

Reversal effect of ginsenoside Rh2 on oxaliplatin-resistant colon cancer cells and its mechanism

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Abstract. Chemotherapy is an important treatment modality for colon cancer, however, drug resistance is the main factor leading to treatment failure. Ginsenoside Rh2 (G-Rh2), the main bioactive metabolite of ginseng, is known to possess the ability to potently induce cell apoptosis, inhibit cell proliferation and reverse multidrug resistance in a variety of cancer cells. The present study examined the effect of G-Rh2 on oxaliplatin (L-OHP)-resistant colon cancer cells and its potential mechanism. L-OHP-resistant colon cancer cells (LoVo/L-OHP) and LoVo cells were used in the present study. The effect of G-Rh2 on LoVo/L-OHP and LoVo cell proliferation was measured using a 3-(4,5 dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide assay. The effects of G-Rh2 on LoVo/L-OHP and LoVo cell apoptosis were detected by flow cytometry. The mRNA and protein expression of apoptosis-related genes Bax, Bcl-2 and caspase-3, drug resistance-related genes P-glycoprotein (P-gp) and Smad4, were determined in LoVo/L-OHP and LoVo cells treated with G-Rh2 by reverse transcription-quantitative polymerase chain reaction and western blot analyses. G-Rh2 treatment significantly inhibited the proliferation and induced the apoptosis of LoVo/L-OHP and LoVo cells. In addition, G-Rh2 treatment

resulted in a significant increase in pro-apoptotic factors, Bax and caspase-3, and decrease in anti-apoptotic factor Bcl-2 in the LoVo/L-OHP and LoVo cells. Furthermore, G-Rh2 treatment significantly decreased the levels of P-gp and increased the levels of Smad4 in the LoVo/L-OHP and LoVo cells. It was found that L-OHP had no significant effects on LoVo/L-OHP cell proliferation or apoptosis, whereas G-Rh2 + L-OHP treatment significantly inhibited LoVo/L-OHP cell proliferation and induced apoptosis. L-OHP had no significant effects on the expression of P-gp, Smad4, Bcl-2, Bax or caspase-3 in LoVo/L-OHP cells. Treatment with G-Rh2 + L-OHP significantly reduced the expression of P-gp and Bcl-2, and enhanced the expression levels of Smad4, Bax and caspase-3. These findings demonstrated that G-Rh2 reversed the drug resistance of LoVo/L-OHP cells to L-OHP, and this may be mediated by inhibiting cell proliferation and promoting apoptosis and regulating the expression of drug resistance genes. These results suggest that G-Rh2 may function as a potent anticancer drug for drug resistance in colon cancer treatment.

Introduction

Colon cancer is one of the most common malignant tumors and the third leading cause of cancer-associated mortality throughout the world (1). The primary treatment of colon cancer usually involves surgical resection and chemotherapy using cytotoxic drugs. Oxaliplatin (L-OHP) is one of the first-line drugs used for the treatment of metastatic colorectal cancer (CRC) (2). However, many cancer patients develop chemotherapeutic drug resistance that can lead to colon cancer treatment failure (3). Therefore, the search for novel effective agents for overcoming drug resistance and colon cancer treatment is required.

Ginseng is a medicinal plant with substantial medicinal effects and has a positive effect in the clinical treatment of cancer. Ginsenosides are the major active ingredients of ginseng and have multiple biological activities, including

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immunomodulatory effects and anti-inflammatory and anti-tumor activities (4,5). A previous study showed that ginsenoside Rh2 (G-Rh2) is one of the main active components of ginseng with antitumor activity (6-8). It has been reported that G-Rh2 exerts anticancer effects in a variety of malignant diseases, including CRC and breast cancer (9,10). G-Rh2 has been found with a potent ability to induce cell apoptosis and inhibit cancer cell proliferation (11).

Apoptosis is a complex process of programmed cell death (12). Two major cysteine-aspartate protease (caspase) activation cascades are involved in cell apoptosis. The first is the extrinsic apoptotic pathway, mediated by the activation of various cell-surface death receptors, including Fas, TNF receptor and DR4, which in turn cleaves and activates three short prodomain caspases, caspase-3, -6 and -7. The other is an intrinsic apoptotic pathway, driven by Bcl-2 family proteins, which leads to mitochondrial outer membrane permeabilization and the release of pro-apoptotic factors, particularly cytochrome *c*, and leads to the activation of caspase-9 (13,14). The abnormal regulation of apoptosis may lead to tumor progression and resistance to chemotherapy (15). It has been demonstrated that G-Rh2 induces human hepatoma cell apoptosis via the Bax/Bak-induced release of cytochrome *c* and activation of caspase-9/caspase-8 (16). G-Rh2 can induce neuroblastoma cell apoptosis through the activation of caspase-1 and caspase-3 and the upregulation of Bax (17). However, the effect and mechanism of G-Rh2 on L-OHP-resistant colon cancer cells have not been clarified.

