Rh2 Synergistically Enhances Paclitaxel or Mitoxantrone in Prostate Cancer Models

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Purpose: We explored the efficacy of the ginsenoside Rh2 and examined its impact on the effective dose of paclitaxel and mitoxantrone in the LNCaP prostate tumor model.

Materials and Methods: Cultured LNCaP cell viability was assessed following treatment (48 hours) with Rh2 (0 to 40 μM) alone or in combination with paclitaxel and mitoxantrone. Synergism or antagonism observed when combining treatment was calculated using CalcuSyn software (Biosoft®). In addition, the inhibition of LNCaP human xenograft tumor growth was examined in vivo when Rh2 treatment was combined with chemotherapy. Harvested tumors were immunohistochemical stained with p27kip and Ki67.

Results: Rh2 and paclitaxel act synergistically in cultured LNCaP cells to lower ED50 and ED75 values. Rh2 and mitoxantrone are also synergistic. However, when combined as ED95, an antagonistic effect was observed in this cell line. Treatment of LNCaP tumors by Rh2 plus paclitaxel produced a significant decrease in tumor growth and serum prostate specific antigen. Immunohistochemical analysis revealed an apparent but nonsignificant effect on proliferation markers in LNCaP tumors. When Rh2 and mitoxantrone were combined in vivo, there was no significant benefit observed.

Conclusions: These results indicate that the combination of Rh2 and paclitaxel has an effect on growth inhibition that is greater and synergistic, as demonstrated in a cultured LNCaP cell line. Conversely combining Rh2 with mitoxantrone appears to elicit no benefit. Therefore, combination therapy using chemotherapy and Rh2 requires further investigation.

Key Words: prostate, prostatic neoplasms, ginsenosides, drug synergism, drug antagonism

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f all of the plants and plant products used in traditional Chinese medicine ginseng has become one of the most well known throughout the world. Characterization of the components found in the root structure has revealed more than 20 ginsenoside compounds. It is these compounds that are believed to impart the medicinal properties of the herb.1 Rh2, a ginsenoside with a dammarane skeleton, has been shown to suppress growth and induce apoptosis in vitro in human prostate cancer cells,2 human melanoma cells3 and human hepatocarcinoma cells.4 Oral administration of Rh2 has inhibited the growth of ovarian xenografts in nude mice.5 When Rh2 and cisplatin were intravenously administered to nude mice bearing ovarian xenografts, a synergistic effect was observed.6 Synergy was also reported when murine hepatoma, sarcoma and melanoma models were treated in vivo and in vitro with Rh2 and paclitaxel or mitoxantrone.7

Earlier studies at our laboratory revealed that 50 mg/kg Rh2 administered orally produces significant growth inhibition of subcutaneous LNCaP tumor in mice.8 The current study builds on and provides an evidence base for this observation and addresses a synergistic phenomenon between ginseng derived compounds and chemotherapeutic compounds. The study presented is an evaluation of the efficacy and toxicity of Rh2 combined with paclitaxel or mitoxantrone9,10 in the LNCaP human prostate cancer model. We determined if concurrent administration of Rh2 has an impact on the tumor growth curve of LNCaP human xenograft tumor by decreasing the effective dose of these chemotherapeutic agents.

MATERIALS AND METHODS

Drugs and Chemicals
Rh2 ginsenoside (purity greater than 99.8%, as determined by high performance liquid chromatography) was provided by Pegasus Pharmaceuticals, Vancouver, British Columbia, Canada. Paclitaxel (Bristol-Myers Squibb, New York, New York) and mitoxantrone (Wyeth-Ayerst Canada, Toronto, Ontario, Canada) were obtained from the pharmacy of the British Columbia Cancer Agency, Vancouver, British Columbia, Canada.
Cell Lines
The LNCaP cell line (ATCC®) was grown as a monolayer in RPMI 1640 medium with 5% fetal bovine serum in a humidified incubator at 37°C in 5% CO₂.

