry gas, 12 ml/min (N₂); dry gas temperature, 350°C; spray capillary voltage, 3500 V; scan range, m/z 100 to 1000; spectra average, 3; and dwell time, 200 ms. For the MS™ spectra, the fragmentation amplitude varied between 0.7 and 1.0 V. The MS® product ion spectra were produced via the collision-induced dissociation of the molecular ions [M + H]⁺ of all analytes at their respective HPLC retention times. Data acquisition was performed in full-scan LC/mass spectrometry (MS) and MS® modes. All data acquired were processed using ChemStation software (revision B.01.03; Agilent Technologies).

Synthesis and Isolation of M1-1, M3-6, and 26-Aldehyde-Rh2. Up to 22 μl (200 μmol) of tert-butyl hydroperoxide was added into a 100-ml flask containing a magnetically stirred suspension of 1.1 (10 μmol) of SeO₂ and 7 mg of (50 μmol) salicylic acid in 25 ml of dichloromethane. The resulting solution was placed in a 30°C water bath, and 40 mg (64 μmol) of Rh2 was introduced. After 30 h, the reaction mixture was filtered and concentrated in a vacuum to obtain a residue. The residue was subjected to semipreparative HPLC (equipped with two Shimadzu LC-6AD pumps and a Shimadzu SPD-20A UV detector; Shimadzu, Kyoto, Japan) for further purification. Chromatographic separation was performed on a YMC-Pack ODS-A column (10 × 250 mm i.d., 5 μm; YMC Company Ltd., Kyoto, Japan) eluted with a methanol/H₂O/formic acid solution (90:10:0.1, v/v/v) at a flow rate of 3 ml/min. The detection wavelength used was 210 nm. The fractions with retention times at 5.4 min (M3-6, 4.7 mg), 7.6 min (M1-1, 15.7 mg), and 8.9 min (26-aldehyde-Rh2, 5.0 mg) were collected.
Synthesis and Isolation of M1-3 and M1-5. m-CPBA (25 mg, 160 μmol) was slowly added to Rh2 solution (20 mg, 32 μmol) in 30 ml of dichloromethane at room temperature. After 15 h of stirring, the reaction mixture was added to sodium carbonate-saturated ice water and extracted three times with ethyl acetate. The organic extract was evaporated to dryness in a vacuum to obtain a residue. The residue was subjected to column chromatography on a C18 reversed-phase silica gel eluted with methanol/H2O (87:13) to produce M1-3 (11.2 mg) and M1-5 (5.4 mg).

Synthesis and Isolation of M3-10 and M3-11. m-CPBA (25 mg, 160 μmol) was slowly added to a M1-1 solution (10 mg, 16 μmol) in 30 ml of dichloromethane at room temperature. The resulting solution was refluxed in a 60°C water bath for 24 h. The reaction mixture was added to sodium carbonate-saturated ice water and extracted three times with ethyl acetate. The organic extract was evaporated to dryness in a vacuum to obtain a residue. The residue was subjected to column chromatography on a C18 reversed-phase silica gel eluted with methanol/H2O (80:20) to produce M3-10 (2.2 mg) and M3-11 (5.5 mg).

Nuclear Magnetic Resonance Instruments. 1H and 13C nuclear magnetic resonance (NMR) spectra (distortionless enhancement by polarization transfer) were recorded with a Varian Mercury 400 spectrometer (400 for 1H and 100 MHz for 13C; Varian, Inc., Palo Alto, CA), and chemical shifts were recorded in parts per million referenced to the solvent signal, respectively. Deuterium pyridine (C5D5N) was the solvent used for Rh2, M1-3, M1-5, M3-10, and M3-11, whereas deuterium methanol (CD3OD) was used for M1-1, M3-6, and 26-aldehyde-Rh2. Rotating-frame Overhauser effect spectroscopy and nuclear Overhauser enhancement difference spectroscopy were performed with a Varian Unity Inova 600 spectrometer (600 MHz; Varian, Inc.).