The purpose of the present study was to investigate the effect of G-Rh2 on the drug resistance of L-OHP-resistant human colon cancer cells (LoVo/L-OHP) and to examine its potential mechanism.

Materials and methods

Reagents and antibodies. G-Rh2 (purity $\geq 98\%$) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). Primary antibodies against Bcl-2, Bax, caspase-3, and P-glycoprotein (P-gp) were obtained from Abcam (Cambridge, UK). Smad4 and GAPDH were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and treatment. The human CRC cells (LoVo) and human L-OHP-resistant CRC cells (LoVo/L-OHP) were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (Gemini Bio-Products) and maintained at 37°C in a humidified atmosphere of 5% CO₂. The cells were treated in the following manner: i) cells were treated with a series of concentrations of G-Rh2 (0, 50, 100, 200 and 250 $\mu\text{g}/\text{ml}$) for 24 h and then subjected to a 3-(4,5 dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay; ii) cells were treated with 250 $\mu\text{g}/\text{ml}$ G-Rh2 for 24 h and then subjected to an MTT assay, flow cytometry (FCM), western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis; iii) cells were

treated with 15 $\mu\text{mol}/\text{ml}$ L-OHP for 24 h and then subjected to an MTT assay, FCM, western blotting and RT-qPCR analysis; iv) cells were treated with 250 $\mu\text{g}/\text{ml}$ G-Rh2 + 15 $\mu\text{mol}/\text{ml}$ L-OHP for 24 h and then subjected to an MTT assay, FCM, western blotting and RT-qPCR analysis. An equal volume of DMSO (final concentration, $<0.1\%$) was used for controls.

Cell viability assay. Cell viability was measured using an MTT assay (Sigma-Aldrich; Merck KGaA) in the present study. Briefly, 1×10^4 cells/well LoVo/L-OHP or LoVo cells were seeded into 96-well plates and allowed to attach for 24 h prior to the addition of drugs, and were then treated with the indicated drugs for 24 h at 37°C with 5% CO₂. An equal volume of DMSO (final concentration, $<0.1\%$) was used as a control. Following treatment, 200 μl MTT (0.5 mg/ml) was added in each well and incubated at 37°C for 4 h following which the supernatant was removed and the cells were incubated with 150 μl DMSO for an additional 30 min in dark at 37°C. The absorbance at 570 nm of each well was recorded using a Synergy HT Multi-Mode microplate reader (Thermo Fisher Scientific, Inc.).

Cell apoptotic analysis. Annexin V-FITC Apoptosis Detection kit (Bioss, Shanghai, China) was used to detect the cell apoptosis according to the manufacturer's instructions. Briefly, the LoVo/L-OHP or LoVo cells (5×10^5 cells/ml) were seeded in 6-well plates and incubated with the gradient concentrations of drugs for 24 h. The treated cells were then harvested and washed with PBS, incubated in 500 μl Annexin-V binding buffer containing 5 μl Annexin-V-FITC for 15 min and then resuspended with 5 μl PI in the dark at room temperature for another 5 min. The percentages of early and late apoptotic cells were quantitatively analyzed using a flow cytometer (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Following treatment, the cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing phosphatase and protease inhibitors at room temperature for 1 min. The protein concentration was quantified using a BCA protein assay kit (BestBio, Shanghai, China). Equal quantities of proteins (50 $\mu\text{g}/\text{lane}$) were separated by 12% SDS-PAGE and then transferred onto PVDF membranes (EMD Millipore, Temecula, CA, USA) followed by blocking with 5% non-fat milk at room temperature for 1 h. Subsequently, the PVDF membranes were incubated with anti-Bax (1:1,000; cat. no. ab32503; Abcam, Cambridge, UK), anti-Bcl-2 (1:1,000; cat. no. ab196495; Abcam), anti-caspase-3 (1:1,000; cat. no. 14220; Cell Signaling Technology, Inc.), anti-P-gp (1:1,000; cat. no. ab103477; Abcam), anti-Smad4 (1:1,000; cat. no. 38454; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074, Cell Signaling Technology, Inc.) at room temperature for 1 h. The immunoreactivity bands were visualized using an enhanced chemiluminescent system (Pierce, Thermo Fisher Scientific, Inc.) and the densitometry of the bands was determined with Gel-Pro Analyzer densitometry software (version 6.3; Media Cybernetics, Inc., Rockville, MD, USA).

