Cancer Chemother Pharmacol (2009) 64:1085–1095
DOI 10.1007/s00280-009-0965-1

ORIGINAL ARTICLE

Pre-clinical evaluation of Rh2 in PC-3 human xenograft model for prostate cancer in vivo: formulation, pharmacokinetics, biodistribution and efficacy

Alain G. Musende · Andy Eberding · Catherine Wood · Hans Adomat · Ladan Fazli · Antonio Hurtado-Coll · William Jia · Marcel B. Bally · Emma Tomlinson Guns

Received: 5 September 2008 / Accepted: 20 February 2009 / Published online: 20 March 2009

Abstract
Purpose This study assesses the pharmacokinetics, biodistribution and efficacy of ginsenoside Rh2 as a single agent administered in a novel oral dosage formulation.
Methods A novel oral dosage formulation of Rh2 has been described. Rh2 levels in blood and tissues following administration to nu/nu nude mice were determined by high performance liquid chromatography tandem mass spectroscopy. Efficacy was determined in an established PC-3 human prostate cancer model.
Results Rh2 administered at a dose of 120 mg/kg exhibited a peak plasma concentration of 19.0 ± 2.0 μg/ml. Rh2 levels were measurable in prostate and tumor tissues, with as much as 0.3% of the administered dose being detected in tumors. This formulation exhibited no measurable toxicity as judged by weight loss or changes in serum levels of aspartate aminotransferase, alanine aminotransferase, and creatinine. This dose engendered a significant delay in PC-3 tumor growth, an increase in apoptotic index, and a decrease in tumor cell proliferation.
Conclusions Rh2 is a stable compound that can be formulated for oral gavage. Pharmacokinetics studies demonstrate its ability to be absorbed following oral administration. Future studies will assess the pharmacokinetics of Rh2 when administered in combination with docetaxel.

Keywords Ginsenoside Rh2 · Prostate cancer · Pharmacokinetics · Biodistribution · Efficacy

A. G. Musende · A. Eberding · C. Wood · H. Adomat · L. Fazli · A. Hurtado-Coll · E. T. Guns (✉)
The Prostate Centre, Vancouver General Hospital, 2660 Oak Street, Vancouver, BC V6H 3Z6, Canada
e-mail: emma.guns@vch.ca

A. G. Musende
e-mail: alainmus@gmail.com

A. G. Musende · M. B. Bally
Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, BC V6T 2B5, Canada

W. Jia
Department of Surgery, University of British Columbia, 910 W. 10th Ave., Vancouver, BC V5Z 4E3, Canada

W. Jia
Brain Research Center, University of British Columbia, 2211 Westbrook Mall, Vancouver, BC V6T 2B5, Canada

M. B. Bally
Department of Advanced Therapeutics, British Columbia Cancer Agency, 675 W. 10th Ave., Vancouver, BC V5Z 1L3, Canada

M. B. Bally · E. T. Guns
Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC V6T 1Z3, Canada

E. T. Guns
Department of Urologic Sciences, University of British Columbia, 3300-950 W. 10th Ave., Vancouver, BC V5Z 4E3, Canada

Introduction

Prostate cancer is the fifth most common neoplasm worldwide, and the second most common cancer among men [1]. Surprisingly, there are really no chemotherapeutic drugs that are considered highly effective when used to treat prostate cancer patients that have relapsed and/or metastatic disease. In recent years, drug combinations involving taxanes have contributed to improvements in treatment outcomes in prostate cancer [2, 3]. In the context of complementary

medicines [4], one working assumption is that combinations of non-toxic effective phytochemicals with established chemotherapy agents could offer the potential of enhanced drug efficacy without adding to the toxicity of the conventional drug. We have a particular interest in assessing the therapeutic potential of Rh2 (Fig. 1a); a biologically active phytochemical extracted from the roots of *Panax ginseng* C.A. Meyer. Rh2 is a tri-terpenoid glycoside saponin with a chemical structure consisting of a steroid nucleus and a glucose sugar moiety. Rh2 has a 20(S)-protopanaxadiol dammarane skeleton aglycone type and has been reported to inhibit the growth of and induce anti-metastatic activity in B16 melanoma and ovarian cancer cells [5–7]. In human breast carcinoma MCF cells, Rh2 was also found to have a G1 phase-specific cell cycle arrest with associated suppression of cdk2 and cyclin E-dependent histone kinase activities, apparently as a consequence of upregulation of p21 which binds and inactivates cdk2 [8]. Recently, Wang et al. investigated possible mechanisms responsible for the antiproliferative, pro-apoptotic, and cell cycle arrest effects of Rh2 against LNCaP androgen-sensitive and PC-3 androgen-insensitive prostate cancer cells. They showed that Rh2 increased the expression of the cdk inhibitor p21 and tumor suppressor protein p53 while decreasing the levels of cdks 2, 4, and 6 [9].

We have previously shown that Rh2 and paclitaxel acted synergistically in cultured LNCaP cells [10]. In the same study, we also reported that oral treatment of LNCaP tumors by Rh2 produced a significant decrease in tumor growth which was accompanied with a significant decrease in serum prostate specific antigen (PSA) when combined with intravenously administered paclitaxel [10]. The present study is the first in a series of investigations exploring the pre-clinical development and efficacy testing of Rh2 in PC-3, an androgen-insensitive model for prostate cancer. Rh2 is thus being evaluated as a potential agent for use in combination with docetaxel for treatment of locally advanced and metastatic prostate cancer. Docetaxel and Rh2 are administered intravenously and orally, respectively. Initially, the solubility and stability of Rh2 in ethanol was determined. The ethanol solubilized drug was formulated for oral gavage with propylene glycol and water. A high performance liquid chromatography tandem mass spectroscopy (LC/MS) method was then developed and validated for the quantitation of Rh2. This assay was used to determine the pharmacokinetic and biodistribution of Rh2 following oral administration to nude mice. The dose used for these preliminary pharmacokinetic studies was determined to be well tolerated and effective when used to treat animals bearing established PC-3 tumors. Serum creatinine levels, as a measure of renal toxicity, were measured using a published HPLC method [11]. Liver toxicity was assessed by measuring serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels [12]. Antitumor activity was associated with an increase in apoptotic cells and a decrease in the proliferation marker Ki-67 [13].

