Synergistic antitumor effect of β-elemene and etoposide is mediated via induction of cell apoptosis and cell cycle arrest in non-small cell lung carcinoma cells

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Abstract. β-Elemene, an anticancer agent, was isolated from the traditional Chinese medicine plant, *curcuma aromatica*. In this study, we investigated the synergistic antitumor effect of β-elemene and etoposide phosphate (VP-16) in A549 non-small cell lung carcinoma cells. The cells were treated with β-elemene (20 or 50 μg/ml), VP-16 (15 μg/ml) or the combination of both for 24 h. Compared to the treatment with β-elemene or VP-16 alone, an increased antitumor activity was observed with the combination of both, which was mediated by the cleavage of PARP, the up-regulation of Bax, p53 and p21, and the suppression of cyclin D1. These results suggest that the combination of β-elemene and VP-16 may be a promising therapeutic option for lung cancer.

Introduction

Lung cancer is one of the leading causes of death, its mortality estimated to reach 1.5 million in 2010. From the overview of cancer statistics in 2009, lung and bronchus cancer in men and women continues to be the most common fatal cancers (1). Non-small cell lung cancer (NSCLC) comprises of 80% of all lung cancers and patients are usually diagnosed at an advanced stage, which can result in poor prognosis (2). Etoposide phosphate (VP-16) is derived from a type of plant alkaloid known as podophyllotoxin. It is commonly used to treat lung, ovarian and testicular cancer. Its cytotoxic effect is produced by inhibiting the enzyme topoisomerase II to break DNA strands (3,4). VP-16 appears to be cell-cycle-dependent and cell-cycle-specific, preferentially killing cells at the S and G_2 phases (5,6). As a chemotherapeutic drug, VP-16 is connected with the risk of damaging normal cells, which leads to the damage of bone marrow stem cells, as well as epithelial, lymphatic and reproductive cells. To overcome this obstacle, combination chemotherapy has attracted attention for the purposes of lowering doses of VP-16 and increasing efficacy by combination with low-toxic agents.

Many kinds of Chinese herbs have been confirmed to have antitumor effects, and clinical studies on the antitumor effects of Chinese herbs have also been reported in recent years (7). Elemene is a broad-spectrum antitumor plant drug extracted from the traditional Chinese medicinal herb, *Rhizoma zedoariae*. The extract of elemene is a mixture of β-, δ- and γ-elemene. The major component, β-elemene, has beneficial clinical therapeutic effects in many types of tumor with minor side-effects (8). β-Elemene was found to induce S and G_2-M phase arrest in H460 NSCLC cells, and to trigger apoptosis in Hep-2 cells (9,10). In addition, many studies have indicated that the effect of β-elemene on cell death may be mediated via a mitochondrial cytochrome c release-dependent apoptotic pathway and the reduction of Bcl-2 protein expression (11). Some other reports have shown that β-elemene can reverse chemotherapeutic resistance and enhance the radiosensitivity in lung carcinoma and renal transplant carcinoma cells (12,13). Thus, the combination of VP-16 and β-elemene may be a possible treatment for lung cancer.

In this study, we examined the synergistic antitumor effect of elemene and VP-16 and investigated the underlying mechanism in human NSCLC A549 cells.

Materials and methods

Cell culture and reagents. Human lung cancer A549 cells were cultured in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml) at 37˚C under an atmosphere of 95% air and 5% CO_2. Cyclin D1, Cdk2, p21, p53, Bcl-2 and PARP

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antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). β-Elemene was obtained from Holley Kingkong Pharmaceutical (Dalian, China).

MTT assay. The capacity for cellular proliferation was measured with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well culture plates and treated with β-elemene, VP-16, or the combination of both for the indicated times. The cells were then incubated with 20 μl of MTT (5 mg/ml) for 4 h at 37˚C and 200 μl of DMSO were added to solubilize the crystals for 20 min at room temperature. The optical density was determined with a spectrophotometer (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

Flow cytometry analysis. The cells were seeded in 6-well plates and treated with β-elemene, VP-16, or the combination of both for the indicated times. The cells were washed twice with ice-cold phosphate buffered saline (PBS), and pelleted in a centrifuge. Cell specimens were fixed in 70% ethanol overnight at 4˚C. The cells were then incubated in 0.5 ml PBS-containing 10 μl Rnase A (0.2 mg/ml) at 37˚C for 30 min, and then stained with propidium iodide (PI) dye. Finally, the samples were evaluated by flow cytometry, and the data were analyzed by WinMDI software.

