Resveratrol down-regulates survivin and induces apoptosis in human multidrug-resistant SPC-A-1/CDDP cells

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Abstract. We studied the effect of resveratrol treatment on multidrug-resistant human non-small cell lung cancer cells. Human multidrug-resistant SPC-A-1/CDDP cells were treated with resveratrol at a concentration of 25, 50, or 100 μM in *in vitro* studies and nude mice were implanted with multidrug-resistant SPC-A-1/and fed a special diet that included resveratrol at a dose of either 1 g/kg/day or 3 g/kg/day in *in vivo* studies. No adverse toxicological effects of resveratrol treatment were observed. The rate of cell proliferation, apoptosis ratio, cell cycle phase distribution, IC_{50} values of cisplatin, gefitinib, and paclitaxel, implanted tumour volume, and expression of survivin in resveratrol-treated and control mice were then determined. Resveratrol significantly inhibited the proliferation of SPC-A-1/CDDP cells, induced apoptosis, arrested the cell cycle phase between G_0-G_1 and S phase or at the G_2/M phase, decreased the IC_{50} values of multiple chemotherapeutic drugs, and showed anti-tumour effects in nude mice that had been implanted with SPC-A-1/CDDP cells. In additional, resveratrol affected the proliferation of SPC-A-1/CDDP cells in a dose- and time-dependent manner. Expression of survivin in SPC-A-1/CDDP cells decreased after they were treated with all concentrations of resveratrol and resveratrol was also found to have a dose-dependent effect on survivin expression. Resveratrol can induce apoptosis in multidrug-resistant human NSCLC SPC-A-1/CDDP cells by down-regulating the expression of survivin.

Introduction

Primary lung cancer is leading cause of cancer-related deaths worldwide and has a five-year overall survival rate of <15%. It is classified into two main histological groups: small cell lung cancer (SCLC) and the more common non-small cell lung cancer (NSCLC), which constitutes ~85% of all lung cancers (1). The poor prognosis of this malignancy may be partially based on the pronounced resistance to multiple chemotherapeutic drugs (multidrug resistance, or MDR) that is intrinsically present in most NSCLCs and is acquired during treatment in many SCLCs that are initially drug-sensitive (2,3). The intrinsic and acquired resistance of cancer cells to chemotherapeutic agents limits the efficacy of cancer treatment (4). MDR is defined as resistance to multiple structurally and functionally unrelated anticancer drugs that occurs after treatment with a single agent (5,6). Multiple mechanisms contribute to chemoresistance and eventually lead to the failure of cancer chemotherapy (7). MDR has become a serious clinical problem, especially in patients with NSCLC, thus the treatment of NSCLC presents a significant clinical challenge (8,9).

Resveratrol (3, 5, 4'-trihydroxy-stilbene) was first isolated from the roots of the white hellebore in 1940 and was later isolated from *Polygonum cupsidatum*, a medicinal plant (10,11) (Fig. 1). From a botanical perspective, resveratrol acts as a phytoalexin, which is a toxic compound produced by plants in response to a parasitic attack or under conditions of stress (12). Starting in the 1980s, resveratrol began attracting special attention because of its diverse pharmacological properties, which included cancer chemoprevention and the prevention of cardiovascular and neurodegenerative diseases (13). Many studies have been published demonstrating the beneficial effects of resveratrol in cellular systems (14). Studies have confirmed that resveratrol has chemopreventative properties for many types of cancer, including breast, prostate, colon, and lung cancers (15). A more recent breakthrough is the discovery that resveratrol can increase the lifespan of yeast. Resveratrol is also able to activate apoptosis and to arrest the cell cycle (16). It has been shown that resveratrol does not have cytotoxic effects in animal models, however, low doses of resveratrol can sensitise cancer cells to low doses of cytotoxic drugs and may provide an innovative strategy to enhance the efficacy of anticancer therapy in various human cancers (17-19). Although resveratrol exerts diverse biological activities that affect both the progression and regression of lung cancers, the chemopreventive potential of this diet-derived agent has not been well-studied in the treatment of

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multidrug-resistant cancers (20). Because of the biological properties described above, resveratrol appears to be a good candidate agent for chemopreventive or chemotherapeutic strategies and is believed to be a potential novel weapon in the treatment of multidrug-resistant NSCLC.

