Osthole induces lung cancer cell apoptosis through inhibition of inhibitor of apoptosis family proteins

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Abstract. In the present study, we investigated the effects and mechanisms of Osthole on the apoptosis of non-small cell lung cancer (NSCLC) cells and its synergistic effect with Embelin. Our results revealed that treatment with both Osthole and Embelin inhibited cell proliferation. Notably, combination treatment of Osthole and Embelin inhibited cell proliferation more significantly compared with monotherapy. In addition, morphological analysis and Annexin V/propidium iodide analysis revealed that the combination of Osthole and Embelin enhanced their effect on cell apoptosis. We further examined the effect of Osthole on the expression of inhibitor of apoptosis protein (IAP) family proteins. That treatment of A549 lung cancer cells with various concentrations of Osthole was observed to decrease the protein expression of X-chromosome-encoded IAP, c-IAP1, c-IAP2 and Survivin, and increase Smac expression in a dose-dependent manner. Furthermore, it was noted that Osthole or Embelin alone increased the expression of BAX, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9, and decreased Bcl-2 levels following treatment. Osthole and Embelin combination treatment had a synergistic effect on the regulation of these proteins. In conclusion, our study demonstrated that Osthole inhibited proliferation and induced the apoptosis of lung cancer cells via IAP family proteins in a dose-dependent manner. Osthole enhances the antitumor effect of Embelin, indicating that combination of Osthole and Embelin has potential clinical significance in the treatment of NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related mortality in the world, with non-small cell lung cancer (NSCLC) accounting for ~80% of cases (1). Despite advances in the management of NSCLC, improvements in survival are marginal and the overall prognosis of patients remains poor (2,3). Defects in the normal apoptosis machinery have been implicated in the resistance of cancer cells to a wide variety of current anticancer drugs (4). Therefore, identifying new agents that induce apoptosis in cancer cells offers novel and potentially useful approaches to improve patient responses to conventional chemotherapy (5,6).

Osthole, 7-methoxy-8-(3-methyl-2-butenyl)coumarin, a natural compound, may be extracted from *Cnidium monnieri* and other medicinal plants (7). Previous studies have revealed that Osthole exhibits various pharmacological activities, including anti-inflammation (8), anti-allergy (9), anti-oxidation (10), estrogen-like (11) and anti-hepatitis (12) effects. Furthermore, accumulating evidence indicates that Osthole confers antitumor effects by inhibiting tumor cell growth and inducing apoptosis (13-15). However, the effects of Osthole on the apoptosis of NSCLC and the possible mechanisms behind it remain unclear.

The inhibitor of apoptosis proteins (IAPs) are significant intrinsic cellular inhibitors of apoptosis (16-20). The human IAP family contains eight proteins: c-IAP1, c-IAP2, NAIP, Survivin, X-chromosome-encoded IAP (XIAP), Bruce, ILP-2 and Livin (21). To date, the overexpression or dysfunction of IAPs have been detected in various cancers (22-24). Therefore, identifying new agents targeting IAPs is essential for cancer drug development. Embelin is one such promising compound targeting XIAP. Embelin, a plant-based benzoquinone derivative (25), has been identified as a cell-permeable, small molecular weight inhibitor of XIAP by virtue of its interaction with the BIR3 domain (26). A number of cancers, including NSCLC (27), express elevated levels of XIAP and become refractory to apoptosis (23,28); however, treatment with Embelin alone or in combination with other anticancer drugs was observed to sensitize them towards apoptosis (26,29).

The present study was performed to evaluate the effects of Osthole on cell viability and apoptosis in NSCLC cells and to determine whether Osthole-mediated apoptosis is dependent on IAP proteins. Furthermore, we evaluated the combined effects of two herbal medicines, Osthole and Embelin, on the apoptosis of NSCLC cells *in vitro*, exploring the possibility of a combined clinical application.

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Materials and methods

Reagents. RPMI-1640, trypsin, penicillin and streptomycin were purchased from Biological Industries (Kibutz Beit Haemek, Israel). Fetal bovine serum (FBS) was purchased from Solarbio Science & Technology (Beijing, China). 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), prop-idium iodide (PI), and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-fluorescein isothiocyanate (FITC) and PI double staining kit were purchased from Key Gene (Nanjing, China). Osthole and Embelin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), Stock solution (50 mM) was prepared by dissolving Osthole or Embelin in DMSO and stored at -20°C. Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other reagents were procured locally.

Cell culture. The human lung cancer cell line A549 was purchased from China Center for Type Culture Collection (Wuhan, China) and cultured in RPMI-1640 medium supple-mented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells were grown on sterile tissue culture dishes and digested using 0.25% trypsin.

