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Effect of etoposide-induced alteration of the Mdm2-Rb signaling pathway on cellular senescence in A549 lung adenocarcinoma cells

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Abstract. The present study aimed to investigate the effect of various concentrations of etoposide (VP-16) on the E3 ubiquitin-protein ligase Mdm2 (Mdm2)-retinoblastoma (Rb) signaling pathway in the cellular senescence of A549 lung adenocarcinoma cells. A549 cells were randomly divided into the following four groups: Control group (no treatment), group 1 (1 μmol/l VP-16), group 2 (5 μmol/l VP-16) and group 3 (25 μmol/l VP-16). Each group was cultured for 48 h after treatment prior to observation of the alterations to cellular morphology. The cell cycle distribution of each group was also detected by flow cytometry. In addition, the activity of cellular senescence-associated β-galactosidase, and the expression of Mdm2 and phosphorylated (p-) Rb protein, was measured. The percentage of senescent cells was significantly higher following VP-16 treatment compared with the control group. The percentage of G_1 phase cells, and p-Rb protein and Mdm2 protein expression were also significantly different following VP-16 treatment compared with the control group. VP-16 increased the activity of β-galactosidase in the A459 cells. VP-16 also decreased the expression level of Mdm2 and p-Rb protein and inhibited cell cycle progression in G_1. These results indicate that VP-16 induces the cellular senescence of A549 cells via the Mdm2-Rb signaling pathway. However, further investigations are required to validate the mechanisms underlying these effects of VP-16.

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Introduction

Lung cancer is a common primary pulmonary malignant tumor, with high incidence and mortality rates worldwide (1). In recent years, the incidence of lung cancer has increased annually and exhibited a trend towards those of younger ages (1). The 5-year survival rate of lung cancer in China is only 10%, since the majority of patients have mid-late stage lung cancer or are no longer eligible for surgery at the time of diagnosis (2). The typical chemotherapeutic approach for lung cancer is to inhibit DNA replication or metabolic enzymes, thus interfering with cell division (3); however, the side effects of these treatments mean that patients do not tolerate them well. Furthermore, the intermittent period between these treatments is long, which may lead to disease recurrence and drug resistance (4). Therefore, it is necessary to develop novel therapeutic strategies for the treatment of lung cancer. Previous studies have suggested that a low-dose and high frequency/long duration of treatment with certain chemotherapeutic drugs may induce cellular senescence, improving the patient's quality of life and prolonging survival; this chemotherapy is called continuous low-dose chemotherapy (LDM) (5,6).

Compared with traditional chemotherapy, LDM has several advantages, including a lower toxicity, lower cost and shorter required intermittent period. This may reduce disease recurrence and drug resistance, in addition to making it easier for patients to receive and accept long-term treatment, thus improving their quality of life and prolonging survival (7,8). However, the mechanism underlying the effects of LDM remains unclear.Induction of cellular senescence may inhibit the cell cycle in G_1 phase (9).

Cellular senescence refers to the transition of a cell from an active growing state to an irreversible growth arrest state (10,11). It was previously demonstrated that senile cells primarily contained G_1 phase DNA, suggesting that they were arrested in G_1 (12). Activation of the retinoblastoma (Rb) protein is associated with G_1 phase cell cycle arrest. It has previously been revealed that negative feedback regulation of the E3 ubiquitin-protein ligase Mdm2 (Mdm2)-Rb signaling pathway serves a role in tumorigenesis (13,14).

Etoposide (VP-16) is as an antitumor drug that specifically targets the cell cycle (15). The present study investigated the effect of various concentrations (1-25 μmol/l) of VP-16 on the Mdm2-Rb signaling pathway and cellular senescence in A549 lung adenocarcinoma cells.

Materials and methods

Cell culture. The A549 lung cancer cell line was obtained from the Cancer Laboratory of the First Affiliated Hospital of Chengdu Medical College (Chengdu, China). A549 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C with 5% CO_2 and saturated humidity. Cells in the logarithmic phase were digested using 0.25% trypsin (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) for 2-3 min and then the cell suspension was obtained.