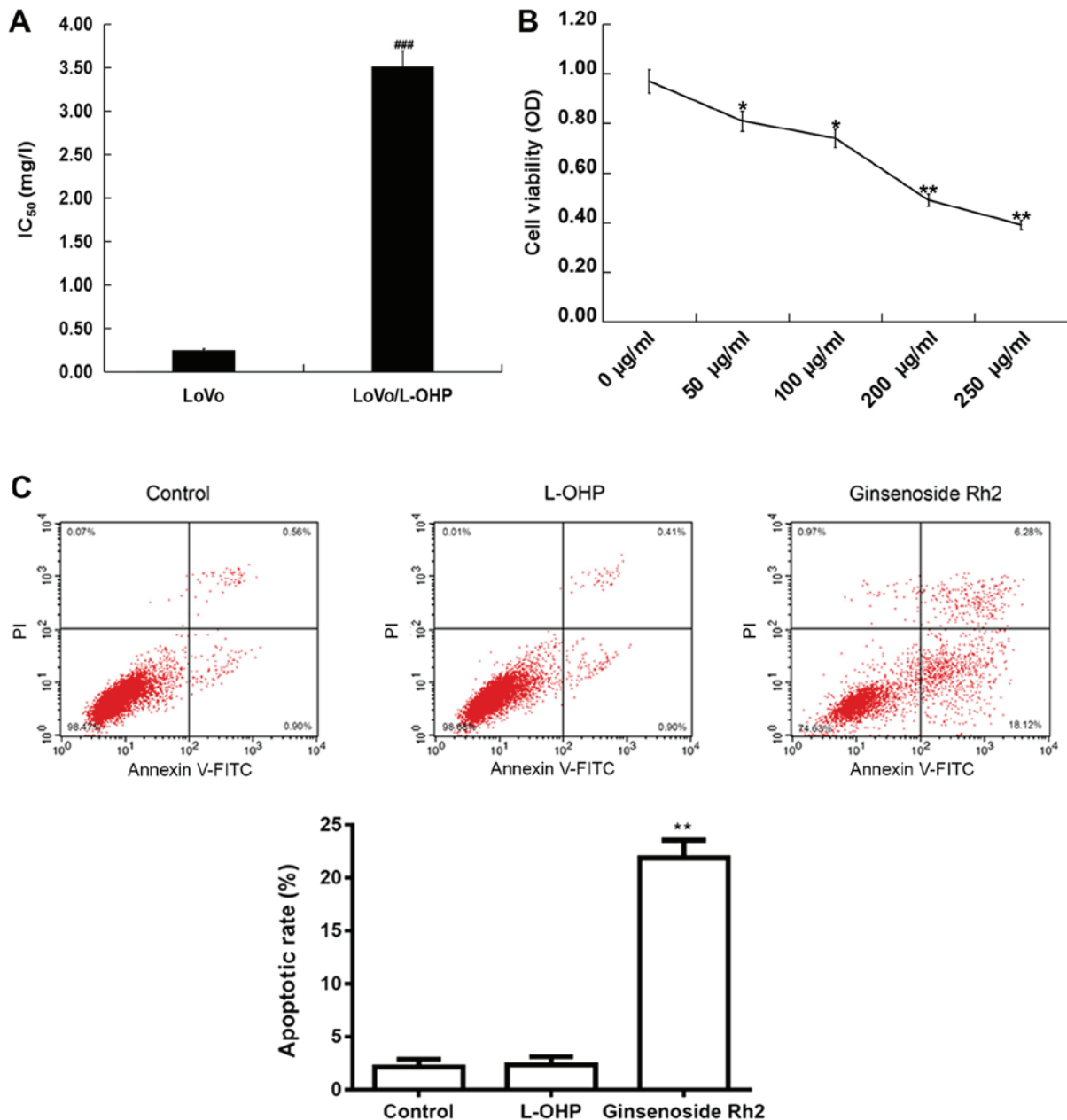


Figure 1. Effect of G-Rh2 on the proliferation and apoptosis of LoVo/L-OHP cells. (A) IC_{50} of L-OHP to LoVo cells and LoVo/L-OHP cells (***P<0.001 vs. LoVo cells). (B) LoVo/L-OHP cells were treated with various concentrations of G-Rh2 (0, 50, 100, 200 and 250 μ g/ml) for 24 h, and cell viability was measured using a 3-(4,5 dimethylthiazol-z-yl)-3,5-diphenyltetrazolium bromide assay (*P<0.05 and **P<0.01 vs. 0 μ g/ml treatment group). (C) LoVo/L-OHP cells were treated with 250 μ g/ml G-Rh2 or 15 μ mol/ml L-OHP for 24 h, and cell apoptosis (early and late apoptotic cells) was determined using flow cytometry (**P<0.01 vs. control group). Data are expressed as the mean \pm SD. G-Rh2, ginsenoside Rh2; L-OHP, oxaliplatin; IC_{50} , half maximal inhibitory concentration.