Isolation and Incubation of M1-4. An up-scaled HLM incubation with Rh2 was performed (1 ml) under similar experimental conditions as described previously under Microsomal and S9 Fraction Incubations of Rh2 and Isolated Metabolites. After 60 min of incubation, 2 ml ice-cold acetonitrile was introduced. This sample was vortex-mixed and centrifuged at 14,000g for 5 min. The resulting supernatant was concentrated under a stream of nitrogen and subjected to HPLC separation under the same chromatography conditions as mentioned previously under Chromatography. The fractions with retention times between 19.8 and 20.5 min (M1-4 and M1-5) were collected and pooled. The pooled fractions containing M1-4 and M1-5 were divided into four equal parts, evaporated to dryness under a stream of nitrogen at 40°C, and individually incubated with one of the following: 1) CyLM and 2 mM GSH; 2) 2 mM GSH; 3) 0.1 M hydrochloric acid; or 4) 100 mM potassium phosphate buffer only (pH 7.4).

Results

Mass Spectral Properties of Rh2. A comprehensive understanding of the fragmentation behavior of the parent compound to be tested can be very helpful in metabolite identification using LC/MS. A higher mass spectrometry signal response was gained by setting the source temperature to 350°C in performing the experiments. Under the experimental conditions, the protonated Rh2 molecule (m/z 623) was not detected under positive scan mode (Fig. 1A). The LC peak at 22.9-min retention time showed typical in-source dissociation fragment ions at m/z 605 ([M + H - H2O]+), 587 ([M + H - 2H2O]+), 425 ([M + H - glucose - H2O]+), and 407 ([M + H - glucose - 2H2O]+), as well as adduct ions at m/z 645 ([M + Na]+). In the MS2 spectrum (Fig. 1B), Rh2 did not display diagnostic fragment ions.

Structure Elucidation of Rh2 Metabolites in HLM and Human Liver S9. Figures 2 and 3 show the total ion and extracted ion chromatograms of the parent drug and its metabolites after 60 min of

![FIG. 2. Extracted ion [M + H]+ and [M + Na]+ chromatograms of Rh2 metabolites in the HLM incubations with NADPH (A) and without NADPH (B). Twenty-three detected metabolites are labeled M1 to M5, and the parent compound is labeled Rh2. These metabolites are numbered according to their chromatographic retention times. The inset is the expanded chromatogram at 14 to 18 min. Intens., intensity; TIC, total ion chromatogram.](https://example.com/fig2.png)
incubation with HLM and human liver S9 fraction, respectively. Compared with the blank sample, 23 and 24 phase I metabolites were observed in HLM and human liver S9 fraction, respectively. These metabolites may be classified into five types: M1 (m/z 639) for the metabolic addition of one oxygen atom (+16 from the parent), M2 (m/z 653) for the addition of two oxygen atoms with dehydrogenation (+30 from the parent), M3 (m/z 655) for the addition of two oxygen atoms (+32 from the parent), M4 (m/z 669) for the addition of three oxygen atoms with dehydrogenation (+46 from the parent), and M5 (m/z 671) for the addition of three oxygen atoms (+48 from the parent). These metabolites were numbered according to their chromatographic retention times, and the structures were elucidated through their mass spectral fragments and NMR data.

**Metabolite M1.** In both HLM and human liver S9 incubations, M1-1, M1-2, M1-3, M1-4, and M1-5 were detected at 16.7, 18.3, 18.9, 20.0, and 20.2 min, respectively, with a protonated molecular weight of 639 (m/z 623), which indicates the addition of one oxygen atom into the molecule. The MS2 spectra (Fig. 4A) of M1-1 and M1-2 showed identical fragment ions at m/z 621 (m/z 18), 603 (m/z 36), 459 (m/z 180), 441 (m/z 180 + m/z 18), and 423 (m/z 180 + m/z 36). The fragment ion of m/z 459 indicated that +16 modification occurred in the dammarane aglycone moiety.

The standard reference of M1-1 was synthesized chemically using tert-butyl hydroperoxide and SeO2. The 1H (Supplemental Fig. 1) and 13C NMR (Table 1) spectra of M1-1 were obtained. A comparison of the 1H and 13C NMR spectra of M1-1 with those of Rh2 (Supplemental Fig. 2; Table 1) indicated the main difference at the side chain. One of the vinyl-methyl proton signals (δH 1.67, s, H3-26; δC 28.50, q, C-26) of Rh2 was replaced by a hydroxymethyl group (δH 3.92, s, H2-26; δC 69.50, t, C-26), suggesting that a terminal hydroxylated metabolite was formed. Furthermore, in the nuclear Overhauser enhancement difference spectrum of M1-1 (Supplemental Fig. 1), H-24 (δH 5.44) exhibited a clear correlation with H2-26 (δH 3.92), suggesting that H-24 and H2-26 are oriented on the same side of the carbon-carbon double bond. Thus, M1-1 was identified as 26-hydroxy Rh2. In addition, M1-2 was tentatively designated 27-hydroxy Rh2.