Cisplatin is an established anti-tumour agent that is used in the treatment of advanced human NSCLC. It is generally used to induce multidrug-resistant lung cancer cell lines in experimental studies to allow investigators to gain a deeper understanding of lung cancer (21). Although the development of resistance to cisplatin is one of the major obstacles faced in the successful treatment of NSCLC, the molecular mechanisms involved in the development of cisplatin resistance remain poorly understood (22). In order to overcome the problems related to drug resistance and to improve the clinical outcomes of patients with NSCLC, we sought to elucidate the effect that resveratrol has on the multidrug-resistant human NSCLC cells with the ultimate goal of identifying a novel therapy for multidrug-resistant NSCLC.

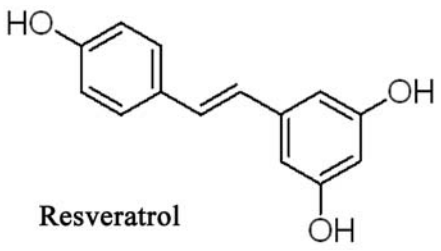

Figure 1. Chemical structure of resveratrol.

Materials and methods

Cell culture. The multidrug-resistant human cell line SPC-A-1/CDDP, which was generated at the Institute of Respiratory Disease of PLA (Xinqiao Hospital, Third Military Medical University, Chongqing, P.R. China), has stable cell biological characteristics and is a reliable model of multidrug-resistant NSCLC. The Institute of Respiratory Disease of PLA (Xinqiao Hospital, Third Military Medical University) provided us with human multidrug-resistant SPC-A-1/CDDP (non-small cell lung carcinoma) cells as a gift. Cells were grown in RPMI-1640 supplemented with 10% foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). The pH of the growth medium was adjusted from a pH of 7.2 to 7.4 by Na_2CO_3, and the cells were kept in a humidified incubator 37˚C that was perfused with a gas mixture containing 1% O_2, 5% CO_2, and 95% N_2. An additional three groups of cells were treated with differing concentrations (25, 50, or 100 μmol/l) of resveratrol (purity >98%, Sigma, USA) and stored in an incubator containing the same atmosphere.

Cell proliferation analysis. Cells were digested with 0.25% trypsin and 0.01% EDTA and then adjusted to be grown at a density of $1x10^4$ cells/ml. Cells were cultured in DMEM medium supplemented with 10% foetal bovine serum (both from Gibco/Invitrogen, NY, USA) in a 37˚C incubator with a humidified atmosphere containing 5% CO_2 and 95% O_2. After 24, 48, 72, and 96 h, the number of cells was determined by haemocytometer. Cell concentration was calculated as follows: cell concentration per millilitre = total cell count in 4 squares x 2500 x the dilution factor.

Cell cycle analysis. The cell cycle distribution of the cells in culture was analysed by flow cytometric analysis as previously described. Briefly, following incubation for 24, 48, or 72 h, cells were digested with 0.25% trypsin (Sigma, St. Louis, MO, USA), counted, centrifuged at 300 x g for 5 min, and fixed in ethanol at 4˚C overnight. Then, the cells were washed and centrifuged. The resulting cell pellets were resuspended in an RNase solution (0.02 mg/ml; Sigma) containing propidium iodide (0.02 mg/ml; Sigma), and incubated at 4˚C for 30 min. The DNA content of approximately $1-2x10^5$ stained cells were analysed using a FACScan flow cytometer equipped with the FACStation data management system running Cell Quest software (Becton Dickinson, San Jose, CA, USA). The results were expressed as a plot of fluorescence intensity versus cell number.

Annexin V/propidium iodide. To determine the number of apoptotic cells present in our samples, Annexin V assays were performed using an apoptosis detection kit (Annexin V-FITC/PI staining kit; Immunotech Co., Marseille, France). Briefly, SPC-A-1/CDDP cells were seeded in 24-well plates and treated with resveratrol at differing concentrations for 24, 48 or 72 h in medium containing 1% FBS. Cells were harvested and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. Next, 5 μl of Annexin V-FITC (BD Pharmingen) and 10 μl of PI (50 μg/ml) (Sigma) were added to 100 μl of resuspended cells, which were then incubated for 15 min with fluorescein-conjugated annexin V and propidium iodide and analysed using the same flow cytometer and software used for the cell cycle analysis. PI-negative, annexin V-positive cells were considered to be 'early apoptotic' cells, while cells that were both PI and annexin V negative were considered normal cells.