RT-qPCR analysis. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A reverse transcription kit (Takara Bio, Inc., Tokyo, Japan) was used for the reverse transcription of RNA to cDNA. Reaction conditions for reverse transcription were: 50°C for 5 min and 80°C for 2 min. qPCR was performed using the SYBR Green real-time PCR kit (Takara Bio, Inc.) on an Applied Biosystems 7500 real-time PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification conditions for qPCR were as follows: 5 min at 95°C; 35 cycles of 95°C for 15 sec, 40 sec at 55°C, and 72 °C for 1 min. The primer sequences for PCR were as follows: P-gp [multidrug resistant 1 (mdr1)a and

mdr1b], mdr1a, forward 5'-CACCATCCAGAACGCAGACT-3' and reverse 5'-ACATCTCGCATGGTCACAGTT-3'; mdr1b, forward 5'-AACGCAGACTTGATCGTGGT-3' and reverse 5'-AGCACCTCAAATACTCCCAGC-3'; Smad4, forward 5'-GACAGCAGCAGAATGGAT-3' and reverse, 5'-CAGGAG CAGGATGATTAGAA-3'; Bax forward, 5'-GGGTTTCAT CCAAGGATCGAGCAGG-3' and reverse 5'-ACAAAGATG GTCACGGTCTGCC-3'; Bcl-2, forward 5'-GAGAAATCA AACAGAGGCCG-3' and reverse 5'-CTGAGTACCTGAACC GGCA-3'; caspase-3, forward 5'-CTGCCTCTTCCCCCA TTCT-3' and reverse 5'-TCGCTTCCATGTATGATCTTT G-3'; GAPDH, forward 5'-CCTCCAAAATCAAGTGGG GCGATG-3' and reverse 5'-CATATTTGGCAGGTTTTT