The MS2 spectra (Fig. 4B) of M1-3 and M1-5 showed identical fragment ions at m/z 621 (m/z 18), 603 (m/z 36), 585 (m/z 54), 459 (m/z 180), 441 (m/z 180 + m/z 18), and 423 (m/z 180 + m/z 36). The fragment ion of m/z 459 indicated that +16 modification occurred in the dammarane aglycone moiety.

The standard references of M1-3 and M1-5 were synthesized chemically using m-cPBA. Comparison of the 1H and 13C NMR spectra of M1-1 with those of Rh2 (Supplemental Fig. 2; Table 1) also revealed the main difference at the side chain, where the trisubstituted carbon-carbon double bond signals of Rh2 (δH 5.32, t, J = 7.1 Hz, H-24; δC 126.29, d, C-24 and 130.74, s, C-25) disappeared, suggesting that the double bond at δ24,25 was formed. One methine signal (δH 88.39, d, C-24; δH 4.17, dd, J = 11.1, 5.2 Hz, H-24) and an OH-bearing tertiary carbon atom (δC 69.64, s, C-25) were observed, indicating the presence of the 20,24-oxide moiety and a hydroxyl group at C-25. Careful comparison of the 1H and 13C NMR spectra of M1-3 and M1-5 showed identical fragment ions at m/z 621 (m/z 18), 603 (m/z 36), 585 (m/z 54), 459 (m/z 180), 441 (m/z 180 + m/z 18), and 423 (m/z 180 + m/z 36). The fragment ion of m/z 459 indicated that +16 modification occurred in the dammarane aglycone moiety.

The standard references of M1-3 and M1-5 were synthesized chemically using m-cPBA. Comparison of the 1H and 13C NMR spectral data of M1-3 with those of Rh2 (Supplemental Fig. 2; Table 1) also revealed the main difference at the side chain, where the trisubstituted carbon-carbon double bond signals of Rh2 (δH 5.32, t, J = 7.1 Hz, H-24; δC 126.29, d, C-24 and 130.74, s, C-25) disappeared, suggesting that the double bond at δ24,25 was formed. One methine signal (δH 88.39, d, C-24; δH 4.17, dd, J = 11.1, 5.2 Hz, H-24) and an OH-bearing tertiary carbon atom (δC 69.64, s, C-25) were observed, indicating the presence of the 20,24-oxide moiety and a hydroxyl group at C-25. Careful comparison of the 1H and 13C NMR spectra of M1-3 and M1-5 showed identical fragment ions at m/z 621 (m/z 18), 603 (m/z 36), 585 (m/z 54), 459 (m/z 180), 441 (m/z 180 + m/z 18), and 423 (m/z 180 + m/z 36). The fragment ion of m/z 459 indicated that +16 modification occurred in the dammarane aglycone moiety.
data of M1-5 and M1-3 (Supplemental Fig. 2; Table 1) revealed similarities, indicating that the two metabolites share the same framework and planar structure. M1-5 differs from M1-3 only in the configuration at C-24. Furthermore, a 24R configuration of M1-5 was supported by rotating frame Overhauser effect spectroscopy. The clear nuclear Overhauser effect cross-peak between H-24 and Me-21 (Supplemental Fig. 3) suggests that both are oriented on the same face of the molecule according to the drawn structure of M1-5. Thus, M1-3 and M1-5 were identified as (20S,24S)-epoxydammarane-12,25-diol-3-D-glucopyranoside and (20S,24R)-epoxydammarane-12,25-diol-3-D-glucopyranoside, respectively.