Cytotoxicity Studies in Prostate Cancer Cell Models
LNCaP cells were quantified and plated onto 96-well microplates at a density of 10⁴ cells per well. Following a 24-hour incubation period the cells were treated for 48 hours in 6 replicate wells per group with Rh2 (0 to 40 μM) dissolved in ethanol, then diluted with RPMI 1640 medium), paclitaxel (0 to 12.5 nM), mitoxantrone (0 to 2 μM), Rh2/paclitaxel (22 μM/0.01 μM) or Rh2/mitoxantrone (22 μM/1 μM). The ethanol concentration in each well was maintained below 0.5%. Cell viability was evaluated using a modified crystal violet assay. Briefly, cells were fixed with 1% glutaraldehyde, stained with 0.5% crystal violet (Sigma Chemical Co., St. Louis, Missouri) and eluted with 100 μl Sorenson’s solution (9 mg trisodium citrate in 305 ml H2O, 195 ml 0.1 N HCl and 500 ml ethanol). Absorbance (A) was measured with a PowerWaveXTM spectrophotometer at 592 nm. Cell viability was determined using the formula, cell viability in percent = A/C/100%, where T represents A₅₉₂ of the treated wells, C represents A₅₉₂ of the control cells and B represents A₅₉₂ of the blank wells.

Assessment of Synergy
A combination index for synergy was determined by comparing the growth inhibition of each compound with that of the combinations of Rh2 and paclitaxel or Rh2 and mitoxantrone. Drug interactions were quantified with CalcuSyn software. CI was calculated at ED₅₀, ED₇₅ and ED₉₅. Synergism, additivity or antagonism of the drugs was indicated by CI, defined as less than 1, 1 and greater than 1, respectively.

Toxicity Study with Rh2, Paclitaxel and Mitoxantrone in Nude Mice
Six groups of 5 male nude mice (Harlan, Indianapolis, Indiana) each were dosed with Rh2, paclitaxel, mitoxantrone, Rh2 and paclitaxel, Rh2 and mitoxantrone or controls. Control group animals were administered corn oil by oral gavage 5 days weekly and saline intravenously on days 1, 5, 15 and 19. Rh2 was administered by oral gavage 5 days weekly at a dose of 50 mg/kg. Paclitaxel and mitoxantrone were administered intravenously via the tail vein on days 1, 5, 15 and 19 at doses of 6 and 2.5 mg/kg, respectively. Mice were monitored daily for 28 days for changes in weight and other signs of toxicity.

Efficacy Study of Rh2, Paclitaxel, Mitoxantrone and Rh2 Combinations as Inhibitors of LNCaP Tumor Growth in vivo
Six groups of 6 male nude mice per group were subcutaneously inoculated with 2 × 10⁶ LNCaP cells at 2 dorsal sites. A caliper was used to measure the 3 perpendicular axes of each tumor twice weekly. The formula, V = π/6 × L × W × H, was used to calculate tumor volume, where L represents length, W represents width and H represents height. When tumors were 150 mm³, treatment with Rh2, paclitaxel and mitoxantrone was initiated. The dosing regimen for each medication was identical to that used in the described toxicity study. Blood was collected by saphenous vein puncture on days 0, 10 and 24 from LNCaP tumor bearing mice. Serum PSA levels were measured using a PSA enzyme-linked immunosorbent assay kit (ClinPro, Union City, California). LNCaP tumors were harvested at the end of the study.

TMA Construction
LNCaP tissues harvested in the efficacy study were formalin fixed and paraffin embedded. Six 0.6 mm diameter cores per tumor were arrayed into a primary recipient block. The recipient block with 312 tumor cores was sectioned at 5 μm at the Morphological Services Laboratory, Department of Pathology and Laboratory Medicine, University of British Columbia. Hematoxylin (Vector Laboratories, Burlingame, California) and eosin staining was performed on sections from above and below those used for IHC staining to confirm the presence of tumor tissue in the experimental sections.