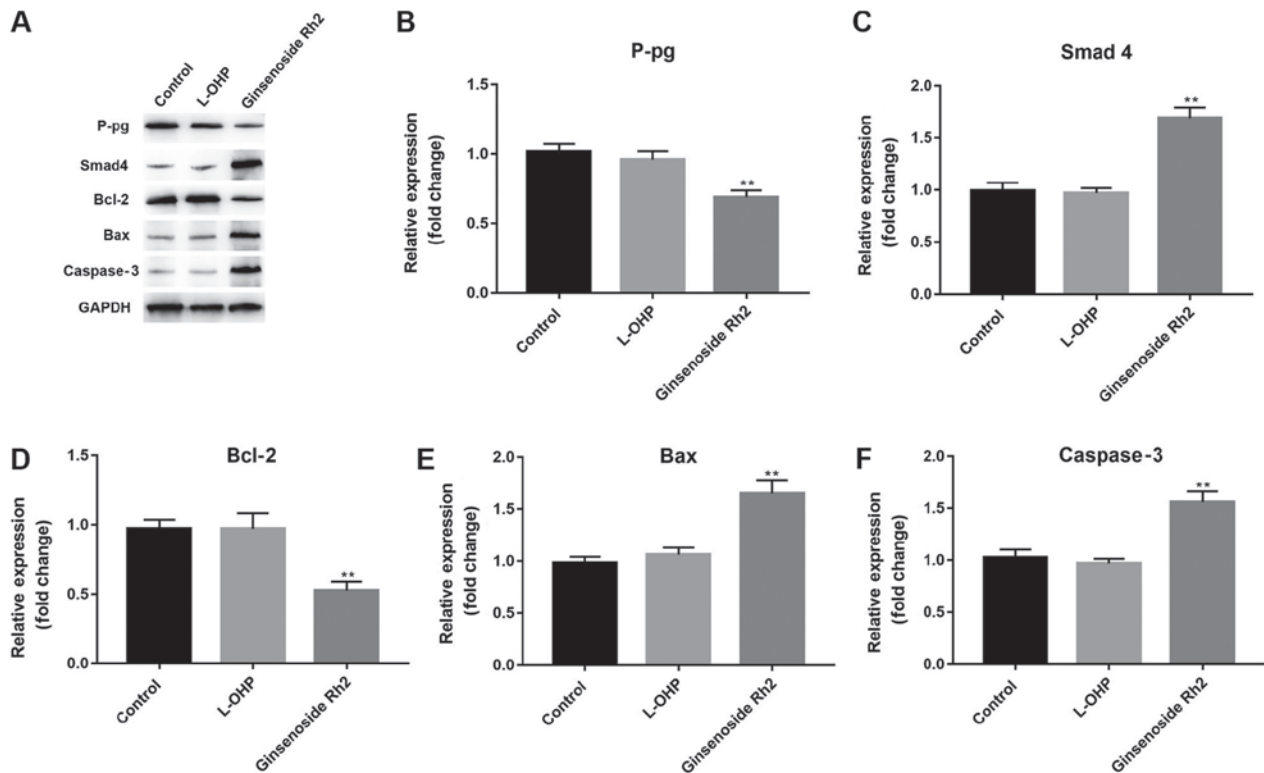


Figure 2. Effect of G-Rh2 on the expression of P-gp, Smad4, Bcl-2, Bax and caspase-3 in LoVo/L-OHP cells. LoVo/L-OHP cells were treated with 250 μ g/ml G-Rh2 or 15 μ mol/ml L-OHP for 24 h, following which (A) protein levels were measured using western blotting. mRNA levels of (B) P-gp, (C) Smad4, (D) Bcl-2, (E) Bax and (F) caspase-3 in LoVo/L-OHP cells were measured using reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm SD. ** P <0.01 vs. control group. G-Rh2, ginsenoside Rh2; L-OHP, oxaliplatin; P-gp, P-glycoprotein.

CTAGAC-3'. The quantitative analysis of mRNA levels was normalized to GAPDH and relative gene expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (18).

Statistical analysis. Statistical analysis was performed with SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Differences among groups were analyzed using Student's *t* test or one way-ANOVA followed by Tukey's post hoc test. Data are presented as the mean \pm SD. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

G-Rh2 inhibits the proliferation and induces the apoptosis of LoVo/L-OHP cells. As shown in Fig. 1A, compared with the half-maximal inhibitory concentration of L-OHP in the parental LoVo cells, the LoVo/L-OHP cells were more resistant to L-OHP.

To examine the effects of G-Rh2 on the proliferation of LoVo/L-OHP cells, an MTT assay was performed to assess the viability of LoVo/L-OHP cells treated with or without G-Rh2 (0, 50, 100, 200 and 250 μ g/ml). The results showed that LoVo/L-OHP cell viability was significantly reduced following treatment with G-Rh2 in a dose-dependent manner (Fig. 1B). The results of FCM showed that the number of apoptotic LoVo/L-OHP cells was significantly increased following treatment with G-Rh2 compared with that in the untreated control group (Fig. 1C). However, 15 μ mol/ml L-OHP had no significant effects on cell apoptosis.

The drug-resistance genes P-gp and Smad4, and apoptosis-related proteins were also determined in the present study. As shown in Fig. 2A-F, compared with the untreated control group, treatment with G-Rh2 markedly decreased the protein and mRNA levels of P-gp and Bcl-2, and increased the protein and mRNA levels of Smad4, Bax and caspase-3 in the LoVo/L-OHP cells. Treatment with 15 μ mol/ml L-OHP had no significant effect on the expression of P-gp, Smad4, Bcl-2, Bax or caspase-3.

Rh2 inhibits the proliferation and induces the apoptosis of LoVo cells. The present study then determined the effect of G-Rh2 on the proliferation and apoptosis of LoVo cells using an MTT assay and FCM. The results showed that 250 μ g/ml G-Rh2 significantly inhibited the proliferation (Fig. 3A) of the LoVo cells and induced cell apoptosis (Fig. 3B). In addition, it was found that 250 μ g/ml G-Rh2 significantly increased the protein (Fig. 3C) and mRNA (Fig. 3D-H) levels of Smad4, Bax and caspase-3, whereas the protein and mRNA levels of P-gp and Bcl-2 were reduced.

G-Rh2 reverses L-OHP resistance in LoVo/L-OHP cells. Treatment with G-Rh2 markedly reversed L-OHP resistance in the LoVo/L-OHP cells (Fig. 4). How G-Rh2 reversed L-OHP resistance in the LoVo/L-OHP cells was subsequently investigated, and cell proliferation and apoptosis were determined. It was found that 15 μ mol/ml L-OHP had no significant effects on LoVo/L-OHP cell proliferation or apoptosis, whereas G-Rh2 + L-OHP treatment significantly

