Treatment with 20(S)-ginsenoside Rg3 reverses multidrug resistance in A549/DDP xenograft tumors

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Abstract. Multidrug resistance (MDR) is an obstacle for cancer chemotherapy. It was reported that 20(S)-ginsenoside Rg3 (hereafter Rg3) was able to regulate MDR in mouse leukemia cells. The present study investigated the effect of Rg3 on the MDR of A549 lung cancer cells. A cell viability assay revealed that Rg3 treatment increased cisplatin (DDP) cytotoxicity in DDP resistant A549 cells (A549/DDP). Furthermore, Rg3 increases the antitumor effect of DDP on A549/DDP xenograft mice. The expression of MDR-mediated proteins, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MPR1) and lung resistance protein 1 (LRP1), was detected in tumor tissue of A549/DDP xenograft mice. The results revealed that Rg3 treatment inhibited the expression of these MDR-associated proteins. Additionally, technetium-99m labeled hexakis-2-methoxyisobutylisonitrile (99mTc-MIBI) single-photon emission computed tomography was used to monitor the effect of Rg3 on cisplatin sensitivity of A549/DDP xenograft tumors. It was observed that uptake of 99mTc-MIBI was increased by Rg3 treatment, which indicated that Rg3 is able to effectively enhance chemotherapy sensitivity of A549/DDP xenograft tumors. Taken together, these results revealed that Rg3 may be able to reverse MDR of lung cancer via the downregulation of P-gp, MPR1 and LPR1.

Introduction

Lung cancer is the most common cause of cancer-associated mortality worldwide (1). The incidence of lung cancer is increasing in China, leading to ~250,000 incidences of mortality each year (2). Although surgical resection is the primary treatment for lung cancer, only 10-15% of patients underwent surgical resection as a result of late diagnosis (3,4). Chemotherapy remains the first-line treatment for patients with lung cancer (5); however, lung cancer exhibits intrinsic multidrug resistance (6). Therefore, the efficacy of chemotherapy is often hampered by multidrug resistance (MDR), which leads to the loss of activity of agents against cancer cells (7).

MDR is common in human cancer, and is a key hindrance to the effectiveness of chemotherapy (8,9). Although the exact mechanisms of resistance remain unclear, a reduction in the intracellular accumulation of drugs was observed in cancer cells exhibiting MDR, which was mediated by increasing drug efflux (8). The phenomenon of drug efflux results from the high expression of ATP-binding cassette (ABC) proteins, which are a drug transporter family that includes P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP1) (10). P-gp and MRP1 affect the intracellular drug concentration and contribute to the MDR of cancer cells via the alteration of drug influx or efflux. It has been reported that P-gp regulates the efflux of various anticancer drugs from cells (11,12). Furthermore, P-gp expression is associated with a poorer prognosis in patients with several types of human tumor including leukemia and lung, bladder, ovarian and breast cancer (13-15). Additionally, the expression of MRP1 is associated with the chemosensitivity of anticancer drugs including paclitaxel and anthracyclines (16). The lung resistance protein (LRP), a major vault protein, mediates MDR via molecular pumping molecules or exocytotic vesicles to increase the drug efflux from intracellular drug targets (16,17). Previous study has reported that LRP serves important functions in the MDR of lung cancer (16).

The ginsenosides are active chemical components extracted from Panax ginseng (18,19). Ginsenosides Rg1, Re, Rc, and Rd were identified to prevent MDR in mouse lymphoma cells by inhibiting the drug efflux pump and increasing drug accumulation (20). 20(S)-ginsenoside Rg3 (Rg3) is a ginsenoside that has attracted attention owing to its inhibitory effect on several types of tumor, including lung, gallbladder, liver, and ovarian cancer (21-24). Furthermore, it has been reported that Rg3 regulates MDR of numerous tumors cells, including human acute myeloid leukemia cells, KBV20C cells and murine
leukemia P388 cells (8.25). However, the effect of Rg3 on the MDR of lung cancer has not been studied.