MTT assay. The number of viable cells was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, logarithmic phase SPC-A-1/CDDP cells were cultured in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of $1x10^4$ cells/well. Cells were treated with cisplatin, gefitinib, or paclitaxel at the indicated concentrations. Each drug was applied to the SPC-A-1/CDDP cells at five different concentrations, each 5-fold larger than the next. After a 48 h incubation period, MTT (Sigma) dissolved in PBS was added to each well at a final concentration of 5 mg/ml and the cells were incubated at 37˚C for 4 h. The water-insoluble dark blue formazan crystals that formed during the cleavage of MTT in cells that were dissolved in DMSO. The optical density was then measured at a wavelength of 550 nm with a Bio-Rad 680 microplate reader (Bio-Rad, CA, USA). All experiments were performed in triplicate. The cell-growth inhibition potency of each chemotherapeutic agent is expressed as an IC_{50} value, which is defined as the concentration of a drug necessary to inhibit the growth of cells by 50%. The IC_{50} of each drug was calculated using a logarithmic plot. The inhibitory rate was

Table I. Effect of resveratrol on SPC-A-1/CDDP cells proliferation (x10^4).

Treatment	0 h	24 h	48 h	72 h	96 h
Control	1.03±0.11	1.52±0.14	2.78±0.23	6.27±0.48	10.17±0.72
Resveratrol (25 μmol/l)	0.98±0.12	1.46±0.16	2.23±0.21[a]	3.77±0.29[a,b]	5.69±0.61[a,b]
Resveratrol (50 μmol/l)	1.00±0.09	1.42±0.10	2.10±0.17[a]	3.59±0.35[a,b]	5.47±0.49[a,b]
Resveratrol (100 μmol/l)	1.01±0.08	1.39±0.13	2.06±0.19[a]	3.14±0.27[a]	4.42±0.37[a]

Cell numbers of SPC-A-1/CDDP were calculated by hemacytometer at 24, 48, 72, or 96 h respectively after treated with resveratrol at 25, 50, or 100 μmol/l. The values are shown as the mean ± SEM. [a]P<0.05, compared to the control group; [b]P<0.05, compared to the group treated with 100 μmo/l resveratrol [least significant difference (LSD) test by ANOVA].

calculated as follows: inhibitory rate = (1-$OD_{drug}/OD_{control}$) x100%.

Mouse xenograft model of multi-drug resistant NSCLC. A solid tumour model was developed from cultured SPC-A-1/CDDP cells. Eighteen female BALB/C nude mice (18-22 g) were randomised to three groups and had a 1 ml suspension of SPC-A-1/CDDP cells (1x10^8/each) injected at flank subcutaneously. The three groups were treated as follows: mice in Group C were free control, mice in Group T1 were treated with 1 g/kg/day resveratrol, and mice in Group T2 were treated with 3 g/kg/day resveratrol. The tumours were measured when the animals were sacrificed on day 28 and tumour volumes were calculated from caliper measurements using the following formula: Vt = [(LxW^2)/2] (L, longest diameter; W, shortest diameter) (23). Food and water were given *ad libitum*. All animal experimental procedures were approved by the Animal Care and Use Committee of Fourth Military Medical University.

Western blot analysis. Protein extracts of the SPC-A-1/CDDP cells were prepared by lysing the cells in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl; pH 8) along with 10 mM EDTA, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonylfluoride (PMSF; all from Sigma) for 30 min at 4˚C. Samples were then centrifuged at 14,000 x g for 25 min at 4˚C. The protein concentration in the supernatant was determined using the BCA protein assay kit (HyClone-Pierce, UT, USA). Equivalent amounts (60 μg protein/lane) of protein lysates were electrophoretically separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (0.22 μm, Invitrogen) in a transfer tank (Bio-Rad) using the submerged method. The membrane was blocked for 2 h at room temperature in PBS containing 0.1% Tween-20 (Sigma) and 5% non-fat dried milk (Carnation, USA), and then incubated with the primary antibody in dilution buffer (1X TBS, 0.1% Tween-20 with 5% BSA) with gentle agitation overnight at 4˚C. The primary antibodies we used were anti-survivin and anti-caspase 3 (diluted 1:400, 1:200, Santa Cruz, CA, USA, respectively), and anti-ß-actin (diluted 1:400, Santa Cruz). Then, the membranes were washed in dilution buffer (1X TBS, 0.1% Tween-20 with 5% BSA) and incubated for 1 h with horseradish-peroxidase-conjugated (HRP-conjugated) secondary antibodies, and finally developed using an ECL system (Cell Signaling Technology, Beverly, MA, USA). The secondary antibodies we used were HRP-conjugated anti-rabbit IgG (diluted 1:2000) and HRP-conjugated anti-mouse IgG (diluted 1:2000, both from Cell Signaling Technology). The Western blots were performed following Laemmli's method and the greyscale values of each band on the blots were measured using BandScan 4.3.