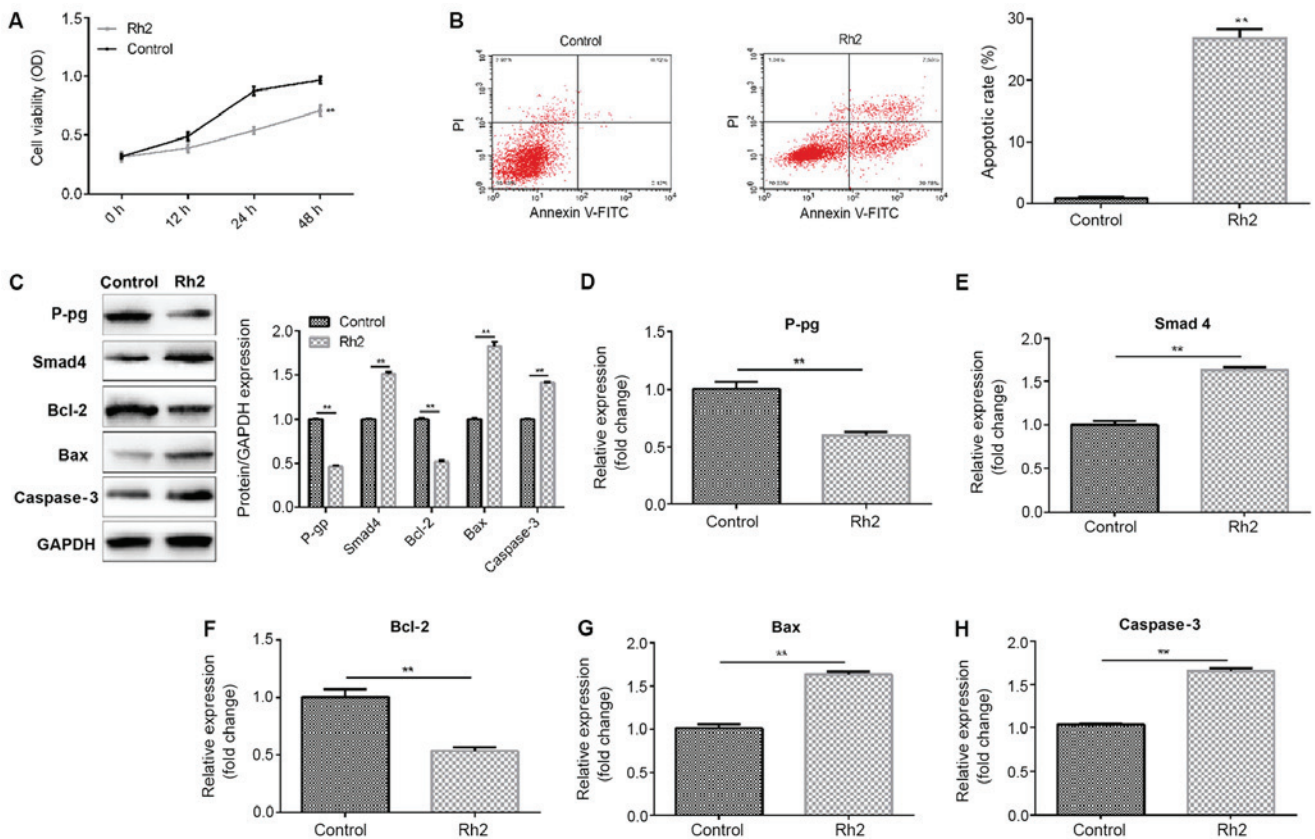


Figure 3. Effect of G-Rh2 on the proliferation and apoptosis of LoVo cells. LoVo cells were treated with or without 250 $\mu\text{g/ml}$ G-Rh2 for 24 h. A 3-(4,5 dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide assay was used to detect (A) cell viability, and (B) flow cytometry was used to detect cell apoptosis (early and late apoptotic cells). (C) Protein levels of P-gp, Smad4, Bcl-2, Bax and caspase-3 in LoVo cells were determined using a western blot assay. mRNA levels of (D) P-gp, (E) Smad4, (F) Bcl-2, (G) Bax and (H) caspase-3 were determined by reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm SD. ** $P < 0.01$ vs. control group. G-Rh2, ginsenoside Rh2; P-gp, P-glycoprotein.

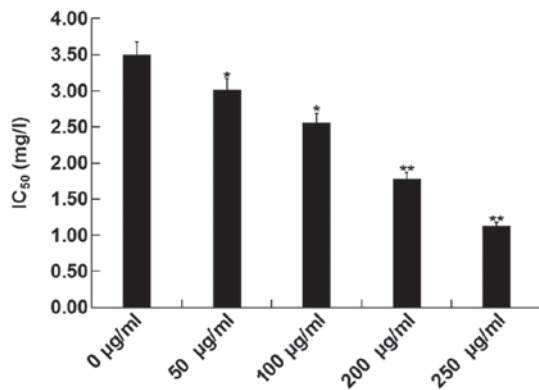


Figure 4. Effect of G-Rh2 on the IC_{50} of L-OHP and LoVo/L-OHP cells. L-OHP and LoVo/L-OHP cells were treated with various concentrations of G-Rh2 (0, 50, 100, 200 and 250 $\mu\text{g/ml}$) and the IC_{50} values were determined. Data are expressed as the mean \pm SD. * $P < 0.05$ and ** $P < 0.01$ vs. 0 $\mu\text{g/ml}$ treatment group. G-Rh2, ginsenoside Rh2; L-OHP, oxaliplatin; IC_{50} , half maximal inhibitory concentration.

inhibited LoVo/L-OHP cell proliferation (Fig. 5A) and induced apoptosis (Fig. 5B). In addition, to investigate the potential molecular mechanisms underlying the reversal of drug resistance by G-Rh2 and the potential mechanism of G-Rh2 on cell proliferation and apoptosis, the effect of G-Rh2 on drug-resistance genes P-gp and Smad4, and apoptosis-related genes were examined. The results (Fig. 5C-H) indicated that

15 $\mu\text{mol/ml}$ L-OHP had no significant effects on the expression of P-gp, Smad4, Bcl-2, Bax or caspase-3 in LoVo/L-OHP cells. However, treatment with G-Rh2 + L-OHP significantly reduced the expression of P-gp and Bcl-2, and increased the expression of Smad4, Bax and caspase-3.