Materials and methods

Test compounds and materials

Ginsenoside Rh2 (M.W. 640.89 g/mol), as a white powder, was purchased from LKT Laboratories Inc., St. Paul, MN (USA) and generously supplied by Panagin Pharmaceuticals Inc, Richmond, BC (Canada). Taxotere® (docetaxel) was purchased from BC Cancer Agency Pharmacy as a 40 mg/ml solution in polysorbate 80, manufactured by Aventis Pharma Inc. Saint-Laurent, Québec (Canada). Cholic acid (M.W. 408.58 g/mol) and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

LC/MS analysis of Rh2

Instrumentation and conditions

The HPLC system consisted of an integrated Waters Alliance LC coupled to a Waters Quattro Micro. Rh2 and cholic acid (internal standard) were resolved on a Waters

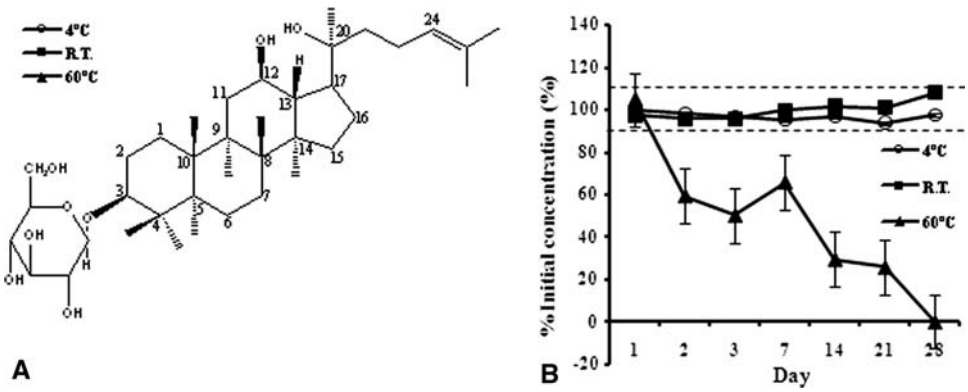

Fig. 1 (**a**) Chemical structure of ginsenoside Rh2, a 20(S)-protopanaxadiol dammarane skeleton aglycone type. (**b**) Stability of Rh2 in 100% ethanol for 28 days. Conditions are 4°C (*empty circles*), room temperature (R.T., *filled squares*), and 60°C (*filled triangles*), $n = 3$

Exterra C18 column (3.5 μm, 50 mm × 2.1 mm) with Mobile Phases A, B, C, and D; H_2O, methanol, acetonitrile, and 10% acetic acid, respectively. The following gradient profile was used: $t = 0$–0.5 min, 40% A, 50% B, 10% D; $t =$ 6–8.5 min, 50% B, 40% C, 10% D, $t = 9$–13 min, 50% B, 50% C; $t = 13.5$–20 min, 40% A, 50% B, 10% D with a flow rate of 0.3 ml min^{-1}. The Quattro Micro detector settings were as follows: capillary: 3.5 kV, desolvation temperature: 300°C, source temperature: 120°C, cone temperature: 20°C, desolvation and cone gas flows: 400 and 20 l/h, respectively, collision cell pressure at ~3e–3 mBar. Data were collected in ES- mode using Multiple Reaction Monitoring (MRM). MRM's of *m/z* 681 > 59 and 681 > 621 were selected for Rh2 with cone and collision energies of 40 V and 22 V, respectively. *m/z* of 407 > 289 was selected for cholic acid with cone and collision energies of 75 V and 40 V, respectively. The retention times were 7.1 and 9.3 min for cholic acid and Rh2, respectively. The 681 ion is the deprotonated Rh2/acetic acid cluster while the 407 is the deprotonated cholic acid. MassLynx version 4.0 (Waters/Micromass) was used to perform peak integration.

Assay validation

Primary standard solutions of Rh2 were prepared in 100% ethanol and spiked with cholic acid (internal standard). Secondary standard solutions were obtained by spiking 120 μl of 95% methanol with 10 μl of the primary standard solution to yield nominal concentrations over a range of 0.01–20.0 μg/ml. All standard solutions were stored at 4°C.

Linearity and range

Five concentrations of Rh2 standard solutions were analyzed ($n = 3$): 0.5, 2.0, 5.0, 10.0, and 20.0 μg/ml. The minimum acceptable correlation coefficient to establish linearity was set at 0.99. The detector response was correlated against analyte concentration by least-squares regression.

Precision and accuracy

Three concentrations of Rh2 standard solutions were analyzed ($n = 6$) over 3 days: 0.5, 2.0, and 10.0 μg/ml. The mean, standard deviation (SD), coefficient of variation (CV), and relative error (RE) were determined for the six replicate runs.

Limit of detection (LOD) and limit of quantitation (LOQ) of Rh2

Stock solutions of Rh2 were prepared in triplicate at 12 concentrations: 0.001, 0.02, 0.05, 0.5, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, and 500.0 μg/ml. We defined the LOD as the minimum concentration that could be detected based on a signal-to-noise (S/N) ratio of 3 to 1. The LOQ was the minimum concentration that could be quantified based on S/N ratio of 10 to 1.

Solubility

Standard solutions of Rh2 in ethanol were prepared between 1 and 10 μM (1, 2.5, 5, 7.5, and 10 μM) and analyzed by HPLC. A calibration curve of peak area versus concentration was generated and a linear relationship shown. Three sets of saturated solutions of Rh2 were then prepared at room temperature by incubating an excess of the compound in 2 ml of 100% ethanol in capped test tubes. The mixture was vigorously mixed and stored at room temperature for 30 min; this time was determined to be sufficient for insoluble materials to settle from the mixture. The clear supernatant was sequentially diluted and the amount of Rh2 in the diluted solutions quantified by HPLC.

Stability of samples at 4°C, room temperature, and 60°C

Rh2 (232 mg) was dissolved in 1.8 ml of 100% ethanol, a concentration based on the maximum solubility of 130 mg/ml determined using the methods described above. The mixture was mixed vigorously and 200 μl pipetted into each of nine eppendorf tubes. One set of three eppendorf tubes was stored at 4°C ($n = 3$), the second set of tubes ($n = 3$) at room temperature, and the last set ($n = 3$) at 60°C. The concentration of Rh2 in these solutions was determined by HPLC on day 1, 2, 3, 7, 14, 21 and 28.

Oral gavage formulation and stability at room temperature

232 mg of Rh2 was dissolved in 1.8 ml of 100% ethanol and mixed vigorously (based on maximum solubility of 130 mg/ml) and stored at 4°C. Prior to administration to mice, 700 μl of ddH_2O was mixed (by vortexing) with 5.5 ml of propylene glycol in a separate container. The propylene glycol–water mixture was then mixed with the Rh2-containing ethanol solution. 500 μl was pipetted into each of twelve eppendorf tubes all stored at room temperature. Each set of three eppendorf tubes was designated for the determination of the concentration of Rh2 by HPLC at a specific time point: 0 (immediately following gavage preparation, $n = 3$), then, 4, 20, and 24 h following gavage preparation ($n = 3$).