Western blot analysis. The cells were washed twice with ice-cold PBS and solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na3VO4 , 1 mM PMSF, 2 μg/ml aprotinin) on ice, then quantified using the Lowry method. Samples (50 μg) of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20) at room temperature for 2 h and incubated at 4˚C overnight with the indicated primary antibodies. After the membranes had been washed with TBST buffer, they were reacted with the appropriate horseradish-peroxidase conjugated secondary antibody for 30 min at room temperature. After the membranes had been extensively washed with TBST buffer, the proteins were visualized with enhanced chemiluminescence reagent (SuperSignal West Pico Chemilunescent Substrate; Pierce, Rockford, IL, USA).

Statistical analysis. All experiments were repeated at least three times. Data are expressed as the means ± SD. Differences in the results for two groups were evaluated with Student's t-test. A P-value of <0.05 was considered to be statistically significant.

Results

Effects of β-elemene on cell viability and cell apoptosis in A549 cells. To examine the effects of β-elemene on cell viability, A549 cells were treated with β-elemene (1-500 μg/ml) for 24, 48 and 72 h. As shown in Fig. 1A, β-elemene caused a dose-dependent inhibition of A549 cells at three time-points. The IC50 doses of β-elemene at 24, 48 and 72 h was 50, 29 and 20 μg/ml, respectively. To examine the effects of β-elemene on cell apoptosis, A549 cells were treated with β-elemene at 50 and 200 μg/ml for 4, 8, 16 and 24 h. The percentage of apoptotic cells was significantly higher with the treatment of 200 μg/ml than with 50 μg/ml β-elemene. In addition, the amount of 200 μg/ml β-elemene caused the time-dependent cell apoptosis (Fig. 1B). These results indicate that β-elemene induces the inhibition of cell proliferation and triggers cell apoptosis in A549 cells.