Discussion

The present study indicated that G-Rh2 inhibited the proliferation and induced the apoptosis of LoVo/L-OHP cells and LoVo cells, and reversed L-OHP resistance in LoVo/L-OHP cells through inhibiting cell proliferation and inducing cell apoptosis via regulating the expression of drug-resistance gene P-gp and Smad4, and apoptosis-related genes. These findings provide a novel strategy and theoretical basis for the treatment of CRC.

G-Rh2, one of the main components of ginseng, has numerous biological activities, and there are no reported side effects in normal cells. A previous study reported that G-Rh2 reduced cell proliferation and sensitized CRC cells to 5-fluorouracil chemotherapy (19). In addition, there is evidence that G-Rh2 may be associated with drug resistance in cancer treatment (20). In the present study, it was demonstrated that G-Rh2 reversed drug resistance in LoVo/L-OHP cells and decreased expression of P-gp. In addition, the results indicated that G-Rh2 had antiproliferative and pro-apoptotic effects on LoVo/L-OHP and LoVo cells.

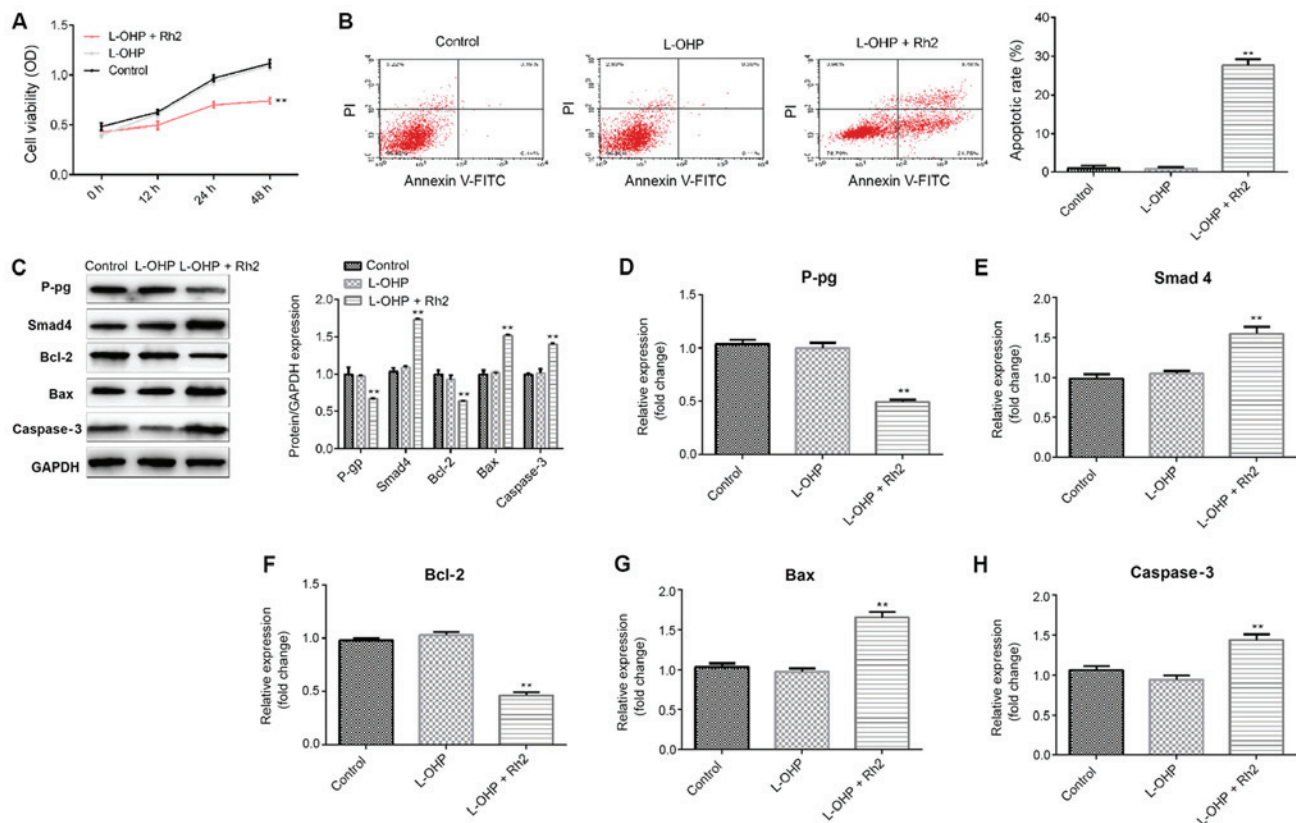


Figure 5. Effect of the combination of G-Rh2 and L-OHP on LoVo/L-OHP cells. LoVo cells were treated with or without 15 $\mu\text{mol/ml}$ L-OHP, or 15 $\mu\text{mol/ml}$ L-OHP + 250 $\mu\text{g/ml}$ G-Rh2 for 24 h. (A) A 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide assay was used to detect cell viability, and (B) flow cytometry was used to detect cell apoptosis. (C) Protein levels of P-gp, Smad4, Bcl-2, Bax, and caspase-3 in LoVo cells were determined using a western blot assay. mRNA levels of (D) P-gp, (E) Smad4, (F) Bcl-2, (G) Bax and (H) caspase-3 were determined by reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean \pm SD. ** $P < 0.01$ vs. control group. G-Rh2, ginsenoside Rh2; L-OHP, oxaliplatin; P-gp, P-glycoprotein.

P-gp is an ATP-dependent efflux transporter, which is expressed at high levels in the gastrointestinal tract and multidrug-resistant tumor cells (21). The inhibition of P-gp can reverse the multidrug resistance induced by chemotherapeutic agents (22). Previous studies have reported that G-Rh2 can reverse multidrug resistance in adriamycin-resistant human breast cancer MCF-7 cells (20) and reverse drug resistance in 5-fluorouracil-resistant LoVo and HCT-8 human CRC cells (19). In a previous study, 20 (S)-Rh2 was shown to inhibit P-gp in multidrug-resistant cancer cells (21). Consistent with previous reports, the results of the present study demonstrated that G-Rh2 reversed drug resistance in LoVo/L-OHP cells and decreased the expression of P-gp.