Pharmacokinetic and biodistribution studies

PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6–8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25–31 g. After 14 days, the developing tumors were measured and mice randomly assigned in different treatment groups based on their weight. Ten groups of three mice were dosed by oral gavage with Rh2 at 120 mg/kg (103–128 μl) (highest achievable dose, limited due to gavage volume limitations (150 μl) imposed by the institutional animal care committee) or the vehicle control at an equivalent volume based on weight. Mice were terminated using CO_2 asphyxiation upon which blood (obtained by cardiac puncture) and major tissues (stomach, small intestine, liver, kidney, spleen, brain, and lung) were promptly collected at 30, 60, 120, and 200 min after administration. Typically, 500–700 μl of blood was obtained and then placed into a Plasma Separator tube (Microtainer®, Becton Dickinson, NJ, USA), mixed, and placed on ice. The prostate and tumors were then collected at 10, 20, 30, 45, 60, 90, 120, 150, and 200 min time points. Tissues were collected and flash frozen, prior to placing the samples in storage at –80°C. To assess Rh2 extraction efficiencies, blank plasma and tissue homogenate samples (obtained from vehicle treated mice) were spiked with Rh2 and cholic acid to achieve three concentrations (0.5, 2.0, and 10.0 μg/ml). The spiked samples were subsequently extracted and analyzed using the HPLC method described above.

Plasma: solid-phase sample preparation

C18 Sep-Pak extraction columns mounted on a vacuum extraction manifold (Waters, Massachusetts, USA) were first equilibrated with 1 ml of 100% methanol and 1 ml of ddH_2O. After loading with 200 μl of plasma spiked with 10 μl of cholic acid (internal standard), each column was subsequently washed with 1 ml of ddH_2O followed by 1 ml of 30% methanol. The column was then eluted twice with 1 ml of 100% methanol into eppendorf tubes, and the eluate was dried in a speed-vac (Labconco Centrivap) at 35°C. The residue was reconstituted with 100 μl of 100% methanol, mixed vigorously, sonicated and transferred into HPLC auto-sampler vials. A non-compartmental method using the nonlinear least squares regression program WinNonlin (Scientific Consulting Inc., Cary, NC, USA) was used to analyze the plasma concentration data at four time points: 30, 60, 120, and 200 min. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinity. Pharmacokinetic parameters were generated including the terminal half-life ($t_{1/2}$) ($n = 3$), the systemic clearance (Cl), the peak plasma concentration (C_{max}), and the time to reach C_{max} following oral administration (T_{max}).

Tissues

Tissue samples were ultrasonicated in 4 × w/v of 0.01 M pH 7.8 phosphate buffer. 200 μl of the homogenate was spiked with cholic acid (internal standard) and mixed for 1 min. Ice cold acetonitrile (800 μl) was added to the homogenate and mixed for 1 min for protein precipitation. The sample was then centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was dried at 35°C, the residue was reconstituted in 200 μl of 100% methanol, vortexed and sonicated, and injected onto the HPLC column. Standard calibration curves were analyzed at three concentrations ($n = 3$): 0.5, 2.0, and 10.0 μg/ml.

Efficacy and toxicity of Rh2

PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6–8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25–31 g. After 14 days, the developing tumors were measured and mice randomly assigned in different treatment groups based on tumor size. $n = 8$ for the Rh2 and docetaxel groups while $n = 6$ for the oral gavage control and saline groups. A caliper was used to measure the three perpendicular axes of each tumor. The formula $V = (L \times W \times H)\frac{\pi}{6}$ where L is the length, W the width, and H the height, was used to calculate the tumor volume. Mice bearing measurable tumors qualified for the study and the average tumor volume when treatment was initiated was 120 mm^3. Mice were weighed 5 days a week and tumors measured twice a week for 25 days. Four different treatment groups were defined: (i) Rh2 solubilized in ethanol:propylene glycol:water (2:7:1 ratio) was administered by oral gavage at a dose of 120 mg/kg (103–128 μl volume range) using a QD × 5 schedule each week for 4 weeks; (ii) vehicle control consisting of ethanol:propylene glycol:water (2:7:1 ratio) given at an equivalent dose of 120 mg/kg (103–128 μl volume range) using a QD × 5 schedule each week for 4 weeks; (iii) docetaxel, used as a positive treatment control, was administered intravenously via the lateral tail vein at a dose of 20 mg/kg (100–124 μl volume range) using a Q7D × 4 dose schedule; and (iv) saline (100–124 μl volume range) given Q7D × 4. The mice were monitored daily for changes in weight and other signs of acute toxicity. Serum creatinine levels were determined using an HPLC system consisting of a Waters 2,695 separations module paired with a Waters 996 photodiode array detector (Waters Corp., Milford, MA). Creatinine was resolved on a 3.0 μm bore diameter 2.1 × 50 mm Waters Atlantis™ HILIC Silica column with a Mobile Phase consisting of

95% acetonitrile solution containing 0.025% ammonium formate. Serum ALT and AST were quantified using standard ALT and AST kinetic assay kits (Stanbio Laboratory, Boerne, Texas) adapted in 96-well plates. For serum creatinine, ALT and AST level determination, Rh2 treated mice were compared to the control and the untreated groups. We have previously published this method [10, 14, 15] and other investigators have used a similar approach [16–21].

Analysis of apoptosis and proliferation markers

At the end of the efficacy studies mice were sacrificed and tumors were excised, formalin-fixed, and then paraffin-embedded. A Tissue Micro-Array (TMA) was constructed by extracting four 600-μm diameter cores of tumor tissue from each paraffin block using a Beecher Instruments tissue core extractor and re-embedding these cores into a gridded paraffin block. After construction, 4-μm tissue sections were cut and adhered to Fisher SuperFrost Plus glass slides. Apoptotic cells were then visualized by terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining which was carried out using the ApopTag® Peroxidase Kit. Tissues were pretreated with proteinase K for 15 min. Subsequently, TdT enzyme was applied and the tissue incubated in a humid chamber at 37°C for 1 h. Anti-digoxigenin peroxidase conjugate was later applied for 30 min, followed by DAB, and counterstain with hematoxylin, as well as Staining Blueing Reagent. For Ki-67, a Mouse-To-Mouse immunohistochemical detection kit (Chemicon®) was used to decrease nonspecific staining. Ki-67 (Dako, Carpinteria, California) (1:200) monoclonal antibodies were used in combination with immunoperoxidase procedures (LSAB + peroxidase kit). Antigen retrial was applied by steaming with citrate buffer for 30 min. The TMA slides were imaged digitally and evaluated by visual scoring of apoptotic and Ki-67 positive cells. Scores from individual tumor cores with significant necrosis were omitted. Scoring was completed by two pathologists blinded to the study groups.