Statistical analysis. All statistical analyses were carried out using SPSS 14.0 statistical software. Data are expressed as the mean ± the standard error of mean (SEM) of separate experiments. Pathological classifications were analysed by the Friedman test and the Q-test. Differences in measured parameters between the groups were analysed using one-factor ANOVA and Fisher's least significant difference test. P≤0.05 was considered statistically significant.

Results

Effect of resveratrol on SPC-A-1/CDDP cell proliferation. The number of viable SPC-A-1/CDDP cells was counted using a haemocytometer after they were incubated with resveratrol for 24, 48, 72, and 96 h (Table I). Compared to untreated samples, those treated with resveratrol had fewer viable cells present, indicating that resveratrol significantly inhibited SPC-A-1/CDDP cell proliferation. Table I shows the number of viable SPC-A-1/CDDP cells present at the indicated time points in the control group and in the groups treated with 25, 50, or 100 μmol/l resveratrol. Resveratrol inhibited SPC-A-1/CDDP cell proliferation in a dose- and time-dependent manner. Cell proliferation did not differ significantly between the control group and the resveratrol-treated groups at 24 h. However, resveratrol was found to inhibit cell proliferation at 48 h (P<0.05), although there were no significant differences observed between the different concentrations of resveratrol used (P>0.05). At 72 and 96 h, there were also found to be fewer viable cells in the resveratrol-treated groups and resveratrol was found to inhibit cell proliferation in a dose-dependent manner: cells treated 100 μmol/l resveratrol exhibited significantly growth inhibition compared with cells treated with 25 and 50 μmol/l resveratrol (P<0.05). No significant differences were observed between cells treated with 25 μmol/l resveratrol as compared to cells treated with 50 μmol/l resveratrol (P>0.05).

Table II. Effects of resveratrol on the 50% inhibitory concentration of cisplatin, gefitinib, and paclitaxel (μg/ml).

Treatment	Cisplatin	Gefitinib	Paclitaxel
Control	0.6824±0.057	0.5713±0.043	0.3812±0.021
Resveratrol (25 μmol/l)	0.4617±0.035[a,b]	0.3269±0.045[a,b]	0.2358±0.025[a,b]
Resveratrol (50 μmol/l)	0.4369±0.041[a,b]	0.2984±0.034[a,b]	0.2157±0.018[a,b]
Resveratrol (100 μmol/l)	0.2975±0.033[a]	0.0871±0.011[a]	0.0949±0.012[a]

Effect of cisplatin, gefitinib, and paclitaxel on IC_{50} of SPC-A-1/CDDP cells were deteced by MTT assay respectively after treated with resveratrol at 25, 50, or 100 μmol/l. The values are shown as the mean ± SEM. [a]P<0.05, compared to the control group; [b]P<0.05, compared to the group treated with 100 μmol/l resveratrol [least significant difference (LSD) test by ANOVA].

Table III. Effect of resveratrol on SPC-A-1/CDDP cells cycle distribution (%).

Treatment	G_0-G_1 (%)	S (%)	G_2-M (%)
Control	58.22±3.12	28.92±2.83	12.86±1.13
Resveratrol (25 μmol/l)	68.77±8.54[a,b]	21.92±2.27[a,b]	9.31±1.01[a,b]
Resveratrol (50 μmol/l)	71.13±7.53[a,b]	20.38±2.39[a,b]	8.49±0.92[a,b]
Resveratrol (100 μmol/l)	81.17±8.22[a]	12.35±1.37[a]	6.48±0.74[a]

SPC-A-1/CDDP cells were treated resveratrol with at various concentrations (25, 50, or 100 μmol/l) for 48 h. Resveratrol caused an accumulation of cells in the G_0-G_1 phases and diminished cells in the S and G_2-M phases compared with control untreated cells. Data are presented as mean ± SE. [a]P<0.05, compared to the control group; [b]P<0.05, compared to the group treated with 100 μmol/l resveratrol [least significant difference (LSD) test by ANOVA].

Table IV. Effect of resveratrol on apoptosis of SPC-A-1/CDDP cells (%).