As stated in our previous article (Li et al., 2011), the metabolic conversion of the carbon-carbon double bond to the 20,24-oxides is very likely involved in the formation of 24,25-epoxide intermediates. Given the intramolecular nucleophilic attack of the hydroxyl group at C-20, the 24R,25-epoxide was quickly transformed...
to 20β,24S-oxide (M1-3). By contrast, considering the steric hindrance posed by the β hydrogen atom at C-24 and the hydroxyl group at C-20, the 24S,25-epoxide was more stable against the intramolecular nucleophilic attack reaction (presumably to form M1-5) than the 24R-epimer; thus, it was detected in the incubation mixture. M1-4 was hypothesized to be 24S,25-epoxide Rh2. We proved our hypothesis by isolating M1-4 (mixed with M1-5) from the HPLC chromatogram peak of M1-4 (Fig. 4B) and its mass spectral response of M1-4 (Fig. 5) compared with the control sample. Thus, M1-4 might rearrange to form M1-5 under acidic conditions. Thus, M1-4 was identified as (24S,25)-epoxydammarane-12,20-diol-3-β-glucopyranoside.

**Metabolite M2.** The protonated molecular weight of M2 was detected at 653, which was 30 higher than that of the protonated parent drug, indicating the introduction of two oxygen atoms with dehydrogenation. M2-1, M2-4, and M2-5 were detected at 12.5, 14.4, and 14.9 min, respectively, in the HLM and human liver S9 incubations. The MS2 spectra (Fig. 4D) showed fragment ions at m/z 635 (−18), 617 (−36), 455 (−180 −18), and 437 (−180 −36), suggesting that +30 modification occurred in the dammarane aglycone moiety.

**Metabolites M2-2 and M2-3 were detected in the HLM and human liver S9 incubations at 13.2 and 14.0 min, respectively. The MS2 spectra (Fig. 4E) showed fragment ions at m/z 635 (−18) and 609 (−44). The fragment ion of m/z 609 was attributed to the neutral loss of one mole of carbon dioxide, which suggests that two carboxylic acid derivatives were
formed. In addition, M2-3 was detected in the HLM and human liver S9 incubations with M1-1. This finding implies that M2-3 was derived from 26-hydroxy Rh2. Thus, M2-3 was tentatively designated as 27-carboxy Rh2.

**Metabolite M3.** M3 was detected with a protonated molecular weight of 655, which was 32 higher than that of the protonated parent drug. This finding indicates that there was an introduction of two oxygen atoms. Ten and 12 independent chromatographic peaks with protonated ions at 655 m/z were detected in the HLM and human liver S9 incubations, respectively.

Metabolites M3-1, M3-2, M3-4, M3-5, M3-8, and M3-12 were detected at 7.3, 7.2, 10.0, 11.0, 13.9, and 17.1 min, respectively. The MS2 spectra (Fig. 4F) showed fragment ions at m/z 637 (−18), 619 (−36), 475 (−180), 457 (−180 − 18), 439 (−180 − 36), 421 (−180 − 54), and 403 (−180 − 72); M3-1, M3-2, M3-4, M3-5, M3-8, and M3-12 were tentatively identified as dioxygenation products of Rh2. Moreover, the two oxygen atoms were introduced into the dammarane aglycone moiety.

Metabolites M3-3 and M3-7 were detected at 9.1 and 13.2 min, respectively. The MS2 spectra (Fig. 4G) showed fragment ions at m/z 637 (−18), 619 (−36), 475 (−180 − 18), 439 (−180 − 36), and 421 (−180 − 54). M3-3 and M3-7 were also identified as dioxygenation products of Rh2. The two oxygen atoms were introduced into the dammarane aglycone moiety.

Metabolite M3-6 was detected at 12.2 min. The MS2 spectra (Fig. 4H) showed major fragment ions at m/z 637 (−18), 619 (−36), 475 (−180 − 18), and 439 (−180 − 36). The standard reference of M3-6 was synthesized chemically using tert-butyldihydroperoxide and SeO2. The 1H (Supplemental Fig. 2) and 13C NMR (Table 1) spectra of M3-6 were obtained. The comparison between the 1H and 13C NMR spectra of M3-6 with those of Rh2 (Supplemental Fig. 2; Table 1) indicated the main difference at the side chain, where two of the vinyl-methyl proton signals (δH 1.67, s, H2-26; δH 2.50, q, H3-26 and H3-27; δH 1.67, q, C=O) of Rh2 were replaced by two hydroxymethyl groups (δH 4.17, t, H2-26 and H2-27; δH 4.09, q, H2-26 and H2-27; δC 56.79, t, C-26 and C-27). This result suggests that a dihydroxylated metabolite was formed. Thus, M3-6 was identified as 26,27-di hydroxy Rh2.