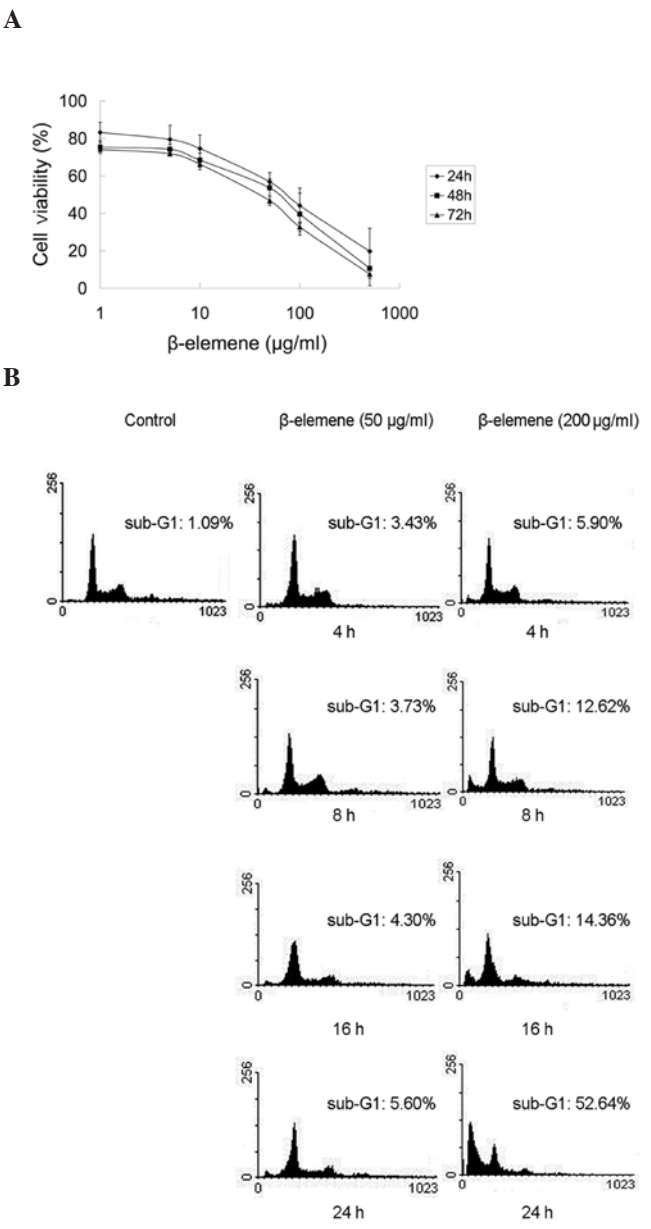

Figure 1. β-Elemene inhibited cell proliferation and induced cell apoptosis in A549 cells. (A) A549 cells were incubated with β-elemene (1, 5, 10, 50, 100 and 500 μg/ml) for different time periods. Cell proliferation was determined by MTT assay. (B) A549 cells were incubated with 50 and 200 μg/ml β-elemene for 0, 4, 8, 16 and 24 h. Cell apoptosis was quantified by flow cytometry.

Effect of β-elemene and VP-16 on cell viability and cell apoptosis in A549 cells. A549 cells were treated with VP-16 (1-500 μg/ml) for 24, 48 and 72 h. VP-16 induced the inhibition of cell proliferation in a dose- and time-dependent manner. The

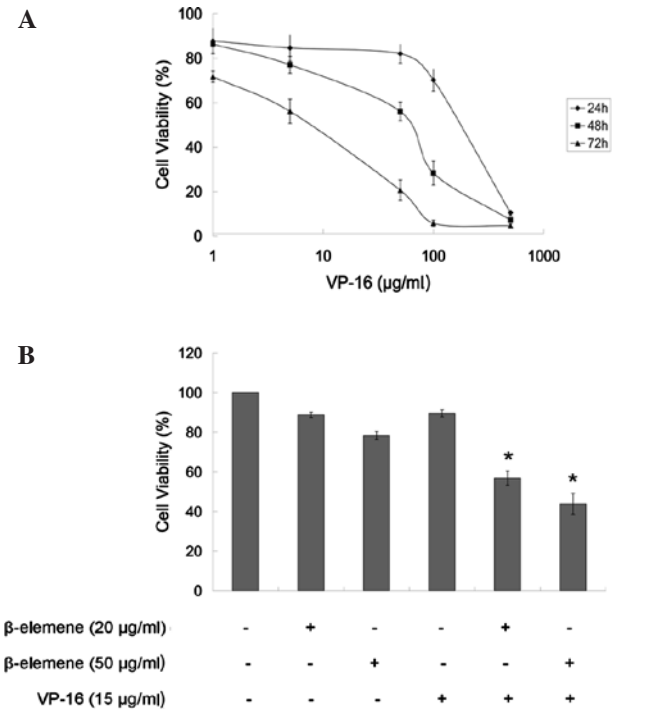

Figure 2. β-Elemene and VP-16 synergistically affected cell proliferation and induced cell apoptosis in A549 cells. (A) A549 cells were incubated with VP-16 (1, 5, 50, 100 and 500 μg/ml) for different time periods. Cell proliferation was determined by MTT assay. (B) A549 cells were incubated with β-elemene (20 or 50 μg/ml) and VP-16 (15 μg/ml) for 24 h. Cell proliferation was determined by MTT assay. *Cells exposed to β-elemene and VP-16 vs. β-elemene or VP-16 alone; P<0.05. (C) Cell apoptosis was quantified by flow cytometry.

IC50 doses of VP-16 at 24, 48 and 72 h were 115, 23 and 3 μg/ml, respectively (Fig. 2A). To reduce the side-effects of VP-16, 15 μg/ml (about 1/8 IC50 dose at 24 h) VP-16 and 20 or 50 μg/ml β-elemene were used in the following experiments. Our results show that the combination of VP-16 and β-elemene induces a more obvious inhibition of cell proliferation compared to that of VP-16 or β-elemene alone, P<0.05 (Fig. 2B). As shown in Fig. 2C, β-elemene resulted in no more than 8% cell apoptosis in A549 cells. Treatment with the combination of 20 or 50 μg/ml β-elemene and 15 μg/ml VP-16 induced a more obvious cell apoptosis than treatment with VP-16 alone (16.57 vs. 6.25%, 21.98 vs. 6.25%, respectively; P<0.05). At the same time, the synergistic effect of β-elemene and VP-16 was also confirmed by Wright-Giemsa staining and Hoechst nuclear staining (data not shown). These results suggest that the synergistic effect of β-elemene and VP-16 might be due, at least in part, to the cell apoptosis in A549 cells.

