

# Emodin sensitizes paclitaxel-resistant human ovarian cancer cells to paclitaxel-induced apoptosis *in vitro*

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**Abstract.** Ovarian cancer has the highest mortality rate among gynecologic malignancies in the world, and the development of drug resistance is a major impediment toward successful treatment of the disease. Emodin has been reported to sensitize human tumor cells to chemotherapeutic agents. The present study investigated whether emodin could overcome chemoresistance of A2780/taxol cells. Cells were treated with different concentration of emodin alone or combined with paclitaxel, then the cell viability was measured by MTT and the apoptosis was determined by flow cytometric analysis. The changes of mRNA and protein were examined by QRT-PCR and Western blotting. The function of P-glycoprotein was also determined by flow cytometry. The results showed that emodin induced apoptosis alone at a high concentration and increased paclitaxel-induced apoptosis at a low concentration. It enhanced the sensitivity of A2780/taxol cells to paclitaxel with down-regulation of P-glycoprotein, XIAP and survivin. Taken together, the results demonstrated a dual role for emodin in the inhibition of drug resistant ovarian tumor growth by increasing paclitaxel cellular concentration and re-sensitizing the resistant cells to paclitaxel. Our results suggest the possibility of an innovative chemotherapeutic strategy that uses emodin in combination with paclitaxel to increase the sensitivity of tumor cells.

## Introduction

Ovarian cancer has the highest mortality rate among gynecologic malignancies in the world (1). The standard treatment for ovarian cancer remains surgical debulking and chemotherapy with carboplatin and paclitaxel (2). However, the majority of ovarian cancer patients have one of the lowest 5-year survival rates (3), and the development of drug resistance is a major impediment toward successful treatment

of the cancer (4). Thus, there have been a number of investigations aimed at understanding the mechanisms of drug resistance, so as to develop strategies to overcome the disease (5,6).

Paclitaxel is an effective chemotherapeutic agent and widely used for the treatment of ovarian cancer, whereas the development of drug resistance limits its usefulness (2). To date, several mechanisms of paclitaxel resistance in ovarian cancer cells have been proposed, including overexpression of ABC/MDR transporter family of proteins to increase the cellular efflux of paclitaxel, delayed G2/M transition, and alterations in apoptosis regulation (7-9).

As a traditional Chinese herbal medicine, emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) has been shown to possess a number of biological activities such as anti-bacterial (10), anti-inflammatory (11), immunosuppressive (12), and anti-tumor activity (13). Previous studies have demonstrated that emodin has anti-proliferative and anti-neoplastic effects in cancer cell lines. For example, emodin was shown to suppress the proliferation of Her-2/neu overexpressing lung cancer cells and sensitizes these cells to chemotherapeutic agents (14). Emodin has also been reported to sensitize different tumor cells to arsenic trioxide, including human esophageal carcinoma EC/CUHK1, human myeloid leukemic NB4, and acute myelocytic leukaemia (AML) U937 cells (15,16). In our previous study, we found that emodin inhibits cell proliferation and induces apoptosis in ovarian cancer cell lines (data not shown), including the paclitaxel-resistant cell line A2780/taxol. Now we investigated whether emodin could sensitize paclitaxel-resistant tumor cells to paclitaxel cytotoxicity and its possible mechanisms.

## Materials and methods

**Chemicals and reagents.** Emodin and paclitaxel were purchased from Sigma (USA) and were dissolved in dimethyl sulfoxide and ethanol, respectively. Antibodies against  $\beta$ -actin, P-gp, XIAP, survivin, caspase-3 were obtained from Santa Cruz.

**Ovarian cancer cell lines.** Paclitaxel-sensitive (A2780) and paclitaxel-resistant (A2780/taxol) human ovarian cancer cell lines were used. A2780 was obtained from China Center for Type Culture Collection (CCTCC), paclitaxel-resistant cell line A2780/taxol was obtained from Cancer Prevention and Treatment Research Institute of Guangxi Medical University

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Table I. Primers used for PCR.

Survivin	Antisense	CAGATTTGAATCGCGGGACCC	208 bp	NM_001168.2
	Sense	CCAAGTCTGGCTCGTTCTCAG		
XIAP	Antisense	CCGTGCGGTGCTTTAGTTGT	292 bp	NM_001167.2
	Sense	TTCCTCGGGTATATGGTGTCTGAT		
MDR-1	Antisense	CCCATCATTGCAATAGCAGG	157 bp	NM_000927.3
	Sense	GTTCAAACCTTCTGCTCCTGA		
$\beta$ -actin	Antisense	TGGCACCCAGCACAATGAA	188 bp	NM_001101.2
	Sense	CTAAGTCATAGCCGCCTAGAAGCA		

(17). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2% sodium pyruvate and 1% penicillin/streptomycin. Culture and drug-treatment of cells were carried out at 37°C in an atmosphere of 95% air/5% CO<sub>2</sub>. Cells were routinely trypsinized (0.05% trypsin/EDTA).