Observation of cell morphology and cell cycle analysis. A549 cells in the logarithmic phase of growth were seeded into 6-well plates ($2x10^5$/well) and cultured at 37˚C for 24 h. Subsequently, 0 (control group), 1 (group 1), 5 (group 2) and 25 (group 3) μmol/l VP-16 (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) was added and the cells were cultured for a further 48 h. The cells were then placed under an inverted microscope. Six fields of view were randomly selected for observation and determination of cell morphologies. The cell cycle distribution was detected by flow cytometry using a FACSCalibur flow cytometer with BD FACStation software (ImagePro-Plus v6.0) (both BD Biosciences, Franklin Lakes, NJ, USA).

Detection of senescence. Prior to cell seeding, 6-well plates were placed on a sterilized tray and $1x10^5$ cells were added to each well. Then the 6-well plates were covered with plastic film, and the cells were cultured overnight at 37˚C. Various concentrations (0, 1, 5 and 25 μmol/l) of VP-16 were added to the culture medium, followed by culture at 37˚C for 48 h. One ml of senescence-associated β-galactosidase fixation fluid (Beyotime Institute of Biotechnology, Haimen, China) was added, followed by incubation at room temperature for 15 min. The senescence-associated β-galactosidase was detected using a previously described method (16) and was used to calculate the percentage of senescent cells in each treatment group.

The use of X-Gal as a substrate for the β-galactosidase enzyme generates deep blue colored products, thus senile cells were defined as those with blue granules when observed under a DVM6 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Immunocytochemistry. Prior to cell seeding, the 6-well plates were placed on a sterilized tray and $1x10^5$ cells were added to each well. The cells were then cultured overnight at 37˚C. Subsequently, 0, 1, 5 and 25 μmol/l VP-16 were added to the culture medium and the cells were cultured for a further 48 h culture at 37˚C. Cells were incubated overnight at 4˚C with the following primary antibodies: Polyclonal rabbit anti-human Mdm2 antibody (catalogue number PAB27165; dilution 1:200; Abzoom Biolabs, Inc., Dallas, TX, USA), polyclonal rabbit anti-human p-Rb antibody (catalogue number FZ200784; dilution 1:100; Shanghai Fuzhong Biological Science Co., Ltd., Shanghai, China). After adding polymer enhancer and PBS washing, 50 μl of goat anti-rabbit IgG horseradish peroxidase-labeled secondary antibody (catalogue number 150077; dilution 1:200; Abcam, Cambridge, USA) was drop wisely added to each section, followed by incubation at 37˚C for 30 min and PBS washing for 3 times. Following coloration, counterstain and mounting, the sections were observed using a Q550CW image acquisition and analysis system (Leica Microsystems GmbH).

Table I. Effect of VP-16 on the cell cycle distribution of A549 cells.

	Percentage of cells		
Group	G_1	S	G_2
Control	56.70±1.17	32.51±2.52	11.01±1.25
Group 1	60.91±0.26	29.21±1.71	9.83±0.78
Group 2	77.35±2.32[a]	12.31±2.79[a]	10.31±0.76
Group 3	46.17±2.73[a]	43.11±2.28[a]	10.71±0.88

[a]P<0.05 vs. the control group.

Statistical analysis. Results are expressed as the mean ± standard deviation. One-way analysis of variance was performed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell morphology changes. Prior to treatment with VP-16, the number of cells and cell morphology of the control and experimental groups was similar. Following a 48 h treatment with VP-16 (Fig. 1), the control group cells grew with uniform sizes and shapes (Fig. 1A), whereas group 2 demonstrated significantly decreased cell numbers and irregular morphology, including increased cell volumes, pseudopodia, large nuclei, reduced cytoplasm and intracytoplasmic vacuoles (Fig. 1C). Groups 1 and 3 demonstrated no notable changes in cellular morphology (Fig. 1B and D, respectively).

VP-16 alters the cycle distribution of A549 cells. The results of flow cytometry analysis demonstrated that, compared with the control group, in group 2 the percentage of cells in G_1 phase was significantly increased and the percentage of cells in S phase was significantly decreased (both P<0.05; Table I). In group 3, the percentage of cells in the G_1 was significantly decreased and the percentage of cells in S phase was significantly increased (P<0.05; Table I). There as no significant differences between the percentage of cells in G_1 or S phases between group 1 and the control group, and no significant difference in the percentage of cells in G_2 phase was revealed between the four groups (Table I).

Table II. Effect of VP-16 on the senescence of A549 cells.

Group	Percentage of senescent cells
Control	1.41±1.06
Group 1	11.03±1.82[a]
Group 2	79.11±6.09[a,b]
Group 3	5.62±1.16[a-c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. group 1; [c]P<0.05 vs. group 2.