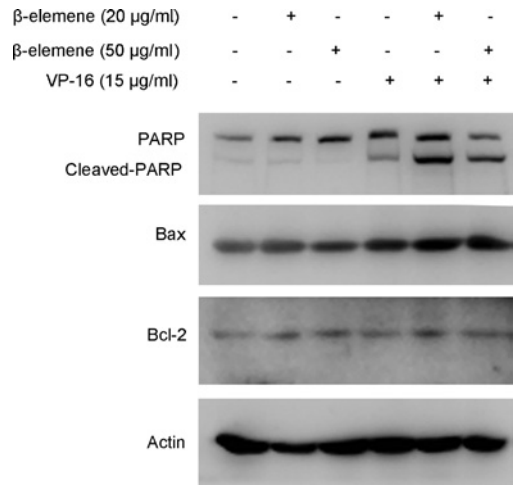

Figure 3. β-Elemene and VP-16 affected the expression of apoptotic regulatory proteins in A549 cells. A549 cells were incubated with β-elemene (20 or 50 μg/ml) and VP-16 (15 μg/ml) for 24 h. The expression of PARP, Bax and Bcl-2 was detected by Western blot analysis.

Effects of β-elemene and VP-16 on the expression of apoptotic regulatory proteins in A549 cells. In order to identify the effect of β-elemene and VP-16 on cell apoptosis, the apoptotic regulatory proteins were detected. As shown in Fig. 3, treatment with the combination of β-elemene and VP-16 for 24 h induced the cleavage of PARP in A549 cells. The protein levels of Bcl-2 were not significantly altered with the combined treatment of β-elemene and VP-16 compared to those with the treatment of β-elemene or VP-16 alone. However, the expression of Bax was up-regulated with the combined treatment. These results indicate that the synergistic antitumor effect of β-elemene and VP-16 might be due to the mitochondrial-dependent apoptotic pathway.

Effects of β-elemene and VP-16 on the cell cycle and the expression of cell cycle-related proteins in A549 cells. As shown in Fig. 4A, VP-16 and the combination of β-elemene and VP-16 arrested A549 cells at the S phase at 24 h. The expression of p53 and p21 was not changed with the treatment of β-elemene alone. However, VP-16 and the combination of β-elemene and VP-16 caused an obvious increase in the expression of p53 and p21 and a decrease in the expression of cyclin D1 in A549 cells. No alteration in CDK2 protein expression was observed (Fig. 4B). These results suggest that the combination of β-elemene and VP-16 causes the S phase arrest by the alteration of cell cycle-related proteins in A549 cells.

Discussion

Most lung cancer patients are diagnosed at an advanced stage, and chemotherapy is one of the limited selective treatments. The effects of VP-16 as a first-line chemotherapeutic agent