MTT assay. A549 cells ($5x10^3$/well) were plated in 96-well plates, cultured overnight, and then treated with Osthole (100 μM) and Embelin (50 μM) alone or in combination for 24 h, respectively. Corresponding DMSO or culture medium was used as an empty control. Briefly, 20 μl 5 mg/ml MTT solution was added to each well and incubated for 4 h at 37°C, then the supernatant was removed from each well, and DMSO (150 μl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm. Data were obtained from triplicate wells per condition and the results are representative of at least three independent experiments.

Flow cytometry. A549 cells ($5x10^5$/well) were seeded in six-well plates and allowed to attach overnight. Then, cells were treated with Osthole (100 μM) and Embelin (50 μM) alone or in combination for 24 h, respectively, and harvested by 0.25% trypsin. The cells were then washed twice with chilled phosphate-buffered saline (PBS), then resuspended in 250 μl binding buffer and adjusted to $1x10^6$/ml. Staining solution containing Annexin V/FITC and PI was added to the cell suspension. Following incubation in the dark for 30 min, the cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Fluorescence microscopy. A549 cells ($5x10^5$) were seeded into six-well plates and cultured overnight, and then incubated with Osthole (100 μM) and Embelin (50 μM) alone or in combina-tion for 24 h, respectively. Cells were washed twice with PBS and fixed with cold methanol and acetic acid (3/1, v/v) at 4°C. Then, cell preparations were washed in PBS and stained with Hoechst 33342 (1 mg/ml) for 30 min in the dark before being washed again in PBS and finally observed with a fluorescence microscope (x400, Nikon Corporation, Tokyo, Japan).
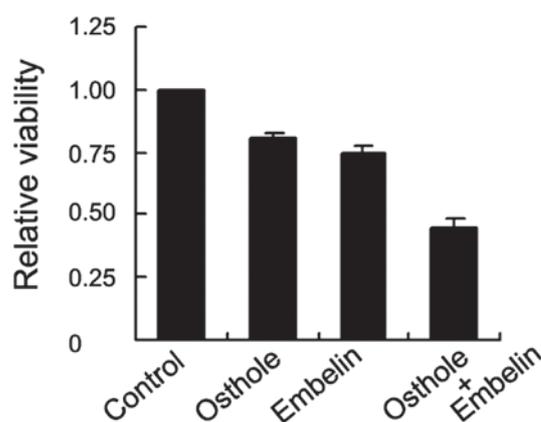

Figure 1. Combined effect of Osthole and Embelin on viability of A549 cells. Osthole (100 μM) and Embelin (50 μM) treatment alone inhibited cell pro-liferation. However, combination treatment of Osthole and Embelin inhibited cell proliferation more significantly compared with monotherapy.

Western blot analysis. Treated cells were analyzed by western blot analysis. Briefly, the cell pellets were resuspended in lysis buffer at 4°C for 1 h. Following centrifugation at 12,000 x g for 20 min, the supernatant was collected and stored at -80°C. A total of 50 μg protein was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk and incubated overnight at 4°C with antibodies against Bcl-2, BAX, caspase-3, caspase-9, cleaved caspase-3, cleaved caspase-9, XIAP, c-IAP1, c-IAP2, survivin and Smac. Following incubation with peroxidase-conjugated anti-mouse/rabbit IgG (Santa Cruz Biotechnology, Inc.) at 37°C for 2 h, proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) and detected using a bioimaging system (UVP Inc., Upland, CA, USA).

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Data are expressed as the means ± standard deviation. Statistical correlation of data was checked for significance by analysis of variance and Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Combined effect of Osthole and Embelin on viability of A549 cells. To evaluate the cytotoxicity of Osthole and Embelin alone and in combination on lung cancer cells, A549 cells were treated with Osthole (100 μM) and Embelin (50 μM) alone or in combination for 24 h, respectively, and the proliferation rate was examined using MTT assay (Fig. 1). Our results revealed that Osthole and Embelin treatment alone inhibited cell proliferation. Notably, however, the combination treatment of Osthole and Embelin inhibited cell proliferation more signifi-cantly compared with monotherapy.