Table III. Effect of VP-16 on the expression of Mdm2 protein in A549 cells.

Group	Percentage of Mdm2 protein-positive cells
Group	90.18±2.38
Group 1	87.03±3.86
Group 2	65.60±6.81[a-c]
Group 3	86.50±4.01

[a]P<0.05 vs. the control group; [b]P<0.05 vs. group 1; [c]P<0.05 vs. group 3. Mdm2, E3 ubiquitin-protein ligase Mdm2.

Table IV. Effect of VP-16 on the expression of phosphorylated Rb protein in A549 cells.

Group	Percentage of phosphorylated Rb protein-positive cells
Control	90.23±2.24
Group 1	86.51±3.43
Group 2	59.12±7.66[a-c]
Group 3	86.15±13.51[c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. group 1; [c]P<0.05 vs. group 3. Rb, retinoblastoma.

VP-1 effects the senescence of A549 cells. Detection of senescence-associated β-galactosidase is an important staining method that can be used to effectively detect senile cells. The lysosome contents of senile cells are increased, which induces an increased expression of the lysosomal enzyme β-galactosidase. Following treatment with 1, 5 or 25 μmol/l VP-16 for 48 h, the cells contained blue particles (Fig. 2), indicating that VP-16 induces the senescence of A549 cells. Group 2 has the highest level of the staining, whereas that in groups 1 and 3 was not as high (Table II). The percentage of senescent cells between the control group and experimental groups, in addition to the comparisons between the experimental groups, were significantly different (P<0.05; Table II).

Impact of VP-16 treatment on Mdm2 protein expression. Mdm2 protein was primarily expressed as brownish-yellow/tan particles in the cytoplasm and nuclei of A549 cells (Fig. 3). Following treatment with various concentrations of VP-16 (0, 1, 5 and 25 μmol/l) for 48 h, group 2 exhibited significantly decreased expression of Mdm2 protein compared with the control group and groups 1 and 3 (P<0.05; Table III). The differences in the expression of Mdm2 between the control group and groups 1 and 3 were not significant, and the difference between groups 1 and 3 was also not significantly different (Table III).

Effect of VP-16 on the expression of p-Rb protein. The Rb protein was primarily identified as a nuclear phosphoprotein inside the nuclei of the A549 cells (Fig. 4). Following treatment with various concentrations of VP-16 (0, 1, 5 and 25 μmol/l) for 48 h, group 2 exhibited a significantly decreased expression p-Rb protein compared with the control group and groups 1 and 3 (P<0.05), whereas the differences between the control group and experimental groups 1 and 3 was not significantly different (Table IV). The difference between groups 1 and 3 was also not significantly different (Table IV).

Discussion

During the 1960 s, Hayflick *et al* (17) demonstrated in a fibroblast culture that normal diploid cells would enter a state of senescence when proliferated for the 50-70th generation *in vitro*. No further subculture could continue while the cells remained in senescence; this phenomenon was named the 'Hayflick limit'. A previous study (18) revealed that cellular senescence is the third most important cancer prevention process, following cell DNA repairing and apoptosis; therefore, it is closely associated with the occurrence, development and treatment strategy for tumors.

It has been demonstrated that senile cells primarily contain G_1 phase DNA, thus it was considered that the senile cells were arrested in phase G_1 and were not able to enter phase S successfully (19). The Mdm2-Rb signaling pathway may induce the arrest the cell in G_1, which can result in cellular senescence (20). A low-concentration of VP-16 may also induce cellular senescence. A low-concentration VP-16 acts on the Mdm2-Rb signaling pathway, increasing the expression of Rb protein, decreasing the phosphorylation of Rb and decreasing the expression of Mdm2, thus promoting the senescence of tumor cells (21). Cellular senescence has various characteristics, including the generation of long pseudopodia and increased β-galactosidase activity (22,23).