Statistical analysis

For each studied variable, mean and standard error of the mean (SEM) were calculated. Statistical significance and differences between the described treatment groups were assessed using the Tukey-Kramer/Student t tests from within One-way Analysis of Variance (ANOVA) analysis. For these tests, the level of significance was set at a p value of at least <0.05. All mean values were reported as mean ± SEM.

Results

Assay validation

The estimated LOD was 2.0 ng/ml while the LOQ that can be reliably and reproducibly measured was estimated to be 5.0 ng/ml. Standard curves for Rh2 showed linearity over the selected concentration range (0.01–20.0 μg/ml). The correlation coefficients were >0.99 throughout the validation procedure. Inter-day precision and accuracy as, expressed by the RSD and the CV and RE, were less than 10%.

Solubility, stability and formulation in the ethanol–propylene glycol–water ternary solvent system

In order to formulate Rh2 for oral gavage, a strategy based on the use of the ethanol–propylene glycol–water ternary solvent system was developed. The first step in this process was to assess the solubility of Rh2 in 100% ethanol at room temperature. The maximum solubility of Rh2, as determined by LC/MS analysis of the supernatant of saturated solutions, was 130.0 ± 2.3 mg/ml. Ethanol solutions of Rh2 were stored at 4°C, room temperature and 60°C over a time frame of 1 month to assess Rh2 stability. The results, summarized in Fig. 1b, suggest that Rh2 is stable at 4°C and room temperature. LC/MS analysis of Rh2 indicated that the drug concentration did not change over the 4 week time period. Rh2 underwent rapid thermal degradation, measured as a loss of intact drug, at 60°C. An approximate decrease of 40% in parent drug concentration was observed within 2 days and the drug was not detectable by day 28 (Fig. 1b). The rate constant for the observed decrease in concentration at 60°C, k (60°C), is 0.14 and the representative compound half-life, $t_{1/2}$ (60°C) was estimated to be 12.5 days. A concentration change of ±15% is generally considered acceptable in stability assays [22], therefore, it was concluded that ethanol solubilized drug solutions could be prepared and stored at 4°C for at least a time frame appropriate for the toxicity and efficacy assessments summarized below. Using the ethanol–propylene glycol–water ternary solvent system diagram designed by Sorby et al. [23], the dielectric constant of the ethanol, propylene glycol and water mixture combined at the 2:7:1 v/v ratio was estimated to be approximately 31. The maximum solubility of Rh2 was achieved at this 2:7:1 v/v ratio. The concentration of Rh2 in the ethanol–propylene glycol–water gavage mixture remained constant over a 24 h period at room temperature, with a relative standard deviation (RSD) of <10% (data not shown). Rh2 formulated in the ethanol–propylene glycol–water mixture was always freshly prepared and administered to animals within 4 h.

Pharmacokinetics and biodistribution

Extraction efficiencies for Rh2 from mouse plasma and tissues at three different concentrations (0.5, 2.0, and 10.0 μg/ml) were determined as described in "Methods" and "Results" summarized in Table 1a. The extraction efficiency from plasma was 65.2 ± 2.4% and this was not influenced by the concentration of Rh2 used in these assays. Extraction efficiencies from tissues varied from 79.6 ± 3.2% in stomach to essentially 100% in the kidney. Tissue standard calibration curves exhibited excellent linearity (correlation factors of ≥0.99) (data not shown).

Within 30 min after administration, Rh2 levels in the plasma peaked at 19 ± 2.0 μg/ml and after 200 min were below the LOQ. Using these data, the pharmacokinetic parameters for Rh2 were estimated and summarized in Table 1b. The half-life ($t_{1/2}$) was 99.6 ± 6.6 min., the clearance (Cl_{oral}) was 46.5 ± 2.7 ml/min/kg while T_{max} was 30.0 min and the AUC was 1884.3 ± 209.2 μg min/ml.

Tissue distribution data have been summarized in Fig. 2. As expected, given the route of administration, Rh2 levels appeared to be highest in the small intestines, with approximately 54% of the intact drug recovered in the small intestines 30 min after the gavage was administered. Approximately 27% of the administered Rh2 dose is found in the liver at the same time point. Rh2 was not quantifiable in the brain at any of the selected time points and most tissues exhibited Rh2 levels below the LOD 200 min after administration. Approximately 0.08% of the administered dose was found in the prostate after 120 min and the Rh2 levels in this tissue remained quantifiable 200 min after administration (Fig. 2e). The percent of administered dose localizing in the PC-3 tumor tissue was 0.3% 90 min following administration (Fig. 2f). Since we lacked a suitable intravenous formulation we could not determine the absolute bioavailability of this formulation, however, the results provided here clearly suggest that Rh2 is bioavailable after oral gavage.

In vivo efficacy and toxicity

The therapeutic activity of Rh2 was determined using mice bearing tumors established following subcutaneous injection of PC-3 human prostate cancer cells. This study included negative control groups (saline and the vehicle control) as well as a positive treatment control (docetaxel administered Q7D × 4 at an established therapeutic dose (maximum tolerated dose (MTD)). The results summarized in Fig. 3a clearly demonstrate that Rh2 was effective in inhibiting PC-3 tumor growth. At the end of the study (39 days after tumor cell inoculation, 25 days after treatment was initiated), the average tumor volume for saline treated and vehicle control treated animals was approximately three times the size of the average tumor volume determined at the time treatment was initiated. For animals treated with Rh2 the tumor volume did not change during this time course. Rh2 treatment group was statistically different from groups treated with the vehicle control ($p = 0.012$) and saline ($p = 0.008$). Lack of tumor progression in animals treated with Rh2 is consistent with the belief that this ginsenoside exerts cytostatic effects rather than cytotoxic effects. In comparison, docetaxel (20 mg/kg Q7D × 4) caused established PC-3 tumors to regress. By day 39, tumors in docetaxel treated animals were still measurable but were 1/4 the size they were when treatment was initiated. On day 39, there was no statistically

Table 1 (a) Average extraction efficiencies of Rh2 in plasma and tissues, (b) pharmacokinetic parameters for Rh2 following oral administration of 120 mg/kg formulated in the ethanol–propylene glycol–water ternary solvent system described in "Methods"

(a)

Plasma (%)	Stomach (%)	Small intestine (%)	Liver (%)	Lung (%)	Spleen (%)	Kidney (%)	Brain (%)	Prostate (%)	Tumour (%)
65.2 ± 2.4	79.6 ± 3.2	99.0 ± 10.7	92.9 ± 2.6	90.3 ± 10.0	96.5 ± 2.7	111.5 ± 7.1	108.0 ± 5.9	86.2 ± 3.1	93.4 ± 3.7

(b)