IHC Analysis of TMAs and Image Processing
Sections from the TMA block were used for IHC staining. Tumor cores were treated with a Mouse-To-Mouse IHC detection kit (Chemicon®) before and after primary antibody incubation to decrease nonspecific staining. Immunoperoxi-
dase procedures (LSAB + peroxidase kit) were used in combination with Ki67 (MIB-1) (Dako, Carpinteria, California) (1:100) and p27kip (DCS-72.F6, NeoMarkers, Lab Vision Corp., Fremont, California) (1:200) monoclonal antibodies. Antigen retrieval was performed by steaming with citrate buffer (Ki67 for 30 minutes and p27kip for 60 minutes)\(^1\). Hematoxylin was used as a nuclear counterstain. The primary antibody was omitted in the negative control sample. A pathologist (LF) examined the stained sections and visually determined the percent of cells positively stained with p27kip antibodies. Ki67 positive and negative staining cells were counted at 40\(^{\times}\) magnification. Ki67 IHC stain intensity was calculated using the formula, positive Ki67 count/(positive Ki67 count plus negative Ki67 count). The final value given for each tumor sample represents the mean of 6 replicates. Scores from individual tumor cores with significant necrosis were omitted.

**Statistical Analyses**
One-way ANOVA and the Tukey/Student-Newman-Keuls tests were used for statistical testing of tumor growth ratio, serum PSA level and IHC scores.

**RESULTS**

**Synergist Effects of Rh2 with Paclitaxel or Mitoxantrone in Cultured LNCaP Cells**
IC\(_{50}\) values for the treatment of LNCaP cells with Rh2 were determined to be 21.2 and 19.0 \(\mu\)M, respectively, as calculated using the median effect plot. Constant IC\(_{50}\) ratio design (1:1) was used to evaluate the synergistic effect of the 2 combination treatments (Rh2 with paclitaxel and Rh2 with mitoxantrone) in the LNCaP cell line.

Figure 1, A shows dose effect plots generated from crystal violet assays. CalcuSyn software was used to calculate ED\(_{50}\), ED\(_{75}\) and ED\(_{95}\) (fig. 1, B). The results of the analysis indicated that Rh2 and paclitaxel acted synergistically in the LNCaP cell line at ED\(_{50}\) (CI 0.88) and ED\(_{75}\) (CI 0.95). In addition, Rh2 and mitoxantrone acted synergistically in the LNCaP cell line at ED\(_{50}\) (CI 0.83) and ED\(_{75}\) (CI 0.84). When combined with paclitaxel and mitoxantrone at ED\(_{95}\), Rh2 produced a noticeable antagonistic effect.

**Acute Toxicity Evaluation in Nude Mice**
Rh2 administered alone at a daily dose of 50 mg/kg orally (5 to 7 days) does not induce acute toxicity in nude mice.\(^6\) The acute toxicity studies described confirmed that paclitaxel (6 mg/kg intravenously twice biweekly) and mitoxantrone (2.5 mg/kg intravenously twice biweekly) dosed in combination with Rh2 did not cause acute toxicity in nude mice and,
therefore, they could be investigated in the efficacy study described. No individual animal lost more than 15% body weight during the observation period (fig. 2).

**Efficacy Study of Rh2, Paclitaxel, Mitoxantrone and Rh2 Combinations as Inhibitors of LNCaP Tumor Growth in vivo**

Male nude mice bearing subcutaneous LNCaP prostate tumors were treated with vehicle, Rh2, paclitaxel, mitoxantrone, or Rh2 combined with paclitaxel or mitoxantrone. Figure 3 shows the effect of treatments on LNCaP tumor growth. Figure 4 shows PSA levels in the groups. The combination of Rh2 and paclitaxel produced significant inhibition of the LNCaP tumor growth rate on day 26 compared with that in the control group (p<0.05). In addition, the same 2 treatment groups had statistically different serum PSA levels on day 24. No other LNCaP bearing treatment group had a statistically significant difference from the control group during this study.

**Effect of Rh2 and Combination Treatment on Mitotic Marker Levels**

The table lists pathological scores describing the percent of positively stained cells on day 26. Figures 5 and 6 show representative IHC stained sections. Slightly higher p27kip scores were observed in the combination Rh2/paclitaxel treatment group compared with the control group. In addition, the Rh2/paclitaxel and Rh2/mitoxantrone groups had a moderately lower Ki67 score than the control group. However, while tumor growth was inhibited by Rh2 plus paclitaxel treatment in the LNCaP model, none of the IHC results showed differences in the proliferation index that were extensive enough to be statistically significant.