Treatment	24 h	48 h	72 h
Control	1.6±0.21	3.4±0.48	6.5±0.71
Resveratrol (25 μmol/l)	5.9±0.68[a,b]	15.9±1.87[a,b]	21.9±2.84[a,b]
Resveratrol (50 μmol/l)	6.6±0.71[a,b]	17.7±2.14[a,b]	24.5±3.27[a,b]
Resveratrol (100 μmol/l)	7.7±0.83[a]	26.9±3.24[a]	42.8±5.63[a]

SPC-A-1/CDDP cells were treated resveratrol with at various concentrations (25, 50, or 100 μmol/l) for 24, 48 or 72 h. Resveratrol caused an increasing of apoptosis compared with control untreated cells. Data are presented as mean ± SE. [a]P<0.05, compared to the control group; [b]P<0.05, compared to the group treated with 100 μmol/l resveratrol [least significant difference (LSD) test by ANOVA].

Effects of resveratrol on the IC_{50} of cisplatin, gefitinib, and paclitaxel. The effects of resveratrol on the IC_{50} of cisplatin, gefitinib, and paclitaxel in SPC-A-1/CDDP cells were assessed by the MTT assay. Table II shows the IC_{50} values for cisplatin, gefitinib, and paclitaxel in untreated SPC-A-1/CDDP as well as cells treated with resveratrol. The IC_{50} of each drug was significantly lower for cells treated with resveratrol as compared to untreated cells (P<0.05). Additionally, resveratrol had a dose-dependent influence on the IC_{50} of each drug. Cells treated with 100 μmol/l resveratrol had significantly lower IC_{50} values for each drug as compared to cells treated with 25 or 50 μmol/l resveratrol (P<0.05). There was no significant difference observed between the IC_{50} values of cells treated with 25 μmol/l resveratrol as compared to cells treated with 50 μmol/l resveratrol (P>0.05).

Effects of resveratrol on the cell cycle distribution of SPC-A-1/CDDP cells. The cellular DNA content of resveratrol-treated cells and untreated cells was analysed by flow cytometry to detect changes in the cell cycle distribution of the SPC-A-1/CDDP cells. DNA histogram analyses showed that resveratrol induced changes in the cell cycle profile of treated cells (Table III). Specifically, resveratrol triggered a significant increase in the number of cells in G_0-G_1 phase and a corresponding decrease in the number of cells in S and G_2-M phase. Similar to the findings of the IC_{50} analysis, resveratrol induced changes in the cell cycle distribution of SPC-A-1/CDDP cells in a dose-dependent manner. Treatment with 100 μmol/l resveratrol was found to induce a significantly larger change in the distribution of cells in the cell cycle as compared treatment with 25 or 50 μmol/l resveratrol (P<0.05). There were no significant differences in cell cycle distribution noted between cells treated with 25 μmol/l resveratrol as compared to cells treated with 50 μmol/l resveratrol (P>0.05).

Effects of resveratrol on induction of apoptosis in SPC-A-1/CDDP cells. To quantify the early apoptotic effects resveratrol had on SPC-A-1/CDDP cells, the cells were stained with annexin V and propidium iodide (PI) following resveratrol exposure. Apoptosis of SPC-A-1/CDDP cells occurred after