Body weight (g)	$t_{1/2}$ (min)	C_{max} (μg/ml)	T_{max} (min)	Cl_{oral} (ml/min/kg)	AUC (μg.min/ml)
25.8 ± 1.3	99.6 ± 6.6	19.0 ± 2.0	30.0 ± 0.0	46.5 ± 2.7	1884.3 ± 209.2

Extraction standards were completed using three concentrations (0.5, 2.0, and 5.0 μg/ml) of Rh2 and each assay was determined in triplicate. Since $n = 3$ per time point, three values were obtained for each parameter listed in the table. Values are reported as mean ± SEM

$t_{1/2}$: Half-life

C_{max}: Peak concentration

T_{max}: Time to peak concentration

Cl_{oral}: Apparent oral clearance

AUC: Area under the plasma concentration versus time curve

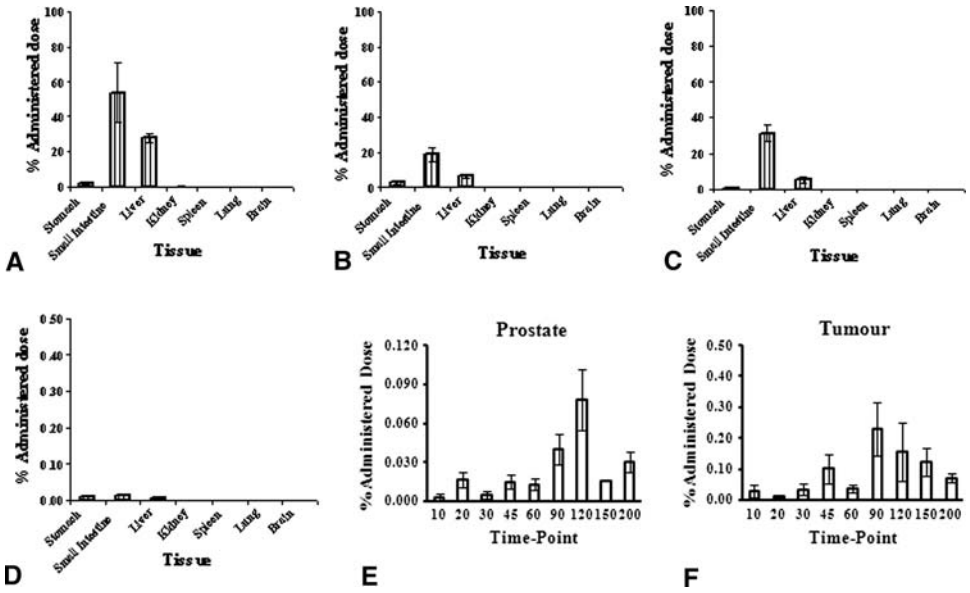

Fig. 2 Percentage of administered dose of Rh2 in specified tissues isolated from mice following oral administration of 120 mg/kg. **a** 30 min **b** 60 min **c** 120 min **d** 200 min. Percentage of administered dose of Rh2 in the prostate (**e**) and tumors (**f**) isolated from mice 10, 20, 30, 45, 60, 90, 120, 150, and 200 min following oral administration of 120 mg/kg. Data were obtained from three animals per time point and results are expressed as mean values ± SEM

significant difference in tumor size in mice treated with Rh2 or docetaxel ($p = 0.179$).

It should be noted that docetaxel administration was associated with almost 20% body weight loss (Fig. 3b, filled squares), and this significant ($p = 0.025$) weight loss is consistent with what would be expected for docetaxel administered at its MTD. In comparison, treatment with Rh2 (Fig. 3b, open triangles) resulted in <8% loss in mean body weight. This toxicity could be attributed to the repeated animal handling required for daily gavages since the vehicle alone (filled circle) engendered similar, albeit not significant, reductions in mean body weight. Interestingly ALT, AST, and ALT/AST levels in the plasma of Rh2 treated animals were significantly lower than those found in plasma of the untreated group ($p = 0.0001$, 0.0149, and 0.0001, respectively) (Table 2). There was no statistical difference in serum creatinine levels between the Rh2 treated animals when compared to the vehicle control ($p = 0.5150$) or untreated ($p = 0.3247$) groups (Table 2). No statistically significant difference in serum creatinine level was found between the control and untreated groups ($p = 0.0869$). In summary, treatment with Rh2 was achieved at a dose which was well tolerated by the animals.

As other measures of therapeutic activity, PC-3 tumors were isolated from mice at the end of the study and prepared for immunohistochemical assessments of apoptosis and inhibition of Ki-67 labeling. The results, summarized in Fig. 3d and e, indicate that tumors from mice treated with docetaxel exhibited the highest apoptotic index. The average apoptotic index for the control was 9.30%. This increased to 14.4% in tumors from animals treated with Rh2 and 22.6% in tumors from animals treated with docetaxel. The apoptotic index observed in the tumors from mice treated with docetaxel was significantly greater than the control ($p = 0.022$) but not significantly different than that observed in tumors from Rh2 treated animals ($p = 0.127$). The apoptotic index in tumors from animals treated with Rh2 were greater than those observed for controls, but this difference was not significantly different ($p = 0.079$) (Fig. 3d). Ki-67 positive cells in tumors from mice treated with Rh2 were lower than that determined for the vehicle control or those tumors derived from docetaxel treated animals. Of the Rh2 tumors, 6.1% were Ki-67 positive whereas the control group exhibited a significantly higher level of 23.4% positive staining ($p = 0.003$) and tumors from the docetaxel treated groups exhibited 14.4% ($p = 0.014$) positive staining (Fig. 3e).

Discussion

We have described a novel oral dosage formulation for ginsenoside Rh2. Pharmacokinetic analysis of Rh2 indicates that Rh2 is absorbed when administered by oral gavage. At the highest achievable dose (120 mg/kg), Rh2 proved to be well tolerated in the nude mouse model and the systemic blood levels achieved were sufficient to prevent progression of established PC-3 tumors. Biodistribution data indicated that intact Rh2 could be isolated from prostate tissue and established PC-3 tumors following oral administration. In