Combined effect of Osthole and Embelin on apoptosis. To investigate the effect of Osthole and Embelin alone and in combination on the apoptosis of lung cancer cells, A549 cells were treated with Osthole (100 μM) and Embelin (50 μM) alone or in combination as indicated above. Morphological
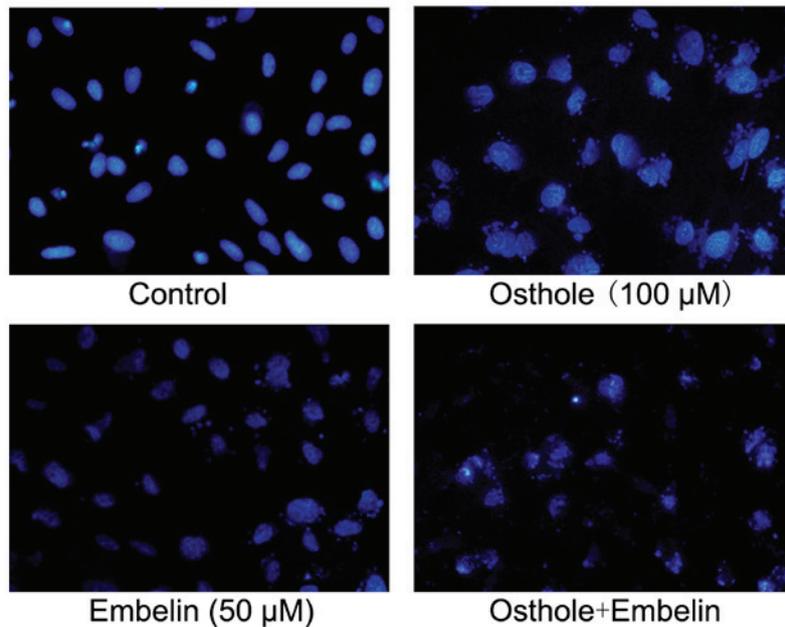
Figure 2. Morphology changes caused by Osthole and Embelin. Osthole or Embelin alone caused morphological alteration in A549 cells. Typical morphological changes including condensation of chromatin, karyopyknosis and nuclear fragmentation were observed. Cell apoptosis was observed by Hoechst 33342 staining. Notably, compared with cells treated with a single agent, cells exposed to combined Osthole and Embelin treatment exhibited a higher apoptosis rate. Magnification, x400.

changes were detected using fluorescence microscopy and the apoptosis rate was evaluated by Annexin V/PI staining.

As shown in Fig. 2, Osthole and Embelin alone caused morphological alteration in A549 cells. Typical morphological changes including condensation of chromatin, karyopyknosis and nuclear fragmentation were observed. Notably, compared with cells treated with a single agent, cells exposed to the combined Osthole and Embelin treatment exhibited a higher apoptosis rate.

Annexin V/PI flow cytometry analysis indicated that cells receiving the combined treatment had higher levels of apoptosis compared with cells treated with Osthole or Embelin alone. As shown in Fig. 3, both Osthole or Embelin increased cell apoptosis. The percentage of apoptotic cells induced by Osthole and Embelin was 4.26±0.41% in the control group, 18.31±2.67% in the Osthole group and 14.76±1.05% in the Embelin group. In contrast, the apoptosis percentage induced by the combination treatment was 34.36±2.98%. These results demonstrated that Osthole and Embelin had a synergistic effect on apoptosis in lung cancer cells.

Osthole regulates the IAP pathway in a dose-dependent manner. The effect of Osthole was further investigated on IAP proteins, which play a significant role in intrinsic programmed cell death. A549 cells were treated with various concentrations of Osthole (0, 50, 100 and 150 μM) for 24 h, and then the expression levels of IAP family members including XIAP, c-IAP1, c-IAP2, Survivin and Smac were determined by western blot analysis. The protein levels of XIAP, c-IAP1, c-IAP2 and Survivin were decreased, while Smac was increased following Osthole treatment (Fig. 4). Notably, the inhibitory effect of Osthole on XIAP, c-IAP1, c-IAP2 and Survivin increased as the dose increased. Conversely, the expression of Smac increased as the dose of Osthole increased.
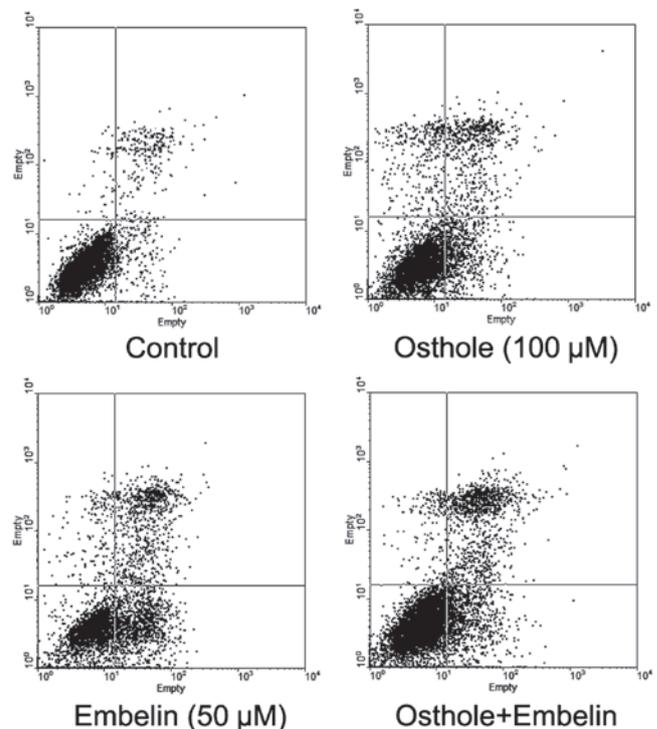