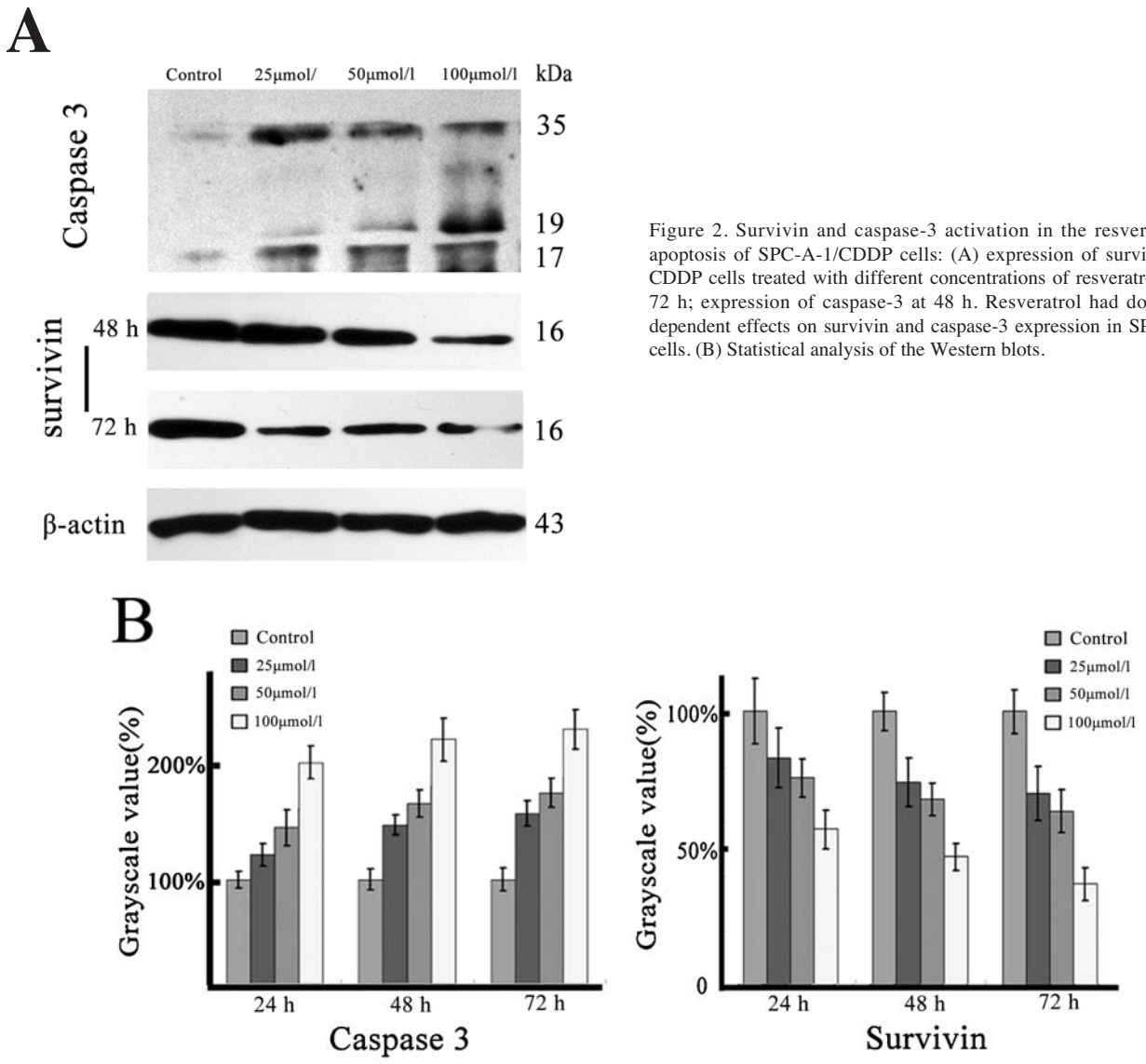

Figure 2. Survivin and caspase-3 activation in the resveratrol-induced apoptosis of SPC-A-1/CDDP cells: (A) expression of survivin SPC-A-1/CDDP cells treated with different concentrations of resveratrol at 48 h and 72 h; expression of caspase-3 at 48 h. Resveratrol had dose- and time-dependent effects on survivin and caspase-3 expression in SPC-A-1/CDDP cells. (B) Statistical analysis of the Western blots.

resveratrol treatment, and the number of apoptotic cells increased in a time- and dose-dependent manner, with all resveratrol concentrations (25, 50, or100 μmol/l) inducing apoptosis at all time points (24, 48, and 72 h) (Table IV).

To investigate the contribution of the extrinsic apoptotic pathway in resveratrol-induced apoptosis, we assessed the activation of caspase-3, and caspase-3 was found to be activated in cells undergoing resveratrol-induced apoptosis. Fig. 2A shows that in SPC-A-1/CDDP cells treated with resveratrol (at all concentrations), caspase-3 was rapidly and substantially cleaved. Additionally, greatly increased caspase-3 activation was observed 72 h after treatment with 100 μmol/l resveratrol as compared to treatment with 25 μmol/l or 50 μmol/l (234±26.7% versus 197±20.4% and 189±16.3%, respectively; $P<0.05$). Thus, our data suggested that the extrinsic apoptotic pathway (mediated by caspase-3) was activated in resveratrol induced apoptosis and that resveratrol had a dose- and time-dependent effect on the expression of caspase-3.