If normal cells did not undergo senescence, it would result in the development of tumors; therefore, inducing tumor cell senescence has become a focus for studies investigating treatments for cancer. Previous studies have demonstrated that appropriate concentrations of DNA replication inhibitor agents, including Adriamycin (24-26), aphidicolin and cisplatin (27) may induce the phenotypes of cellular senescence. Furthermore, ionizing radiation, cytarabine, etoposide, paclitaxel, vincristine, hydroxyurea (28,29), camptothecin (30) and bromodeoxyuridine (31,32) may also be able to induce senescence. The most extensively researched agent is Adriamycin, a topoisomerase inhibitor. Elmore *et al* (33) reported that among 14 cell lines derived from solid human tumors, adriamycin induced senescent phenotypes in 11 cell lines,

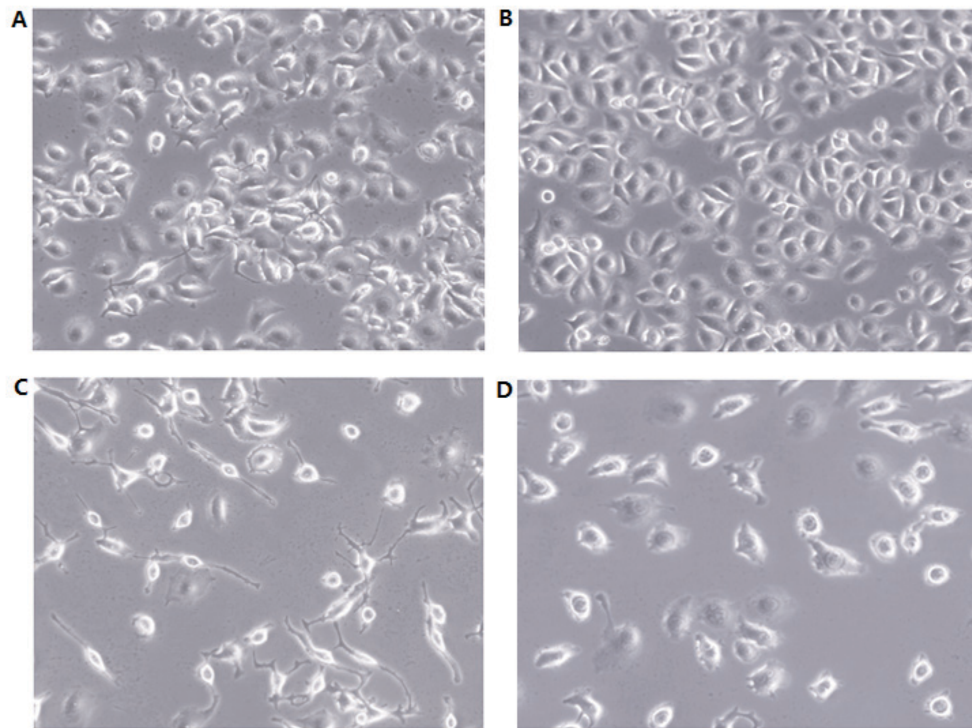

Figure 1. VP-16 alters the morphology of A549 cells. (A) Control group (no treatment). (B) Group 1 (1 μmol/l VP-16). (C) Group 2 (5 μmol/l VP-16). (D) Group 3 (25 μmol/l VP-16). Magnification, x200.