Figure 3. Combined effect of Osthole and Embelin on apoptosis. Annexin V/propidium iodide flow cytometry analysis indicated that the percentage of apoptotic cells induced by Osthole or Embelin was 4.26±0.41% (control group), 18.31±2.67% (Osthole group) and 14.76±1.05% (Embelin group). The apoptosis percentage induced by the combination treatment was 34.36±2.98%.

Combined effect of Osthole and Embelin on apoptosis-related proteins. To explore the possible mechanisms by which Osthole and Embelin regulate apoptosis, a panel of apop-
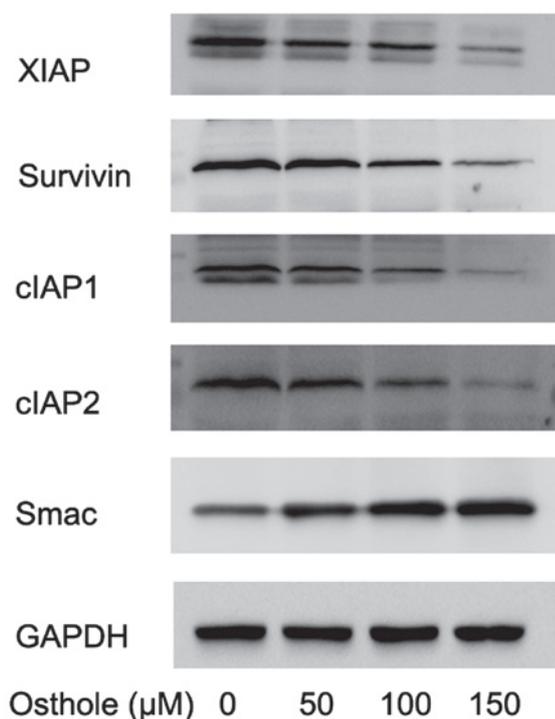
Figure 4. Osthole regulates inhibitor of apoptosis protein (IAP) family proteins. The protein levels of XIAP, c-IAP1, c-IAP2 and Survivin were decreased while Smac was increased following Osthole treatment in a dose-dependent manner.
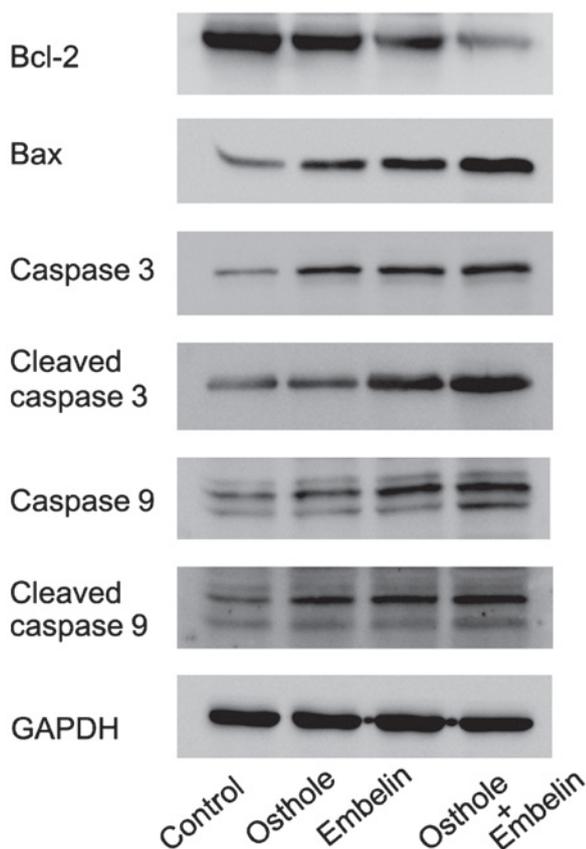

Figure 5. Combined effect of Osthole and Embelin on apoptosis-related proteins. Osthole or Embelin alone increased the expression of BAX, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9, and decreased Bcl-2 levels following treatment. The Osthole and Embelin combination treatment had a synergistic effect on the regulation of these proteins.

tosis-related proteins were screened following treatment with Osthole (100 μM) and Embelin (50 μM), alone or in combination. Compared with the control group, Osthole or Embelin alone increased the expression of BAX, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9, while Bcl-2 levels were decreased following treatment (Fig. 5). Notably, the Osthole and Embelin combination treatment had a synergistic effect on the regulation of these proteins.