Survivin expression in SPC-A-1/CDDP cells treated with resveratrol. To determine the involvement of survivin in resveratrol-induced apoptosis of SPC-A-1/CDDP cells, the steady-state levels of survivin were measured by Western blot analysis (Fig. 2A). Survivin expression decreased significantly after treatment in all cell samples treated with resveratrol (at concentrations of 25, 50 or 100 μmol/l) 24, 48, and 72 h after treatment as compared to the control group. Additionally, resveratrol inhibited survivin expression in a dose-dependent manner. Treatment of SPC-A-1/CDDP cells with 100 μmol/l resveratrol decreased survivin expression significantly more than treatment with 25 μmol/l or 50 μmol/l resveratrol ($P<0.05$). For example, the ratio of survivin expression in resveratrol-treated cells as compared to controls was 74.0±9.2%, 67.8±6.1%, and 46.9±5.8% in cells treated with 25, 50 or 100 μmo/l resveratrol, respectively, at 48 h.

Western blot analysis revealed that survivin levels were decreased in SPC-A-1/CDDP cells undergoing resveratrol-induced apoptosis, indicating that inhibition of surviving expression is the mechanism underlying resveratrol-induced apoptosis.

Anti-tumour effects of resveratrol in nude mice. Having shown the anti-proliferative activity of resveratrol *in vitro*, we went on to evaluate the anti-tumour properties of resveratrol

Table V. Anti-tumor effect of resveratrol on SPC-A-1/CDDP cells in nude mice.

Treatment	Tumour size (mm^3)	Tumour weight (g)
Control	895.67±157.23	0.72±0.17
1 g/kg/day	527.5±75.7[a,b]	0.41±0.13[a,b]
3 g/kg/day	287.9±81.2[a]	0.23±0.04 [a]

Nude mice with SPC-A-1/CDDP transplant tumor were treated with (orally) resveratrol 1 g/kg or 3 g/kg every day for 28 days. Data are presented as mean ± SE. [a]P<0.05, compared to the control group; [b]P<0.05, compared to the group treated with 3 g/kg/day resveratrol [least significant difference (LSD) test by ANOVA].

in nude mice that had been implanted with SPC-A-1/CDDP cells. Compared to the control group, the group treated with resveratrol (either 1 g/kg/day or 3 g/kg/day) demonstrated significantly inhibited tumour growth, both in terms of tumour size and weight (Table V and Fig. 3). Additionally, resveratrol was found to inhibit the tumour growth in a dose-dependent manner (P<0.05). No adverse effects of resveratrol were observed in treated mice.

Discussion

Multidrug resistance is the major cause of chemotherapy failure in non-small cell lung cancer patients (24). The causes of MDR-induced chemotherapy failure are complex and may be determined by multiple tumour characteristics, such as the proportion of tumour cells that are not actively dividing, the adequacy of the tumour's blood supply, as well as the multidrug resistance phenotype (25). Several mechanisms are responsible for the development of MDR, including the overexpression of transmembrane transporter molecules, specifically resistance P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP), which have been defined as cellular drug targets of chemotherapeutic agents (26). However, several MDR cell lines have been described that do not overexpress either Pgp or MRP, indicating that alternative mechanisms of MDR must be occurring (27). In lung cancer, the role that the different forms of multidrug resistance play is complex and only partially understood. Therefore, it is critical to identify proteins or genes that can be used to predict chemotherapy success and that can be used in the development of chemo-prevention strategies for multidrug-resistant NSCLC (28,29).

It has been revealed that resveratrol has the ability to suppress growth of many chemically induced and spontaneously occurring tumours. Furthermore, resveratrol has been shown to inhibit the local growth and dissemination of several transplantable tumours in laboratory animals (30). Due to its valuable biological properties and lack of significant side effects even after long-term administration, it seems that resveratrol is one of the most promising agents that could be applied in the future in cancer prevention strategies and cancer therapy in humans (31).

In this study, we examined the effect of resveratrol on multidrug-resistant human NSCLC cells (SPC-A-1/CDDP

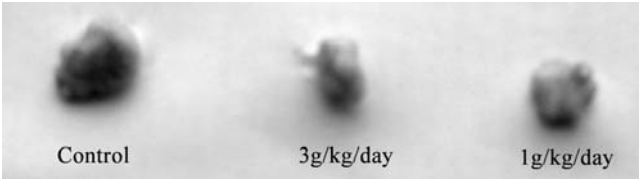

Figure 3. Anti-tumour effect of resveratrol on SPC-A-1/CDDP cells in nude mice: dose-dependent effects of resveratrol on tumour volume and tumor weight in nude mice injected with SPC-A-1/CDDP cells.

cells) both *in vitro* and *in vivo*. We found that resveratrol significantly inhibited SPS-A-1/CDDP cell proliferation at each concentration we tested, but the effect was most pronounced in cells treated with 100 μM resveratrol at 96 h compared with free-control cells. In the following experiment, we found that resveratrol could also induce apoptosis and arrest the cell cycle at G_0-G_1 and could do so in a time- and dose-dependent manner, with the 100 μmo/l concentration having the largest effect on apoptosis and cell cycle distribution. Thus, resveratrol had dose- and time-dependent effects on the SPC-A-1/CDDP cell proliferation, apoptosis and cell cycle phase distribution. These findings suggest that high-dose resveratrol may be more effective than low-dose resveratrol in the treatment of drug-resistant human non-small cell lung cancer SPS-A-1/CDDP cells and that resveratrol may be a novel way to treat actual patients' tumours.