The aim of this study is to investigate the effect of Rg3 on the MDR of A549 lung cancer cells. A cell viability assay was used to detect the effect of Rg3 on cisplatin (DDP)-induced A549/DPD cells cytotoxicity. The antitumor function of Rg3 was tested in A549/DDP xenograft mice. Western blot analysis was performed to detect the expression of MDR-mediated proteins, including P-gp, MPR1 and LR1, in the tumor tissue of A549/DDP xenograft mice with or without Rg3 treatment. Technetium-99m-labeled hexakis-2-methoxyisobutylisonitrile (99mTc-MIBI) single-photon emission computed tomography (SPECT) was used to monitor the effect of Rg3 on cisplatin sensitivity of A549/DDP xenograft tumors. Our results suggested that Rg3 increased chemosensitivity of the DDPP-resistant lung cancer A549/DDP cell line via the downregulation of P-gp, MRP1 and LR1.

Materials and methods

Cell line and cell culture. The human lung cancer A549 and DDP-resistant A549/DDP cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidity incubator with 5% CO2. To maintain drug resistance, DDP-resistant A549 cells were cultured with 5 µg/ml DDP (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and then cultured further in DDP-free RPMI-1640 medium for two days prior to starting the experiment.

Chemicals and drug preparations. Ginsenoside Rg3 was purchased from Zhejiang Yatai Pharmaceutical Co., Ltd. (Shaoxing, China). Purity quotient of Rg3 was ≥99.5%. Cisplatin (DDP) was obtained from Sigma-Aldrich (Merck KGaA). The compounds were dissolved in dimethyl sulfoxide to make stock solution (Rg3, 15 mM; and DDP, 100 mM). These two compounds were kept at -80°C as aliquots.

Cell viability assay. For the cell viability assay, 5×103 A549/DDP cells were seeded into each well of the 96-well microplate overnight. The cells were treated with various concentrations of DDP with or without Rg3 (40 µM). The final concentrations were 0.02, 0.2, 2, and 20 µg/ml, which are 10 times of the human peak serum doses for DDP, respectively. The final concentrations of DDP and Rg3 were 2.0 µg/ml (resistant cells). Measurement of DDP was performed on a microplate reader (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The half-maximal inhibitory concentration (IC50) of DDP was then calculated. The reversed effect of Rg3 on IC50 was presented as reversal fold, [Reversal fold=IC50 (resistant cells)/IC50 (reversal cells)].

Flow cytometry. Following 99mTc-MIBI SPECT analyses, tumor tissue was isolated and digested. The tissues were digested with 2 mg/ml collagenase I (cat. no. C0130; Sigma-Aldrich; Merck KGaA) and 2 mg/ml hyaluronidase.
Protein extraction and western blot. Protein extraction and western blot analysis were performed as previously described (30). Briefly, total proteins were extracted from tumor tissue using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with Complete EDTA-free Protease Inhibitor cocktail tablets (Roche Applied Science, Penzberg, Germany), according to the manufacturer's protocol. Protein concentrations were tested with a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.) at room temperature following three washes with Tris-buffered saline with 0.1% Tween-20 (Ameresco, Inc., Framingham, MA, USA). The data was analyzed by FlowJo software (version 7.6; FlowJo LLC., Ashland, OR, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from A549/DDP tumor tissues using TRIzol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was acquired from 1 µg RNA using reverse transcription for 1 h at 37°C (OmniScript RT kit, Qiagen) with oligo (dT) primers (Oligo (dT)20 primer; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturers protocol. Following RT, qPCR was conducted using 2X TransStart Green qPCR SuperMix (TransGen Biotech Co., Beijing, China) on an ABI 7500 instrument, as previously described (31). PCR thermocycling parameters included: Initial denaturation for 3 min at 94°C; 40 total cycles of denaturation 30 sec at 94°C; annealing for 30 sec at 60°C, extension for 30 sec at 72°C; and a final extension step of 2 min at 72°C. The primers as previously described (32,33) were as follows: P-gp forward, 5’-AAAAAGATCAACTCG TACACTC-3’ and reverse, 5’-GCACAAAAATACACACAACA A-3’; MRP1 forward, 5’-TCAAATACCTGCTGTTG-3’ and reverse, 5’-TGAAGTCTGGTCTGATGCGAT-3’; LRP1 forward, 5’-GTGCGGTTGAGATGAGTG-3’ and reverse, 5’-CGAGATGGCAGAGGGG-3’; and β-actin forward, 5’-GCAACCAGTGAAGATGACC-3’ and reverse, 5’-GCCACGGGCAGCAT-3’. The relative expression quantity of P-gp, MRPI and LRP1 mRNA were calculated with the 2ΔΔCq method (34) and normalized to the expression of β-actin. All the above assays were repeated three times.