Metabolite M3-9 was detected at 15.6 min. The MS2 spectra (Fig. 4I) showed major fragment ions at m/z 637 (−18) and 619 (−36). M3-9 was detected in the HLM and human liver S9 incubations with M1-1. M1-1 oxidation with m-CPBA also can produce M3-9 (data not shown). Thus, M3-9 was tentatively identified as (24S,25S)-epoxydammarane-12,20, 26-triol-3-carboxylic acid derivative M2-3 (not detected because of the quick rearrangement). M1-1 underwent extensive oxidative metabolism in HLM incubations and major metabolic pathways, including hydroxylation, to form M3-3, M3-6, and M3-7. In addition, it underwent epoxidation to form M3-9 and its 24-epimer (not detected because of the quick rearrangement) and their rearrangement products M3-10 and M3-11. A small amount of M5-2 generated from the hydroxylation of M3-11 and a carboxylic acid derivative M2-3 was also detected. The metabolites from M1-3 may be classified into the following three types: M2-1 and M2-4 from hydroxylation with dehydrogenation; M3-1, M3-2, M3-4, M3-5, and M3-10 from hydroxylation; and M5-1 from dihydroxylation. Hydroxylation and dehydrogenation were the major metabolic pathways observed for M1-5 to generate metabolites M2-5, M3-8, M3-11, M3-12, and M4. No obvious oxidative metabolites were detected in the HLM incubation with M3-6, M3-10 and M3-11 only underwent hydroxylation to produce M5-1 and M5-2 in the HLM incubations, respectively.

**Identification of P450 Enzymes Responsible for Rh2 Metabolism.** P450 enzymes responsible for the oxidative metabolism of Rh2 were probed through coinubation with isoenzyme-selective inhibitors, including α-naphthoflavone (CYP1A2), ticlpidine (CYP2B6 and CYP2C19), sulfaphenazole (CYP2C9), quinidine (CYP2D6), chlormethiazole (CYP2E1), or ketoconazole (CYP3A3), with HLM in the presence of NADPH. The formations of M1-2, M1-3, and M1-4 + M1-5 were monitored because of their higher MS responses compared with the sequential oxidative metabolites. Among the inhibitors used, only ketoconazole showed a significant inhibitory effect on the oxidative metabolism of Rh2. No such inhibition was observed for the other inhibitors (Fig. 6). P450 3As were probably the major enzymes responsible for the oxidative metabolism of Rh2.

Catalytic activities of 12 individual recombinant human P450s, including CYPIA2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP4A11, in Rh2 biotransformation were evaluated. As expected, the oxidative metabolites of Rh2 were detected in the incubations only with CYP3A4 and CYP3A5 to a minor extent. The phase I metabolite profile of Rh2 in CYP3A4 was the same as that in HLM, whereas CYP3A5 produced only small amounts (less than 5% that of CYP3A4, respectively) of M1-1, M1-2, and M1-3 (Table 2). Other
isomers that were tested did not exert a catalytic activity toward Rh2 biotransformation.

Formation of M2-3 from M1-1. Different M2-3 concentrations were detected in the incubations of M1-1 with NADPH-fortified CYP3A4, HLM and human liver S9, and NAD⁺-supplemented human liver cytosol. The metabolic conversion was NADPH- and NAD⁺-dependent because the incubations lacking the corresponding cofactor did not produce M2-3 (data not shown). The metabolic conversion of M1-1 to M2-3 was catalyzed by both CYP3A4 and cytosol enzymes.

Three specific chemical inhibitors (4-methylpyrazole for alcohol dehydrogenase, raloxifene for aldehyde oxidase, and disulfiram for aldehyde dehydrogenase) were incorporated into the pooled human liver cytosol incubations with M1-1 to characterize cytosol enzymes that participated in the M1-1 oxidation. 4-Methylpyrazole, raloxifene, and disulfiram inhibited M2-3 formation better than the control samples that contain no chemical inhibitors by approximately 90, 18, and 60%, respectively, on the basis of LC/MS detection (Fig. 7A). Both alcohol dehydrogenase and aldehyde dehydrogenase are involved in the metabolism of M1-1 to M2-3, whereas the involvement of aldehyde oxidase is ambiguous. In addition, the expected oxidative intermediate 26-aldehyde-Rh2 was not detected in all incubation mixtures.