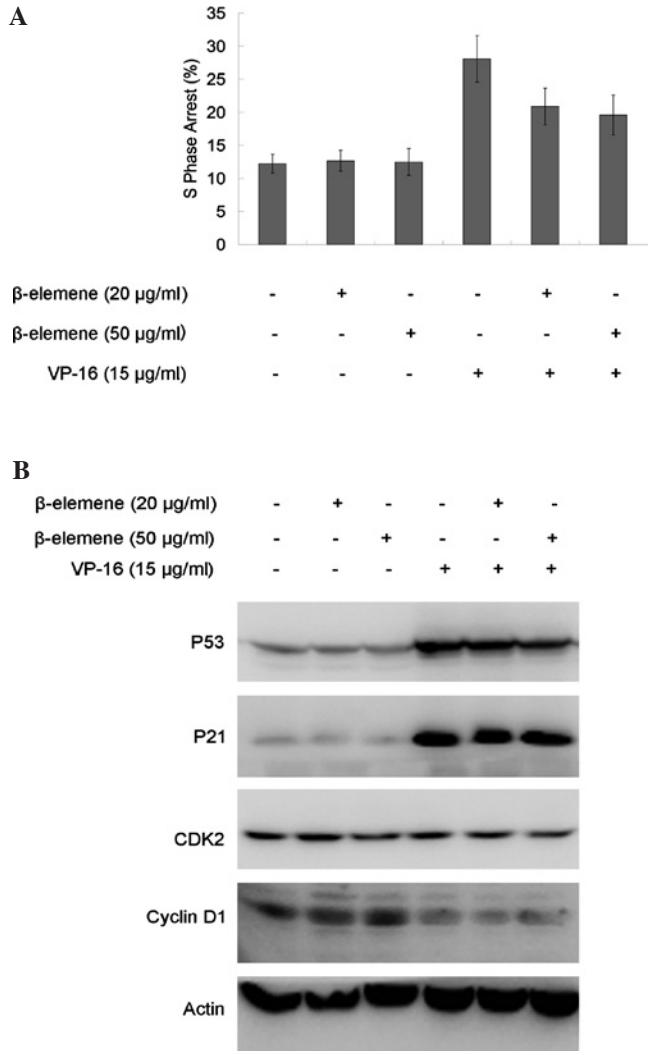

Figure 4. β-Elemene and VP-16 affected the cell cycle and the expression of cell cycle-related proteins in A549 cells. (A) A549 cells were incubated with β-elemene (20 or 50 μg/ml) and VP-16 (15 μg/ml) for 24 h. S phase arrest was quantified by flow cytometry. (B) The expression of p53, p21, CDK2 and cyclin D1 was detected by Western blot analysis.

are satisfactory, although there are certain side-effects (14). Therefore, new approaches, such as novel drugs with mild toxicities or excellent combination regimens, are needed for the treatment of lung cancer. β-Elemene, a type of anticancer drug with a lower cytotoxicity, has been shown to exhibit anticancer effects in many cancer cells, especially lung cancer cells (12,15). In this study, β-elemene inhibited cell proliferation in a dose-dependent manner in A549 cells. The combination of β-elemene and VP-16 at a low concentration was superior to either drug being used alone in inhibiting the survival of A549 cells.

It is known that cell death is mediated via the mitochondrial-dependent and mitochondrial-independent apoptotic pathway. Caspases are the central executors of the apoptotic process, and the apoptotic pathway is activated through the cleavage of PARP (16). Bcl-2 plays a pivotal protective role by preserving mitochondrial structure and function. Bax, a dominant-negative inhibitor of Bcl-2, induces a mitochondrial permeability transition and promotes apoptosis (17,18). In this study, β-elemene or VP-16 alone led to a small amount of cell apoptosis, respectively, while their combination effectively and significantly increased cell apoptosis in A549 cells. Moreover, the cleavage of PARP and up-regulation of Bax were detected in A549 cells co-treated with VP-16 and different doses of β-elemene. These results suggest that β-elemene combined with VP-16 has synergistic apoptotic effects on A549 cells. The synergetic antitumor effect of their combination may be mediated via a the mitochondrial-dependent apoptotic pathway.

The cell cycle is regulated by cyclins, cyclin-dependent kinase (CDK) and cyclin-dependent kinase inhibitors (CKIs). As a primary responder to the damage, p53 involves the trans-activation of CKI-p21, which may be combined with CDK to form a quaternionic complex that is capable of stopping the cell circle and suppressing cell growth (19,20). Additionally, cyclin D1 plays a crucial role in accelerating cell cycle progression (21). In this study, the effect of β-elemene and VP-16 on the growth control in A549 cells was associated with cell cycle arrest at the S phase. Furthermore, the treatment of VP-16 alone and the combination of β-elemene and VP-16 enhanced the expression of p53 and p21 and decreased the cyclin D1 level, whereas no alteration was found in cultures treated with β-elemene alone. These data suggest that the effect of the combined treatment (β-elemene and VP-16) on A549 cells may rely on p53-associated S phase arrest.

In conclusion, a subtoxic concentration of VP-16 and β-elemene has a synergistic cytotoxic effect on A549 cells. The synergistic effect was partly due to the induction of the mitochondrial-dependent apoptosis pathway and the trigger of the p53-regulated cell cycle arrest. Our results indicate that the combination of β-elemene and VP-16 may be a promising therapy for lung cancer.