Statistical analyses. The data are presented as the mean ± standard deviation. Semi-quantitative analysis of 99mTc-MIBISPECT was compared with the expression of P-gp. The Pearson’s correlation coefficient was used for statistical analysis. Student’s t-test was performed to assess differences between two groups. One-way analysis of variance and the post-hoc Bonferroni test was conducted to assess differences among multiple groups. All statistical calculations were performed using GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

Rg3 increases the sensitivity to DDP in A549/DDP cells. The present study determined the effect of Rg3 on the sensitivity of the cells to DDP. As presented in Fig. 1, the IC50 value of the Rg3 treated cells was lower than that of the parental A549/DDP cells (IC50, 8.14±0.59 vs. 11.97±0.71 µg/ml; P<0.01). The reversal fold of Rg3 treatment was 1.29-fold.
Table II. Percentage of positive cells expressing P-gp in the DDP and DDP + Rg3 group and their Pearson’s correlation coefficients with the ER, DR and WR values.

| Variable                  | ER±         | DR±         | WR±         | P-gp-positive cells%, % |
|---------------------------|-------------|-------------|-------------|-------------------------|
| DDP group                 | 1.08±0.08   | 1.04±0.06   | 0.03±0.07   | 71.62±10.52²            |
| Pearson’s correlation coefficient ⁷ | 0.555       | -0.323      | 0.780       |                         |
| P-value                   | 0.096       | 0.363       | 0.008       |                         |
| DDP + Rg3 group           | 1.90±0.53   | 1.91±0.54   | -0.07±0.40  | 45.40±8.65²            |
| Pearson’s correlation coefficient ⁷ | 0.145       | -0.129      | 0.858       |                         |
| P-value                   | 0.690       | 0.722       | 0.002       |                         |

²Expressed as mean ± standard deviation. ⁷Pearson correlation coefficients of P-gp positive cells and ER, DR, WR value in the control group. ³Percentage of P-gp positive cells in the control group. ⁴Pearson correlation coefficients of P-gp positive cells and ER, DR, WR value in the DDP + Rg3 group. ⁵Percentage of P-gp positive cells in control group in the DDP+Rg3 group; P-gp, P-glycoprotein; ER, early tumor uptake ratio; DR, delayed tumor uptake ratio; WR, washout rate; DDP, cisplatin.

Thus, these results indicated that Rg3 is able to increase the DDP sensitivity of the A549/DDP cells.

**Rg3 increases the antitumor effect of DDP on A549/DDP xenograft mice.** A549/DDP xenograft mice were used to assess whether the addition of Rg3 increased the sensitivity of A549/DDP cells to DDP *in vivo*. Mice were intraperitoneally injected twice weekly for 4 weeks with DDP (7.5 mg/kg in 0.2 ml of normal saline) and/or Rg3 (15 mg/kg). The mice that received DDP-Rg3 combination therapy exhibited significantly reduced tumor volumes on day 29 (39.5% reduction; P=0.0006) compared with animals treated using DDP alone (Fig. 2). Furthermore, the weight of tumors in the DDP-Rg3 combination group was decreased compared with that of the DDP group (85% reduction; P=0.003). Taken together, these results indicated that Rg3 can increase the sensitivity of lung cancer cells to DDP *in vivo*.

**99mTc-MIBI SPECT monitoring of the effect of Rg3 on the sensitivity of A549/DDP to DDP.** 99mTc-MIBI SPECT imaging was used to monitor the effect of Rg3 on the DDP sensitivity of A549/DDP. 99mTc-MIBI data are summarized in Table I. The results revealed that ER and DR in the DDP-Rg3 combination therapy group were higher than that of DDP group (ER, 1.90±0.53 vs. 1.08±0.08; P<0.001; DR, 1.91±0.54 vs. 1.04±0.06; P<0.001). The WR did not differ significantly between the groups. These results indicated that Rg3 treatment increased uptake of 99mTc-MIBI.