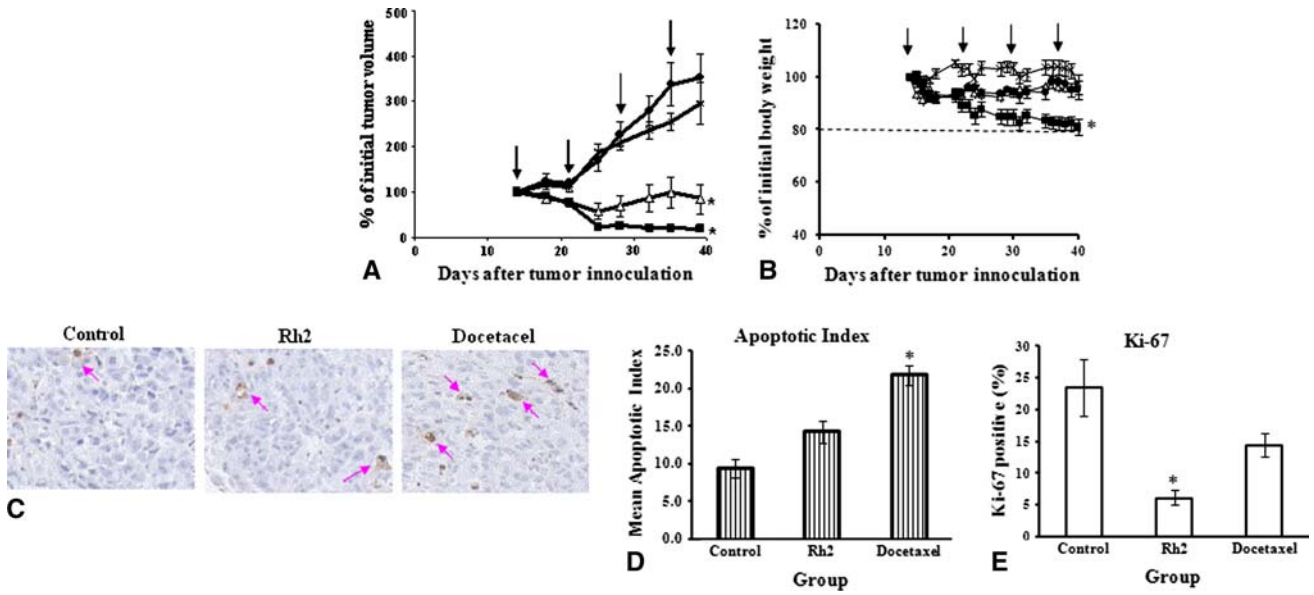

Fig. 3 **a** In vivo efficacy of Rh2 in PC-3 bearing nude mice. Changes in tumor volume were followed over time for animals treated with the oral gavage vehicle control (ethanol, propylene glycol and water, *filled circle*), saline (*x*), Rh2 (120 mg/kg QD × 5, *empty triangle*), and docetaxel (20 mg/kg Q7D × 4, *filled square*). Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post PC-3 cells inoculation. Volumes were calculated using $V = (L \times W \times H)/(\pi/6)$. Mean value ± SEM is shown with error bars. A statistically significant difference was found between Rh2 and the control ($p = 0.0122$) and saline ($p = 0.0081$) on day 39. No statistical difference between Rh2 and docetaxel ($p = 0.1787$). $n = 8$ for Rh2 and docetaxel. $n = 6$ for the oral gavage control and saline. **b** In vivo toxicity as assessed by decreases in mean body weight. Body weight loss of >20% is considered severe enough to warrant termination of the animals. No animals showed any signs of toxicity other then weight loss in these studies. Docetaxel showed a significant weight loss compared to saline ($p = 0.0246$). $n = 8$ for Rh2 and docetaxel. $n = 6$ for the oral gavage control and saline. **c** Representative tissue microarray spots for PC-3 tumors: immunostained using a TUNEL assay by enzymatically labeling the free 3′-OH terminal generated on DNA fragments of 180–200 bp. Using this method, apoptotic cells could be identified in areas of viable tissue, as indicated by the *arrows*. **d** Apoptotic index summarized from ApopTag® staining of tissue microarrays. **e** Summarized Ki-67 staining of tissue microarrays. Mean scores were determined as a percentage of the total number of cells. Four cores of tissue were extracted per tumor and one tumor per animal. *n* (total number of cores) = 24 for the control and saline and 32 for Rh2 and docetaxel. Mean value ± SEM is shown with error bars. *Signifies statistically significant difference from the control

Table 2 Secondary measures of toxicity in mice serum ($n = 4$)

Group	ALT (alanine aminotransferase) (U/L)	AST (aspartate aminotransferase) (U/L)	ALT/AST	Creatinine (μg/ml)
Rh2	19.57 ± 0.2*	48.39 ± 0.4**	0.36 ± 0.003*	1.11 ± 0.14***
Control	23.09 ± 0.2	54.60 ± 0.3	0.48 ± 0.005	1.21 ± 0.07
Untreated	24.63 ± 0.2	52.06 ± 0.6	0.47 ± 0.006	0.84 ± 0.22

Values are reported as mean ± SEM

U/L: Units per liter

P values—Rh2 compared with the untreated group

* $P = 0.0001$

** $P = 0.0149$

*** $P = 0.3247$

aggregate, the results presented here provide strong support for our efforts to develop Rh2 for clinical evaluations and we are particularly interested in the potential of using this agent in combination with docetaxel for treatment of relapsed and hormone insensitive metastatic prostate cancer.

As noted in Fig. 1b, accelerated stability studies with Rh2 in ethanol at three temperatures suggested that Rh2 could be stored at 4°C or room temperature for at least 1 month without any significant change in concentration. Using the ethanol–propylene glycol–water ternary solvent system diagram designed by Sorby et al. [23], the dielectric constant of the formulated Rh2 oral gavage mixture was estimated to be 31 (24.6–30.0 and 80.1 for ethanol and water, respectively [24, 25]), ideal for the solubility of Rh2.

The concentration of Rh2 in the formulated mixture was stable for at least 24 h at room temperature.

As summarized earlier, the LOD was estimated to be 2.0 ng/ml while the LOQ value was 5.0 ng/ml. Xie et al. estimated that rat plasma concentrations of Rh2 could be reliably quantitated between 2 and 100 ng/ml [26]. We have since shown that the method developed here can reliably be used for the determination of Rh2 in mouse plasma and tissues, including prostate and tumor tissues (correlation factors of $\geq$0.99 for tissue calibration). Since we lacked a suitable intravenous formulation we could not determine the absolute bioavailability of this formulation, however, the results provided here clearly suggest that Rh2 is bioavailable after oral gavage. These results are consistent with a previous study of ginsenoside K (one glucose moiety at C-20 instead of C-3), a compound of similar structure to Rh2. Following a 20 mg/kg oral administration of ginsenoside K in rats, the T_{max} was reached at 30 min (comparable to the data presented here for Rh2) while the AUC obtained was 341.0 (μg min/ml) [27]. The Rh2 AUC determined in mice (Table 1b) was six times that value, however, the dose administered here was six times greater than that used by Paek et al. Thirty minutes following administration, <2% of the administered dose could be found in the stomach, with 54% and 27% in the small intestine and the liver, respectively (Fig. 2a). Considering that Rh2 is being investigated for the treatment of prostate cancer, mouse prostates and tumors were harvested at more frequent intervals, compared to the other tissues. Approximately 0.08% of the administered dose could be isolated from prostate tissue 120 min after administration (Fig. 2e). Similarly, in the subcutaneous PC-3 tumors, approximately 0.3% of the administered dose could be isolated from the tumor 90 min after administration (Fig. 2f). The biodistribution data presented here are consistent with the conclusion that Rh2 administered orally in the ethanol, propylene glycol and water formulation is readily absorbed and accesses key target tissues in an intact, unmetabolized form. Paek et al. determined the transport rate of ginsenoside K in Caco-2 cell monolayers to be 3–6 $\times$ 10^{-6} cm/s and concluded that it should be well absorbed by the intestine [28]. The biodistribution data summarized in Fig. 2 is consistent with the ginsenoside K results. In fact, Hasegawa et al. [29] showed that ginsenoside K also accumulated in the liver following oral administration. They concluded that this accumulation was due, in part, to the presence of hepatocyte receptors capable of recognizing and binding the associated glucose moiety.