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References

1. Jha P: Avoidable global cancer deaths and total deaths from smoking. Nat Rev Cancer 9: 655-664, 2009.
2. Beadsmoore CJ and Screaton NJ: Classification, staging and prognosis of lung cancer. Eur J Radiol 45: 8-17, 2003.
3. Baldwin EL and Osheroff N: Etoposide, topoisomerase II and cancer. Curr Med Chem Anticancer Agents 5: 363-372, 2005.
4. Sung PA, Libura J and Richardson C: Etoposide and illegitimate DNA double-strand break repair in the generation of MLL translocations: new insights and new questions. DNA Repair (Amst) 5: 1109-1118, 2006.
5. Schonn I, Hennesen J and Dartsch DC: Cellular responses to etoposide: cell death despite cell cycle arrest and repair of DNA damage. Apoptosis 15: 162-172, 2010.
6. Shiozawa K, Nakanishi T, Tan M, *et al*: Preclinical studies of vorinostat (suberoylanilide hydroxamic acid) combined with cytosine arabinoside and etoposide for treatment of acute leukemias. Clin Cancer Res 15: 1698-1707, 2009.
7. Lu Y, Li CS and Dong Q: Chinese herb related molecules of cancer-cell-apoptosis: a minireview of progress between Kanglaite injection and related genes. J Exp Clin Cancer Res 27: 31, 2008.

8. Peng X, Zhao Y, Liang X, *et al*: Assessing the quality of RCTs on the effect of beta-elemene, one ingredient of a Chinese herb, against malignant tumors. Contemp Clin Trials 27: 70-82, 2006.
9. Wang G, Li X, Huang F, *et al*: Antitumor effect of beta-elemene in non-small-cell lung cancer cells is mediated via induction of cell cycle arrest and apoptotic cell death. Cell Mol Life Sci 62: 881-893, 2005.
10. Tao L, Zhou L, Zheng L, *et al*: Elemene displays anticancer ability on laryngeal cancer cells in vitro and in vivo. Cancer Chemother Pharmacol 58: 24-34, 2006.
11. Li X, Wang G, Zhao J, *et al*: Antiproliferative effect of beta-elemene in chemoresistant ovarian carcinoma cells is mediated through arrest of the cell cycle at the G2-M phase. Cell Mol Life Sci 62: 894-904, 2005.
12. Li QQ, Wang G, Zhang M, *et al*: β-Elemene, a novel plant-derived antineoplastic agent, increases cisplatin chemosensitivity of lung tumor cells by triggering apoptosis. Oncol Rep 22: 161-170, 2009.
13. Zhao J, Li QQ, Zou B, *et al*: *In vitro* combination characterization of the new anticancer plant drug beta-elemene with taxanes against human lung carcinoma. Int J Oncol 31: 241-252, 2007.
14. Soués S, Wiltshire M and Smith PJ: Differential sensitivity to etoposide (VP-16)-induced S phase delay in a panel of small-cell lung carcinoma cell lines with G1/S phase checkpoint dysfunction. Cancer Chemother Pharmacol 47: 133-140, 2001.
15. Yang L, Zhu R, Zhu Q, *et al*: Influence of β-elemene on the secretion of angiotensin II and expression of AT1R in hepatic stellate cells. Front Med China 3: 36-40, 2009.
16. Degterev A, Boyce M and Yuan J: A decade of caspases. Oncogene 22: 8543-8567, 2003.
17. Decker P and Muller S: Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. Curr Pharm Biotechnol 3: 275-283, 2002.
18. Festjens N, van Gurp M, van Loo G, *et al*: Bcl-2 family members as sentinels of cellular integrity and role of mitochondrial intermembrane space proteins in apoptotic cell death. Acta Haematol 111: 7-27, 2004.
19. Bode AM and Dong Z: Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer, 4: 793-805, 2004.
20. Chiu CC, Lin CH and Fang K: Etoposide (VP-16) sensitizes p53-deficient human non-small cell lung cancer cells to caspase-7-mediated apoptosis. Apoptosis 10: 643-650, 2005.
21. Mejlvang J, Kriajevska M, Vandewalle C, *et al*: Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. Mol Biol Cell 18: 4615-4624, 2007.