**DISCUSSION**

We have previously reported that, when cultured in the presence of Rh2, LNCaP human prostate cancer cell growth can be significantly inhibited. The in vitro experiments in this study revealed that Rh2 has an apparent chemosensitizing effect in the micromolar range in LNCaP human prostate cancer models. These results mirror those seen in other cell lines. The median effect method of Chou and Talalay was used to evaluate any synergistic effect produced when Rh2 and paclitaxel or Rh2 and mitoxantrone were administered in combination. In an in vitro environment LNCaP cells treated with a 1:1 IC50 ratio of Rh2 and paclitaxel were synergistically inhibited. Rh2 and mitoxantrone treatment of LNCaP cells yielded similar results. These results were partially substantiated by the in vivo efficacy study. Combination treatment of subcutaneous LNCaP tumor xenografts with Rh2 and paclitaxel produced a statistically significant effect on tumor volume and serum PSA concentrations. Considering cell culture and xenograft results it is evident that Rh2 synergistically improved the inhibitory effect of the chemotherapeutic agent paclitaxel in the LNCaP human prostate cancer model. However, the in vitro results indicate that an antagonistic effect was produced by dosing at ED50 concentrations. Because synergism was seen at the lower dose level, it may be that combination treatment with Rh2 could cause a decrease in the effective dose of paclitaxel. If this benefit is transferable to the clini-
cal setting, lower doses of paclitaxel could be administered that continue to elicit optimal treatment efficacy, while decreasing or perhaps eliminating adverse toxic side effects. An apparent problem is that there was observed antagonism seen at higher doses of Rh2 plus paclitaxel in vitro. Clinically this could result in a smaller margin of safety for the cytotoxic drug. Further research is required to determine whether the antagonism observed at ED95 concentrations can be eliminated by altering the ratio of Rh2 and cytotoxic drug applied.

There is an obvious trend in the data on Rh2 and mitoxantrone combination treatment that indicates a divergence from the control group. However, no significant difference could be established due to attrition from within the group. Rh2 alone appeared to impact the growth rate of LNCaP xenografts, albeit at levels below significance. Previous studies performed at our laboratory with this tumor model have shown statistically significant growth inhibition by Rh2 when the treatment duration was extended.10 The study presented was terminated at 4 weeks in an attempt to avoid the extensive necrosis that is characteristic of LNCaP tumors. Necrosis would have adversely affected subsequent IHC investigation of the tumor tissue.

The p27kip and Ki67 TMA immunohistochemical analysis of the LNCaP tumors was performed to identify changes in mitotic arrest and progression following treatments with Rh2, paclitaxel and mitoxantrone. No significant changes in the intracellular cyclin dependent kinase inhibitor p27kip and the mitotic biomarker Ki67 were observed, which may have been affected by the mentioned early termination. However, a visually apparent increase in p27kip positive staining in the Rh2 plus paclitaxel treatment group was observed compared to that in the control group. Others have reported that p27kip is increased in SK-HEP-1 human liver adenocarcinoma cells after Rh2 treatment.5 The percent of cells that stained positively for Ki67 was slightly decreased in the 2 combination treatment groups. This suggests that the in vivo tumor growth inhibition effect might be related to G1/S, arrest as previously reported,18 and through this action chemosensitize tumor cells to paclitaxel/mitoxantrone treatment.

American ginseng extracts have previously been shown to inhibit MCF-7 cell growth via apparent estrogenic effects. However, total ginsenoside accounts for only 7.9% in American ginseng extract and of them 6.9% are due to Rb1, Re, Rc, Rd, Rg1 and Rh2.20 Therefore, we believe that it is unlikely in this model that Rh2 was also acting through an estrogenic pathway.

Overall we noted that Rh2 yields a synergistic effect when combined with paclitaxel in cultured LNCaP cells. In addition, a significant chemosensitizing effect was seen when Rh2 and paclitaxel were administered to mice bearing the subcutaneous LNCaP prostate cancer human xenograft. These results imply that further research into the mechanisms and development of Rh2 as a chemosensitizing agent is warranted. Our studies also show that the dose combination ratio is critical and the antagonistic effects observed in vitro also warrant further investigation.

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