It should be noted that others have shown [30] and suggested [31] that Rh2 could be metabolized through deglycosylation to aPPD, its aglycone derivative. Secondary metabolites of ginsenosides such as Rh2 have also been suspected of forming in the liver and being responsible for the observed pharmacological activities [29]. Importantly, the LC/MS method developed here could also detect aPPD (results not shown) yet there was no evidence of aPPD in the plasma or tissues extracted from Rh2 treated mice. We have demonstrated the presence of intact, unmetabolized Rh2 in the blood and every harvested tissue examined. Future investigation might involve detection and quantitation of possible esterified secondary metabolites from the liver of Rh2 treated mice.

Importantly, the formulation developed here was well tolerated. The mice monitored in our study showed no acute signs of toxicity as determined by body weight loss (Fig. 3b) and this observation is consistent with other studies [32] that suggest that ginsenosides in general, and Rh2 in particular, cause negligible toxicity, even at the maximal achievable dose. As shown in Table 2, secondary measures of liver toxicity also suggest the drug is well tolerated. Serum ALT and AST activity following Rh2 administration were not increased. Serum ALT and AST values for all three groups were within the expected range for normal mice (ALT $\leq$ 50 U/l and AST $\leq$ 150 U/l) [33]. Serum creatinine levels were determined as a measure of kidney toxicity and the results suggest that there was no statistical difference in serum creatinine levels between the Rh2 and the control ($p = 0.5150$) or untreated group ($p = 0.3247$) (Table 2). The untreated group was also not statistically different from the control group ($p = 0.0869$). Despite the apparent difference in serum creatinine level between the untreated and control group, values for all three groups were within the expected range for normal mice (0.05–0.15 mg/dl) [11], suggesting no significant toxic effects of Rh2 on this organ.

Consistent with previous findings [6], Rh2 appears to exhibit therapeutic activity when used to treat established tumors derived following subcutaneous injection of PC-3 cells (Fig. 3a) and the results suggest that the activity, albeit lower, was comparable to that achieved when treating mice with docetaxel administered at its MTD. Nakata et al. demonstrated that a daily oral administration of Rh2 in nude mice bearing HRA human ovarian cancer xenograft caused an inhibitory effect comparable to a weekly administration of 4 mg/kg cisplatin [6]. Rh2 treated mice showed no signs of toxicity while nearly half of cisplatin-treated mice had to be terminated due to acute body weight loss. Interestingly, Nakata et al. attributed the therapeutic effects of Rh2 to enhanced NK activity, however, our work was completed in immunocompromised mice and this suggests that additional mechanisms must contribute to the compounds therapeutic effects [6]. To better understand the nature of Rh2 mediated therapeutic effects, PC-3 tumors were isolated at the end of the treatment phase of the study and measures of apoptosis (TUNEL staining) and proliferation (Ki-67) were made. The apoptotic index of the

docetaxel group was significantly higher than the control ($p = 0.022$) while the value determined in tumors from animals treated with Rh2 group were 50% greater than that seen for control tumors, this difference was not statistically significant ($p = 0.079$) (Fig. 3d). Others have shown that ginsenosides are strong promoters of apoptosis [6, 8, 34–37]. The fact that we did not observe significant increases in the apoptotic index may be related to the time point used in our study. We demonstrated that Rh2 significantly inhibited cell proliferation as measured by Ki-67 labeling (Fig. 3e) and the extent of suppression was significantly lower than what was observed in tumors isolated from animals treated with docetaxel.

In summary, the formulation that we describe here is well tolerated and effective when used to treat established PC-3 tumors. Pharmacokinetic and biodistribution studies suggest that Rh2 is well absorbed. Future studies will assess the activity of this ginsenoside when used in combination with conventional cytotoxic/cytostatic drugs, in particular docetaxel, in vitro and in vivo.

Acknowledgments The authors wish to thank Panagin Pharmaceuticals Inc, Richmond, BC (Canada) for generously supplying Rh2. We also would like to recognize the staff of the Jack Bell animal resource center for providing outstanding care for the animals used in these studies. This research was supported in part by grants from the Canadian Institutes of Health Research (MBB) and Health Canada (EG).