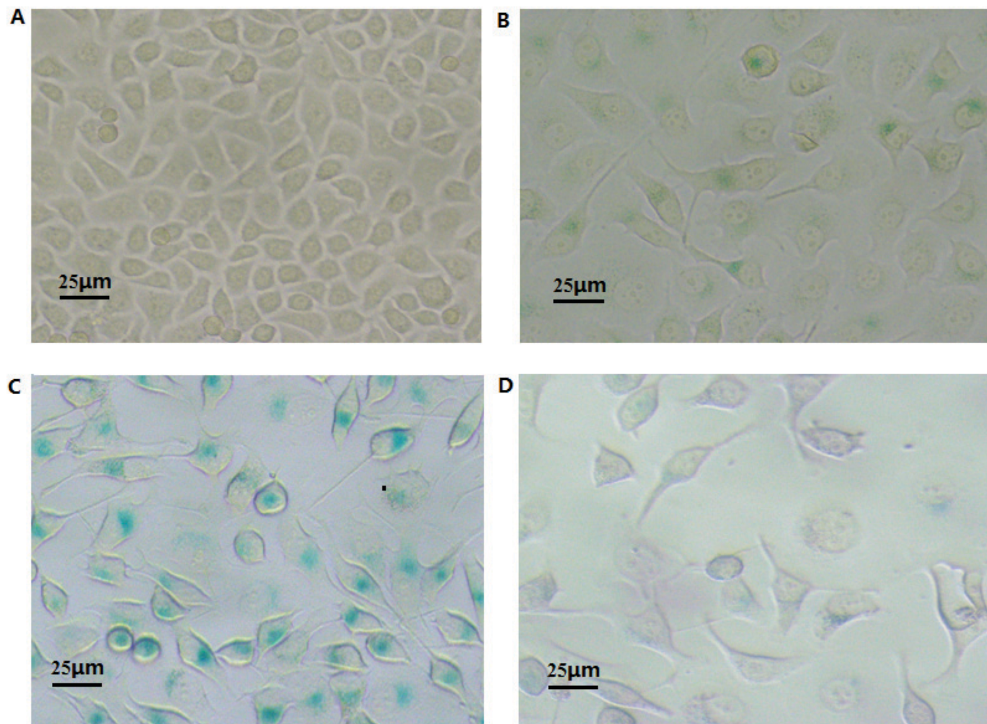

Figure 2. VP-16 affects the senescence of A549 cells. (A) Control group (no treatment). (B) Group 1 (1 μmol/l VP-16). (C) Group 2 (5 μmol/l VP-16). (D) Group 3 (25 μmol/l VP-16). Magnification, x400.

and it was confirmed that the drug-induced senescent phenotypes were not associated with the shortening of telomeres, so could not be inhibited by the overexpression of telomerase. However, in certain cell lines, Adriamycin was not able to induce the characteristics of cellular senescence; therefore, it was suggested that the cellular senescence of tumor cells may occur spontaneously or be associated with the cellular microenvironment (24). Drug-induced cellular senescence has been identified to be associated with p21, p16 and cellular tumor antigen p53 (p53) (34).

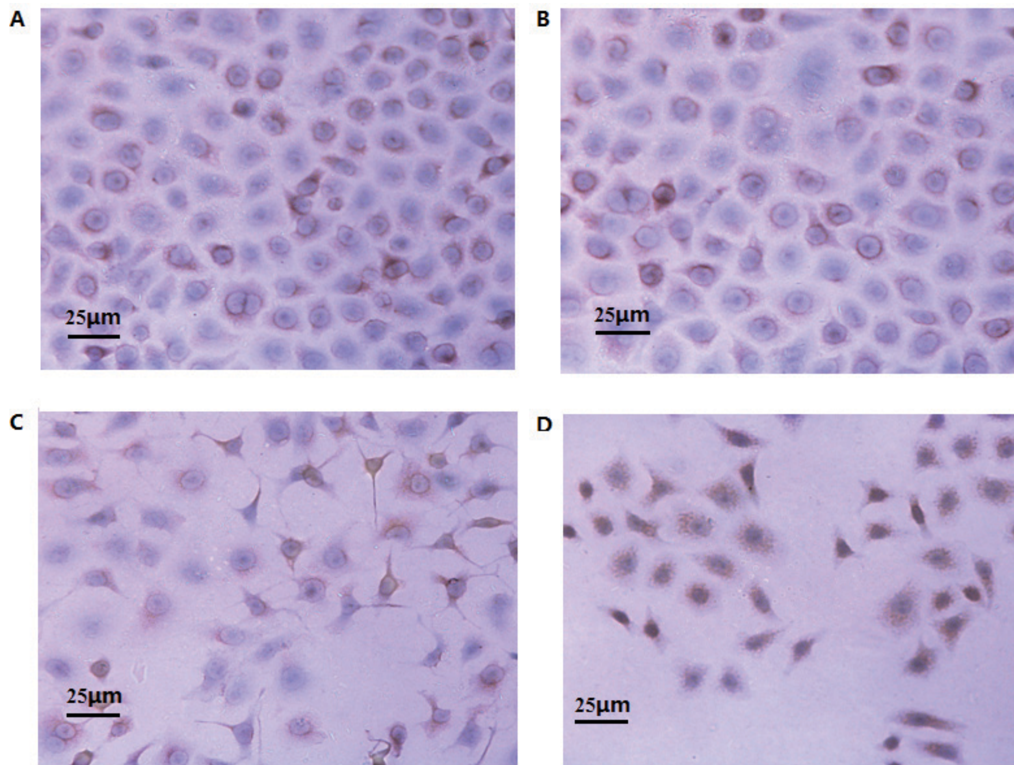

Figure 3. Effect of VP-16 on the expression of Mdm2 protein in A549 cells. (A) Control group (no treatment). (B) Group 1 (1 μmol/l VP-16). (C) Group 2 (5 μmol/l VP-16). (D) Group 3 (25 μmol/l VP-16). Magnification, x400.