The expression of the MDR-associated protein P-gp was analyzed using flow cytometry analysis. Compared with the control group, the proportion of P-gp-positive cells was significantly reduced in the DDP/Rg3 group (71.62±10.52 vs. 45.40±8.65; P<0.01). Moreover, the proportion of P-gp positive cells was positively associated with WR values (Table II), which suggested that the drug clearance detected by 99mTc-MIBI imaging is positively correlated with P-gp expression in A549/DDP xenograft mice. Thus, 99mTc-MIBI SPECT can reveal the MDR state of tumors and also dynamically monitor the effect of Rg3 on the reversion of MDR of A549/DDP-xenograft mice.

**Discussion**

Lung cancer represents a considerable threat to global health and human life (35). Over half of patients with lung cancer are diagnosed late, resulting in a minimal opportunity for patients to undergo surgical resection (36). Currently, chemotherapy is the primary therapeutic treatment (37,38). DDP is the most common chemotherapy drug used, which causes the death of tumor cells by induction of DNA damage (39). DDP-based combination chemotherapies are widely used for patients with lung cancer (40). However, the occurrence of chemotherapy resistance in lung cancer prevents successful treatment (41).
Thus, MDR in lung cancer is a widespread problem that remains unresolved.

Rg3 is a pharmaceutical ingredient extracted from the Chinese herb *P. ginseng*. It has been reported that ginsenosides such as Rg1, Re, Rc and Rd exert an inhibitory effect on drug efflux pumps, thus preventing MDR in lymphoma (20). In the present study, a cell viability assay revealed the increased DDP cytotoxicity of A549/DDP cells via when treated with Rg3, which indicated that Rg3 may increase the sensitivity of lung cancer cells to DDP *in vitro*. Furthermore, Rg3 increases the antitumor effect of DDP on A549/DDP xenograft mice. These results indicated that Rg3 may reverse MDR of lung cancer. Consistent with the results of the present study, previous studies reported that Rg3 was able to reverse MDR in oral cancer KBV20C cells in a dose-dependent manner (9,25).
The efflux of drugs from cancer cells is one of the mechanisms involved in drug resistance (42,43). Increases of drug efflux from cancer cells are mediated by the upregulation of ABC proteins, including the membrane transporters P-gp and MRP1, in MDR cells (44-46). LPR is also an important MDR-mediated protein (47). It has also been reported that Rg3 treatment is able to decrease MDR by altering the function of P-gp (9). In the present study, the expression of these proteins was evaluated in A549/DDP cells treated with Rg3. The results revealed that Rg3 treatment significantly decreased the mRNA and protein levels of P-gp, MRP1 and LRP in A549/DDP cells. Accordingly, a previous study reported that Rg3 treatment reversed the MDR of A549/DDP cells by the downregulation of membrane transporters (48).

\( ^{99m}\text{Tc-MIBI} \) is a well-documented tumor-seeking radiotracer, which is a substrate of P-gp, which is widely used for in vivo imaging, including cardiac breast, thyroid, bones, central nervous system and lung visualization (7,29,49,50). The accumulation of \( ^{99m}\text{Tc-MIBI} \) in tumor cells is increased by preventing the efflux transport function (51). In the present study, \( ^{99m}\text{Tc-MIBI} \) SPECT was used to monitor the reversed effect of Rg3 on P-gp-associated MDR in A549/DDP xenograft mice. The uptake ratio detected by \( ^{99m}\text{Tc-MIBI} \) imaging is positively associated with P-glycoprotein (P-gp) expression in A549/DDP xenograft tumors, which indicated that \( ^{99m}\text{Tc-MIBI} \) SPECT may be a valuable diagnostic imaging technique for assessing P-gp-associated MDR.

The study of MDR is valuable for identifying novel strategies to overcome the treatment failure of lung cancer. In the present study, Rg3 increased the sensitivity of A549/DDP cells to DDVP via downregulation of MDR-mediated proteins including P-gp, MRP1 and LRP. These results indicating that Rg3 may possess the potential to be used in reversing the MDR of lung cancer in patients experiencing treatment failure.

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