Conflict of interest statement The authors declare that they have no competing financial interests.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55:74–108
2. De Wit R (2008) Chemotherapy in hormone-refractory prostate cancer. BJU Int 101(Suppl 2):5–11
3. Oh WK (2003) The evolving role of chemotherapy and other systemic therapies for managing localized prostate cancer. J Urol 170:S28–S32 discussion S33-4
4. Richardson MA, Sanders T, Palmer JL, Greisinger A, Singletary SE (2000) Complementary and alternative medicine use in a comprehensive cancer center and the implications for oncology. J Clin Oncol 18:2505–2514
5. Jia WW, Bu X, Philips D, Yan H, Liu G, Chen X, Bush JA, Li G (2004) Rh2, a compound extracted from ginseng, hypersensitizes multidrug-resistant tumor cells to chemotherapy. Can J Physiol Pharmacol 82:431–437
6. Nakata H, Kikuchi Y, Tode T, Hirata J, Kita T, Ishii K, Kudoh K, Nagata I, Shinomiya N (1998) Inhibitory effects of ginsenoside Rh2 on tumor growth in nude mice bearing human ovarian cancer cells. Jpn J Cancer Res 89:733–740
7. Wakabayashi C, Hasegawa H, Murata J, Saiki I (1997) In vivo antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. Oncol Res 9:411–417
8. Oh M, Choi YH, Choi S, Chung H, Kim K, Kim SI, Kim DK, Kim ND (1999) Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. Int J Oncol 14:869–875
9. Wang W, Wang H, Rayburn ER, Zhao Y, Hill DL, Zhang R (2008) 20(S)-25-methoxyl-dammarane-3beta, 12beta, 20-triol, a novel natural product for prostate cancer therapy: activity in vitro and in vivo and mechanisms of action. Br J Cancer 98:792–802
10. Xie X, Eberding A, Madera C, Fazli L, Jia W, Goldenberg L, Gleave M, Guns ES (2006) Rh2 synergistically enhances paclitaxel or mitoxantrone in prostate cancer models. J Urol 175:1926–1931
11. Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K (2004) Utility of endogenous creatinine clearance as a measure of renal function in mice. Kidney Int 65:1959–1967
12. Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, Jia G, Gao Y, Li B, Sun J, Li Y, Jiao F, Zhao Y, Chai Z (2007) Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol Lett 168:176–185
13. Kameyama K, Takami H, Umemura S, Osamura YR, Wada N, Sugino K, Mimura T, Ito K (2000) PCNA and Ki-67 as prognostic markers in human parathyroid carcinomas. Ann Surg Oncol 7:301–304
14. Eberding A, Madera C, Xie S, Wood CA, Brown PN, Guns ES (2007) Evaluation of the antiproliferative effects of Essiac on in vitro and in vivo models of prostate cancer compared to paclitaxel. Nutr Cancer 58:188–196
15. Low JC, Wasan KM, Fazli L, Eberding A, Adomat H, Guns ES (2007) Assessing the therapeutic and toxicological effects of cesium chloride following administration to nude mice bearing PC-3 or LNCaP prostate cancer xenografts. Cancer Chemother Pharmacol 60:821–829
16. Shevrin DH, Gorny KI, Kukreja SC (1989) Patterns of metastasis by the human prostate cancer cell line PC-3 in athymic nude mice. Prostate 15:187–194
17. Wang Y, Corr JG, Thaler HT, Tao Y, Fair WR, Heston WD (1995) Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. J Natl Cancer Inst 87:1456–1462
18. Asgari K, Sesterhenn IA, McLeod DG, Cowan K, Moul JW, Seth P, Srivastava S (1997) Inhibition of the growth of pre-established subcutaneous tumor nodules of human prostate cancer cells by single injection of the recombinant adenovirus p53 expression vector. Int J Cancer 71:377–382
19. O'Reilly T, McSheehy PM, Wenger F, Hattenberger M, Muller M, Vaxelaire J, Altmann KH, Wartmann M (2005) Patupilone (epothilone B, EPO906) inhibits growth and metastasis of experimental prostate tumors in vivo. Prostate 65:231–240
20. Li GX, Lee HJ, Wang Z, Hu H, Liao JD, Watts JC, Combs GF Jr, Lu J (2008) Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite. Carcinogenesis 29:1005–1012
21. Newman RA, Yang J, Raymond M, Finlay V, Cabral F, Vourloumis D, Stephens LC, Troncoso P, Wu X, Logothetis CJ, Nicolaou KC, Navone NM (2001) Antitumor efficacy of 26-fluoroepothilone B against human prostate cancer xenografts. Cancer Chemother Pharmacol 48:319–326
22. USP C (2000) USP Pharmacopeia & National Formulary. Board of Trustees of the USP Pharmacopeial Convention, Inc., Rockville, MD
23. Sorby DL, Bitter RG, Webb JG (1963) Dielectric constants of complex pharmaceutical solvent systems. I. Water–ethanol–glycerin and water–ethanol–propylene glycol. J Pharm Sci 52:1149–1153
24. Martin A (1993) Physical Pharmacy. Lippincott Williams & Wilkins, Baltimore, Maryland

25. James KC (1986) Solubility and related properties. M. Dekker, New York
26. Xie HT, Wang GJ, Sun JG, Tucker I, Zhao XC, Xie YY, Li H, Jiang XL, Wang R, Xu MJ, Wang W (2005) High performance liquid chromatographic-mass spectrometric determination of ginsenoside Rg3 and its metabolites in rat plasma using solid-phase extraction for pharmacokinetic studies. J Chromatogr B Analyt Technol Biomed Life Sci 818:167–173
27. Akao T, Kanaoka M, Kobashi K (1998) Appearance of compound K, a major metabolite of ginsenoside Rb1 by intestinal bacteria, in rat plasma after oral administration–measurement of compound K by enzyme immunoassay. Biol Pharm Bull 21:245–249
28. Paek IB, Moon Y, Kim J, Ji HY, Kim SA, Sohn DH, Kim JB, Lee HS (2006) Pharmacokinetics of a ginseng saponin metabolite compound K in rats. Biopharm Drug Dispos 27:39–45
29. Hasegawa H, Lee KS, Nagaoka T, Tezuka Y, Uchiyama M, Kadota S, Saiki I (2000) Pharmacokinetics of ginsenoside deglycosylated by intestinal bacteria and its transformation to biologically active fatty acid esters. Biol Pharm Bull 23:298–304
30. Ota T, Maeda M, Odashima S (1991) Mechanism of action of ginsenoside Rh2: uptake and metabolism of ginsenoside Rh2 by cultured B16 melanoma cells. J Pharm Sci 80:1141–1146
31. Xie HT, Wang GJ, Chen M, Jiang XL, Li H, Lv H, Huang CR, Wang R, Roberts M (2005) Uptake and metabolism of ginsenoside Rh2 and its aglycon protopanaxadiol by Caco-2 cells. Biol Pharm Bull 28:383–386
32. Chen SE, Sawchuk RJ, Staba EJ (1980) American ginseng. III. Pharmacokinetics of ginsenosides in the rabbit. Eur J Drug Metab Pharmacokinet 5:161–168
33. Saad AJ, Domiati-Saad R, Jerrells TR (1993) Ethanol ingestion increases susceptibility of mice to Listeria monocytogenes. Alcohol Clin Exp Res 17:75–85
34. Kim HE, Oh JH, Lee SK, Oh YJ (1999) Ginsenoside RH-2 induces apoptotic cell death in rat C6 glioma via a reactive oxygen- and caspase-dependent but Bcl-X(L)-independent pathway. Life Sci 65:PL33–PL40
35. Fei XF, Wang BX, Tashiro S, Li TJ, Ma JS, Ikejima T (2002) Apoptotic effects of ginsenoside Rh2 on human malignant melanoma A375-S2 cells. Acta Pharmacol Sin 23:315–322
36. Park JA, Lee KY, Oh YJ, Kim KW, Lee SK (1997) Activation of caspase-3 protease via a Bcl-2-insensitive pathway during the process of ginsenoside Rh2-induced apoptosis. Cancer Lett 121:73–81
37. Kim YS, Jin SH, Lee YH, Park JD, Kim SI (2000) Differential expression of protein kinase C subtypes during ginsenoside Rh2-induced apoptosis in SK-N-BE(2) and C6Bu-1 cells. Arch Pharm Res 23:518–524