Formation of M2-3 from 26-Aldehyde-Rh2. The standard reference of 26-aldehyde-Rh2 was chemically synthesized using tert-butyl hydroperoxide and SeO₂. The chemical structure was characterized by ¹H, ¹³C NMR, and nuclear Overhauser enhancement difference spectrum (Supplemental Fig. 4; Table 1). The biotransformation of 26-aldehyde-Rh2 to M2-3 was also investigated in a pooled human cytosol with or without specific chemical inhibitors. Inhibitions of approximately 24, 22, and 45% were determined for 4-methylpyrazole, raloxifene, and disulfiram, respectively, with respect to the formation of M2-3 (Fig. 7B) by comparing them with the control samples. Aldehyde dehydrogenase was the major enzyme responsible for the conversion of 26-aldehyde-Rh2 to the corresponding carboxylic acid derivative M2-3. In addition, there was a minor contribution from both alcohol dehydrogenase and aldehyde oxidase.

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+, metabolite detected; –, metabolite not detected.
fortified HLM, DLM, RLM, MLM, and human liver S9 incubations with Rh2 in the presence of GSH, GEE, and NAC (data not shown).

One peak (M6) responsible for the corresponding molecular ion of the GSH conjugate (946 m/z) was detected by LC/MS at a retention time of 10.6 min only in the incubation mixture with CyLM (Fig. 8A). M6 formation in the incubations was NADPH-dependent, as illustrated by the representative chromatograms from the CyLM incubations (Fig. 8B). The presence of 1 μM ketoconazole in the CyLM incubations caused a dramatic reduction (85% according to MS peak areas) in M6 formation, as shown in Fig. 8C. The MS2 spectra of M6 (Fig. 9A) showed fragment ions at m/z 928 (H2O), 910 (H2O), 892 (H2O), 853 (H2O - glycine), 799 (H2O - pyroglutamate), 781 (H2O - pyroglutamate), 766 (H2O), 763 (H2O), 748 (H2O), 730 (H2O), 712 (H2O), 639 (H2O), 621 (H2O), 603 (H2O), 585 (H2O), 423 (H2O), 405 (H2O), 387 (H2O), 369 (H2O), 351 (H2O), 333 (H2O), 315 (H2O), 307 (H2O), 289 (H2O), 271 (H2O), 253 (H2O), 235 (H2O), 217 (H2O), 199 (H2O), 181 (H2O), 163 (H2O), 145 (H2O), 127 (H2O), 109 (H2O), 91 (H2O), 73 (H2O), 55 (H2O), 37 (H2O), 19 (H2O), 8 (H2O), 6 (H2O), 4 (H2O), 2 (H2O), 0 (H2O).

One peak (M7) responsible for the corresponding molecular ion of the GEE conjugate (974 m/z) was detected by LC/MS at a retention time of 12.9 min in CyLM (Fig. 8D) when GEE was used as an exogenous agent under the same incubation conditions. M9 formation was also found to be NADPH-dependent, as illustrated by the representative chromatograms from the CyLM incubations (Fig. 8E). The MS2 spectra of M7 (Fig. 9B) showed fragment ions...

Fig. 7. Effects of chemical inhibitors on the formation of the carboxylic acid metabolite M2-3 in human liver cytosol incubations with 10 μM M1-1 as a substrate (A) and with 10 μM 26-aldehyde-Rh2 as a substrate (B). Data are reported as the mean of two separate determinations.

Fig. 8. Extracted ion [M + H]+ chromatograms of Rh2 metabolites in CyLM incubations trapped with GSH with NADPH (A), trapped with GSH without NADPH (B), trapped with GSH with NADPH and 1 μM ketoconazole (C), trapped with GEE with NADPH (D), and trapped with GEE without NADPH (E). Two detected conjugates are labeled M6 (946 m/z) and M7 (974 m/z). Intens., intensity.
at 956 (H2O), 938 (2H2O), 871 (glycine ethyl ester), 853 (H2O – glycine ethyl ester), 827 (H2O – pyroglutamate), 809 (2H2O – pyroglutamate), 791 (3H2O – pyroglutamate), 673 (GEE – H2S), 621 (GEE – H2O), 603 (GEE – 2H2O), 441 (glucose – GEE – 2H2O), 423 (glucose – GEE – 3H2O) and 405 m/z (glucose – GEE – 4H2O). Thus, M7 was identified as the GEE conjugate of M1-4. LC/MS analyses showed that no conjugates were formed in the GSH- and NADPH-fortified HLM and human liver S9 incubations with M1-1.