Discussion

Despite therapeutic advances, the high mortality rate of patients with NSCLC has not been substantially reduced over the past years. In order to improve the prognosis and survival rate, intensive efforts have been made to identify novel anticancer agents, and much attention has been drawn to herbal medicines, owing to their wide range of biological activities, low toxicity and minimal side effects. In the present study, we identified Osthole, a natural derivative of coumarin, as a novel antitumor agent in NSCLC. Moreover, we observed that Osthole had a synergistic effect on Embelin, which is another promising antitumor agent extracted from herbal medicines.

Osthole has long been used in traditional Chinese medicine for the treatment of eczema, cutaneous pruritus, trichomonas vaginalis infection and sexual dysfunction. Numerous previous studies have confirmed that Osthole possesses antitumor activity. It was reported that Osthole inhibited migration and invasion of breast cancer cells via suppression of matrix metalloproteinase (MMP)-2 (30). In addition, Osthole suppresses the migratory ability of human glioblastoma cells via the inhibition of focal adhesion kinase-mediated MMP-13 expression (31). Moreover, several studies have suggested that Osthole suppresses cell growth and induces apoptosis in leukemia and hepatocellular and cervical carcinoma cells (13-15,32). We have previously reported that Osthole suppressed migration and invasion, and induced apoptosis in A549 lung cancer cells (33,34). In addition, we observed that Osthole enhanced the anticancer effect of cisplatin in lung cancer cells *in vitro* (35). However, the possible mechanisms behind this remained unclear. In the present study, we demonstrated that Osthole induced apoptosis of A549 lung cancer cells via IAP inhibition.

IAPs are a group of structurally related proteins that were initially identified in baculoviruses (36). Mammalian IAPs block apoptosis either by binding and inhibiting caspases or through caspase-independent mechanisms (22). c-IAPs, XIAP and melanoma IAP bind caspase-3, -7 and -9 via the BIR domains (37-40), and induce their ubiquitination or neddylation via the RING domain (41,42). Moreover, c-IAPs are positive regulators of cell proliferation (43), and the nuclear expression of c-IAP1 has been associated with advanced disease stages and poor patient prognosis in human cervical and esophageal squamous cell carcinomas and bladder cancers (44-46). To date, the overexpression of several IAPs has been detected in various cancers including NSCLC (22-24,27,47), and IAPs are significant targets for therapeutic intervention. It was previously reported that IAP-targeting therapy induces apoptosis and enhances chemotherapeutic activity against human lung cancer cells *in vitro* and *in vivo* (27,47). In the present study,

we evaluated the effect of Osthole on the IAPs by measuring the protein levels of XIAP, c-IAP1, c-IAP2 and Survivin. We observed that treatment of A549 lung cancer cells with various concentration of Osthole decreased the protein expression of XIAP, c-IAP1, c-IAP2 and Survivin, and increased Smac expression in a dose-dependent manner. These results indicate that Osthole induced apoptosis via regulation of IAP family proteins in a dose-dependent manner in NSCLC.

Embelin, a plant-based benzoquinone derivative, serves as a novel antitumor compound by inhibiting the activity of XIAP (25,26,48-51). More recently, it was reported that Embelin induced apoptosis in NSCLC cells (52). Considering that Osthole and Embelin are low-toxicity natural compounds regulating the apoptosis of NSCLC, we questioned whether these two agents would have a synergistic effect on cancer therapy. We hence evaluated the combination effect of Osthole and Embelin on A549 cell apoptosis, and revealed that combination treatment exhibited a stronger apoptosis-inducing effect compared with monotherapy. In addition, compared with single-agent treatment, Osthole and Embelin combination treatment caused a greater change in apoptosis-related proteins including Bcl-2, BAX, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9. These results indicated that Osthole and Embelin have a synergistic effect on NSCLC treatment.

In conclusion, the present study demonstrated that Osthole inhibited proliferation and induced apoptosis in A549 lung cancer cells via the IAP family proteins in a dose-dependent manner. In addition, Osthole enhances the antitumor effect of Embelin. The present study indicates that the combination of Osthole and Embelin has potential clinical significance in the treatment of NSCLC.

Acknowledgements

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