Increasing studies have shown that apoptosis is an important mechanism through which chemotherapeutic agents kill susceptible cells (23). During the process of programmed cell death, the Bcl-2 protein interferes with the activation of caspases by preventing the release of cytochrome c, which can be induced by Bax (24). A previous study reported that G-Rh2 induces apoptosis via the activation of caspase-1 and -3 and upregulation of Bax in human neuroblastoma (17). G-Rh2 can inhibit the proliferation of the A549 human lung adenocarcinoma cell line in a dose-dependent manner by activating caspase-8/3 to promote apoptosis (25). In the present study, the effects of G-Rh2 on cell apoptosis-related molecules were investigated. The results demonstrated that G-Rh2 decreased the expression of Bcl-2 and increased the expression of Bax,

caspase-3 and Smad4 in LoVo/L-OHP and LoVo cells. As reported previously, G-Rh2 inhibits tumor cell growth and prevents cells from entering the growth phase, including the G2/M, G1/S and S phases, respectively (25,26). G-Rh2 can inhibit A172 human glioma cell proliferation and induce cell cycle arrest status (27). In the present study, it was also found that G-Rh2 treatment markedly inhibited the proliferation and induced the apoptosis of LoVo/L-OHP and LoVo cells. In addition, G-Rh2 + L-OHP treatment significantly inhibited LoVo/L-OHP cell proliferation and induced apoptosis. To investigate the potential molecular mechanisms underlying the reversal of drug-resistant by G-Rh2 and the potential mechanism underlying the effects of G-Rh2 on cell proliferation and apoptosis, the present study also determined the effect of G-Rh2 on drug-resistance genes P-gp and Smad4, and apoptosis-related genes. It was found that G-Rh2 + L-OHP treatment significantly reduced the expression of P-gp and Bcl-2, whereas the expression levels of Smad4, Bax and caspase-3 were enhanced. L-OHP alone had no significant effect on the LoVo/L-OHP cells.

In conclusion, the results of the present study showed that G-Rh2 effectively reversed drug resistance in LoVo/L-OHP cells and its potential mechanism involved inhibiting cell proliferation and promoting apoptosis and changes in drug resistance genes. These results indicate that G-Rh2 may be a promising therapeutic approach for drug resistance in CRC chemotherapy. However, the present study is a preliminary investigation on the

effect of G-Rh2 on oxaliplatin-resistant colon cancer, and the role of G-Rh2 on L-OHP-resistant colon cancer requires extensive investigation. For example, whether G-Rh2 has an effect on other L-OHP-resistant colon cancer cell lines requires investigation, and *in vivo* experiments should be performed. These issues will be investigated in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JM and GG contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. HL, DF, LL, GW and AC contributed to data interpretation, statistical analysis and literature search. YY, HZ and JH contributed to statistical analysis and literature search. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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