**Discussion**

Rh2-containing products are widely used in Asia, Europe, and North America. However, a comprehensive metabolism study is generally lacking. The extremely limited metabolism information is not sufficient to understand its clinical effectiveness and ensure its safe use. A published article by Yang et al. (2011) assigned Rh2 as a Biopharmaceuticals Classification System class 4 compound on the basis of the fact that Rh2 has a low water solubility and permeability. Class 3 and class 4 compounds are primarily eliminated into the urine and bile in an unchanged form, whereas class 1 and class 2 compounds are eliminated primarily via metabolism (Wu and Benet, 2005). This assignment is inconsistent with observations in the animal experiments, wherein Rh2 was mainly eliminated by extensive metabolism. In addition, the reasonable oral bioavailability (55%, 8 mg/kg) in dogs suggests that Rh2 can be well absorbed. Rh2 is possibly more appropriate to be classified in the Biopharmaceutics Drug Disposition Classification System class 2 compounds, designated as a poor-solubility, high-permeability drug (Wu and Benet, 2005; Benet et al., 2011). The permeability criterion used is responsible for the discrepancy because Rh2 was found to have a high intrinsic permeability (Pmbar, value up to 3 × 10^-6 cm/s) versus the apparent low permeability due to efflux by P-gp (Yang et al., 2011). As a highly permeable lipophilic compound (cLog P = 4.0), Rh2 is very likely a good substrate for P450 enzymes (Smith, 1994). This finding is consistent with the results from the animal experiments.

In the present study, the oxidative metabolism of Rh2 was systematically investigated in a complementary set of in vitro models, including liver microsomes, S9 fraction, and cytosol. This study focused mainly on the oxidative metabolism of Rh2 because there were no conjugates derived from Rh2 and its oxidative metabolites were detected in animal studies. However, a certain contribution from phase II enzymes cannot be excluded because some sulfate and glucuronate conjugates of the oxidative metabolites of 20(S)-protopanaxadiol, which is the hydrolysis product of Rh2, were detected both in vivo (Deng et al., 2009) and in vitro (Li et al., 2011). Three different concentrations (3, 10, and 50 μM) were selected to perform preliminary experiments because the actual concentration of Rh2 in the human liver was not available. The Rh2 metabolite profiles in HLM were almost the same at the three concentrations used. The final incubation concentration chosen was 10 μM to facilitate MS detection and avoid potential saturation and inhibition of drug-metabolizing enzymes at a high substrate concentration (Yao et al., 2007).

A total of 25 oxidative metabolites were detected and characterized by their mass spectral fragmentation patterns using LC-electrospray ionization MS². All oxidative modifications occurred at the dammarane aglycone moiety. The eight-carbon side chain was determined to be the metabolic soft spot of Rh2. Epoxidation of the trisubstituted carbon-carbon double bond and hydroxylation of the two vinyl methyl groups were the initial steps in Rh2 biotransformation. The former produced a 24,25-epoxide derivative M1-4 and its 24-epimer (undetectable because of the quick rearrangement), followed by rearrangement to generate 20,24-oxide metabolites M1-5 and M1-3, respectively. The latter yielded two hydroxylated metabolites M1-1 and M1-2, followed by dehydrogenation and oxygenation to generate two carboxylated derivatives M2-3 and M2-2, respectively. In addition, double-bond epoxidation followed by rearrangement to produce...
24,25-epoxide derivatives (M3-9 and its isomer) and the corresponding 20,24-oxide metabolites (M3-10 and M3-11) of M1-1 were also confirmed. M1-2 possibly undergoes biotransformations similar to those of M1-1. However, this phenomenon was not proven by the present study because of lack of reference standard. Three monoxygenated metabolites (M1-1, M1-3, and M1-5), three dioxygenated metabolites (M3-6, M3-10, and M3-11), and a metabolic intermediate (26-aldehyde-Rh2) of Rh2 were chemically synthesized. In addition, their structures were rigorously characterized by NMR experiments. The oxidative metabolic pathways of Rh2 were proposed with the aid of individual incubations of the isolated metabolites with HLM, as depicted in Fig. 10.