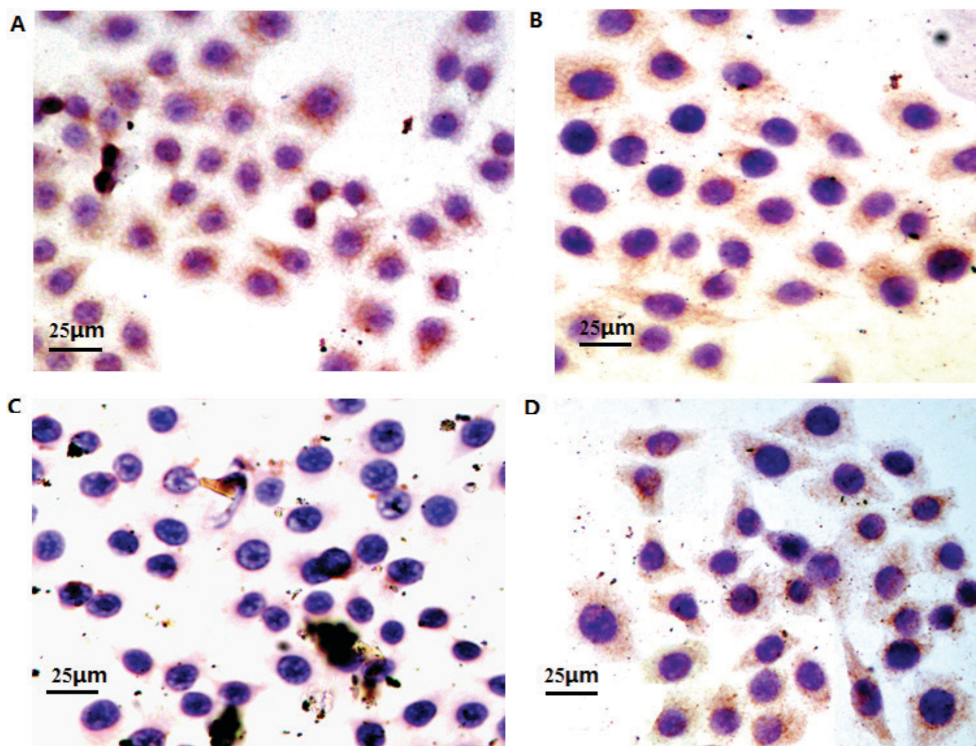

Figure 4. Effect of VP-16 on the expression of phosphorylated Rb protein in A549 cells. (A) Control group (no treatment). (B) Group 1 (1 μmol/l VP-16). (C) Group 2 (5 μmol/l VP-16). (D) Group 3 (25 μmol/l VP-16). Magnification, x400.

VP-16 is a cell cycle-specific antitumor drug that inhibits cells in the S/G_2 phase. VP-16 acts on DNA topoisomerase II, forming a stable drug-enzyme-DNA complex, thus interfering with DNA repairing (35). Therefore, the present study used 1, 5 and 25 μmol/l of VP-16 for 48 h treatments. The results demonstrated that 5 μmol/l VP-16 significantly induced

cellular senescence of A549 cells, 1 µmol/l only slightly induced cellular senescence and 25 µmol/l VP-16 did not significantly induce cellular senescence. This suggests that a very specific concentration of VP-16 is required to induce cellular senescence in A549 cells. Therefore, the optimal concentration of VP-16 that induced cellular senescence in A549 cells was 5 µmol/l.

When treated with 5 µmol/l VP-16, p-Rb and Mdm2 protein expression was significantly reduced in the A549 cells, whereas no obvious change was observed when 1 and 25 µmol/l treatments were administered. This indicates that low-concentration treatments of VP-16 reduced the levels of p-Rb protein, resulting in the release of transcription factor E2F, which prevents cell cycle progression and downregulates the expression of Mdm2, and thus its binding to p53 and Rb.

In conclusion, low-concentration treatments of VP-16 may effect the Mdm2-Rb signaling pathway, reducing the expression of Mdm2 protein and thus its binding to Rb. Therefore, non-phosphorylated Rb protein expression was increased, whereas the p-Rb protein expression was reduced, increasing the amount of functional Rb and resulting in the induction of cellular senescence. However, as this is a preliminary study, future investigations are required to further reveal the mechanisms underlying the effects of VP-16.

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