In the next set of experiments, we studied the effect of resveratrol on the IC_{50} of the anti-tumour drugs cisplatin, gefitinib and paclitaxel. Results showed that the treatment of SPS-A-1/CDDP cells with resveratrol significantly decreased the IC_{50} of each of the anti-tumour drugs we tested, suggesting that resveratrol increased the chemosensitivity SPC-A-1/CDDP cells. Therefore, resveratrol may be a good adjuvant treatment for patients suffered from multidrug-resistant NSCLC. Additionally, resveratrol had a dose-dependent effect on the IC_{50} of the various chemotherapeutic drugs that were tested, with 100 μmol/l resveratrol causing the largest reduction in the IC_{50} values. In our *in vivo* studies, we observed that resveratrol had anti-tumour effects on nude mice that had been injected with SPC-A-1/CDDP cells. Resveratrol led to significantly lower tumour volumes in mice either that were fed either 1 or 3 g/kg/day resveratrol as compared to untreated mice. The effect was especially marked in the mice treated with the highest dose of resveratrol, thus again demonstrating that resveratrol has a dose-dependent effect on tumours. These findings suggest that we should seek to identify the maximal safe dose of resveratrol both *in vitro* and *in vivo*.

We next examined survivin expression in resveratrol-treated and control SPC-A-1/CDDP cells. Survivin is a member of the inhibitor of apoptosis protein (IAP) family that inhibits apoptosis and regulates cell division (32). Survivin has been implicated in both the control of cell division and the inhibition of apoptosis (33). Specifically, its anti-apoptotic function seems to be related to the ability to directly or indirectly inhibit caspases. Survivin is expressed in embryonic tissues as well as in the majority of human cancers, but is not expressed in most normal adult tissues and is associated with chemotherapy resistance (34), and patients with survivin-expressing

tumours have poorer survival (35). Studies have found that patients with tumours that do not express survivin or only express it in low levels have more favourable outcomes. Conversely, high survivin expression was found to be correlated with unfavourable outcomes (36). The cancer-specific expression of survivin in adults, coupled with its importance in inhibiting cell death and in regulating cell division, makes it a useful diagnostic marker for human cancers as well as a potential target for cancer treatment. Based on these findings, survivin has been proposed as an attractive target for new anticancer interventions (37,38). In our last experiment, we found that the expression of survivin in SPC-A-1/CDDP cells decreased after treatment with resveratrol (at any concentration). Resveratrol was found to decrease survivin levels in a time- and dose-dependent manner. We also examined another pathway involved in apoptosis, the caspase cascade, which cleaves key cellular components, including cytoskeletal proteins and nuclear proteins, and leads to an altered cellular morphology, which we observed in resveratrol-treated SPC-A-1/CDDP cells.

Our study showed that caspase-3 was activated immediately after exposure to resveratrol. However, inhibition of survivin expression and initiation of the caspase cascade occurred at almost the same time, so the caspase cascade may in fact be activated by the inhibition of survivin. We have not excluded a caspase-independent mechanism for resveratrol-induced apoptosis. However, we do think that both the survivin-mediated intrinsic pathway, as well as the caspase-3-mediated extrinsic pathway, play an important role in resveratrol-induced apoptosis. Although there is a lot of work yet to be done to study the mechanism by which resveratrol exerts an effect on survivin, survivin may be a novel target for chemotherapeutic drugs and resveratrol may play an important role in the treatment of multidrug-resistant human NSCLC.

In conclusion, resveratrol exerted dose- and/or time-dependent effects on the inhibition of SPC-A-1/CDDP cell proliferation and the induction of apoptosis in these cells. It also arrested the cell cycle at phase G_0-G_1, decreased the IC_{50} values of different anti-tumour drugs, and decreased survivin expression in mice injected with SPC-A-1/CDDP cells (thereby exerting an anti-tumour effect) in a dose- and time-dependent manner.

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