Both chemical inhibition studies and recombinant enzyme screening results showed that CYP3A4 was the major P450 isoform involved in the oxidative metabolism of Rh2. In addition, the same phase I metabolite profiles of Rh2 in CYP3A4 and in HLM indicated that CYP3A4 contributed to the formation of all oxidative metabolites of Rh2. Rh2 is also a good substrate of P-gp (Yang et al., 2011) and has inhibitory effects on the activities of both CYP3A4 (Yao et al., 2007) and P-gp (Zhang et al., 2010). In this context, significant drug-drug interactions such as transporter-enzyme interplay in both the intestine and liver should be taken into serious consideration when inhibitors (for P-gp, CYP3A, or both) are combined to increase the oral bioavailability of Rh2 (Benet et al., 2011).

Results from human cytosol incubations with specific chemical inhibitors clearly demonstrated that alcohol dehydrogenase plays a predominant role in the metabolic conversion of M1-1 to the corresponding carboxylic acid derivative M2-3. Meanwhile, aldehyde de-
hydrogenase mainly catalyzed the oxidation of 26-aldehyde Rh2 to form M2-3 followed by aldehyde oxidase. 26-Aldehyde-Rh2 was not detected in all of the incubation mixtures when M1-1 was used as a substrate, even when the presence of disulfiram showed an inhibition rate of 60% on M2-3 formation. These findings may be a result of the covalent binding of 26-aldehyde-Rh2 to cytosol proteins through a possible Michael addition or Schiff base formation mechanism; this is unlikely to be a significant concern because the activities of enzymes involved in the oxidation of aldehydes will be inhibited simultaneously in very rare cases.

Three thiol-containing nucleophilic compounds were chosen in performing trapping experiments to further confirm the formation of reactive metabolites. The rationale for choosing GEE is based on its ability to increase MS signal intensity compared with GSH in detecting reactive metabolites (Wen and Fitch, 2009). On the other hand, NAC was used to probe whether GSH adduct formation depends on glutathione transferases (GSTs) or on a direct chemical reaction because NAC is likely to be a poorer cofactor than GSH for GSTs, except for microsomal GST1, which is the only GST that can use NAC as a selective substrate in place of GSH (Weinander et al., 1994). The GSH conjugate M6 and GEE adduct M7 were generated after NADPH-fortified CyLM incubations with Rh2 in the presence of GSH and GEE, respectively. By contrast, none of the NAC conjugates were found in the trapping experiments under the same incubation conditions as those of GSH and GEE. In addition, the GSH-supplemented CyLM incubations with purified M1-4 also led to the formation of M6, proving that M1-4 is the precursor of M6. It was anticipated that the carbons of epoxides in M1-4 would react with the sulfur atom of the hydrosulfide group in both GSH and GEE via nucleophilic addition reaction in the presence of GSTs. Unfortunately, the limited quantity of the resultant conjugates caused difficulty in performing NMR experiments to determine the conjugation site. It was proposed that the sulfur atom is linked to the C-24 of Rh2 in M6 and M7 mainly on the basis of steric hindrance of considerations (Chen et al., 2006). Furthermore, inconsistencies observed between the present in vitro trapping experiments and the in vivo animal data (Deng et al., 2009) strongly suggested that the bioactivation potential of Rh2 in humans can be underestimated when it is only based on in vitro data.

In conclusion, Rh2 is a good substrate of CYP 3A4 and can undergo sequential oxidative metabolism under CYP3A4 catalysis. Double-bond epoxidation followed by rearrangement and vinyl-methyl group hydroxylation represent the initial metabolic pathways. The formation of reactive epoxide metabolites in humans is strongly indicated even if no GSH conjugates have been detected in the trapping studies. Monkey might be a better nonrodent safety species than dog because GSH conjugate M6 and GEE adduct M7 mainly in vivo animal data (Deng et al., 2009) Furthermore, inconsistencies observed between the present in vitro trapping experiments and the in vivo animal data (Deng et al., 2009) strongly suggested that the bioactivation potential of Rh2 in humans can be underestimated when it is only based on in vitro data.

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Authorship Contributions
Participated in research design: Li, Chen, Zhou, and Zhong.
Conducted experiments: Li and Zhou.
Contributed new reagents or analytic tools: Li, Chen, Zhou, and Zhong.
Performed data analysis: Li, Chen, Zhou, and Zhong.
Wrote or contributed to the writing of the manuscript: Li, Chen, and Zhong.