*Cell viability assay.* Cells were plated in 96-well plates at a density of 4000 cells per well. Cells were incubated overnight and then treated with different concentration of emodin or/and paclitaxol for 24 h, respectively. All experiments were performed in triplicate and repeated at least three times. Emodin was dissolved in DMSO at a concentration of 100 mmol/l and added into the culture. To prevent crystallization, the plate was shaken well immediately after emodin was added. Paclitaxel was dissolved in ethanol at a concentration of 2 mmol/l for storage. The final DMSO concentration never exceeded 0.1%, and this condition was used as the control in each experiment. At the end of incubation, the cells were washed with phosphate-buffered saline (PBS), and then 20  $\mu$ l of 5 mg/ml of MTT (Sigma) solution was subsequently added to each well with 180  $\mu$ l RPMI-1640. After further incubation for 4 h at 37°C, culture medium was removed and 150  $\mu$ l of DMSO was added to dissolve the resulting formazan crystals, and thoroughly mixed for 10 min. The absorbance of samples was then measured by spectrometric absorbance at 570 nm with background subtraction at 630 nm on an automatic multiwell spectrophotometer (Bio-Rad-Coda, Richmond, CA).

*Apoptosis analysis.* Apoptotic rates were assessed with flow cytometry using the Annexin V-fluorescein isothiocyanate/propidium iodide (PI) kit (Bipecc Biopharma Corp, USA). Samples were washed with ice-cold PBS twice and resuspended in binding buffer at a density of 1x10<sup>6</sup> cells/ml. The cells were stained with Annexin V-FITC and gently vortexed. After 15 min incubation at 4-8°C in the dark, PI was added to the cells for another 5 min incubation at 4-8°C in the dark. The results were analyzed by flow cytometry (FACScan, BD, USA). Annexin V-positive, PI-negative cells were scored as early apoptotic. Double-stained cells were considered as late apoptotic, and Annexin V-negative, PI-positive cells were considered as necrotic. The apoptosis rates estimated in the present study included all Annexin V-positive populations.

*Rhodamine 123(Rh123) efflux assay.* Studies were carried out in A2780/taxol cells. Cells (1x10<sup>6</sup>) were seeded in 6-well plates and cultured for 48 h. A2780/taxol cells were pretreated with 10  $\mu$ M emodin or/and 1  $\mu$ M paclitaxel for 72 h, respectively. After pretreatment, the cells were incubated with 200 ng/ml Rh123 at 37°C for 60 min in the dark. After incubation, cells were washed twice with PBS, then the cells were incubated with RPMI-1640 with 10% FBS at 37°C for 30 min and harvested for the measurement of Rh123 efflux. The sample was subjected to flow cytometry to measure the percentage of cells that did not emit fluorescence. All analyses were performed in triplicate in three separate experiments and the results are expressed as the mean percentage of the cells effluxing the dye, which reflects the efflux activity of P-glycoprotein (P-gp). The green fluorescence of Rh123 was excited by 488 nm wavelength and measured by a 530 nm band-pass filter.

*Quantitative reverse transcription and polymerase chain reaction (QRT-PCR) analyses.* Total RNA was extracted from cells by the TRIzol reagent (Invitrogen, USA), according to the supplier's protocol. Total RNA (3  $\mu$ g) samples were reverse transcribed to a final volume of 20  $\mu$ l, using 50 pM oligo(dT)-primer (Takara, Japan), 1 mM dNTP mix (Takara), 200 U reverse transcriptase (Promega, USA), and 5 x buffer 4  $\mu$ l. RT reactions were performed on Mastercycler (Eppendorf, Germany).

QRT-PCR analysis was performed on Light Cycler (Roche Applied Science, USA) and on a volume of 20  $\mu$ l containing 1  $\mu$ l of cDNA, 10  $\mu$ l of x SYBR Green PCR Master Mix (Toyobo, Japan) 0.5  $\mu$ l of each primer (10 pM) and 8  $\mu$ l of DEPC-treated water. The sequences of the primers are shown in Table I.

The 20  $\mu$ l volume PCR reaction mixture was pre-heated at 95°C for 10 sec, followed by 45 cycles at 95°C for 5 sec, annealed for 10 sec at 60°C (survivin and XIAP)/55°C (MDR-1) and elongated for 10 sec at 72°C. The results were normalized to the relative amounts of  $\beta$ -actin.

*Western blotting.* Cells were washed twice with PBS and lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton-X100, 2 mM EDTA (pH 8.0), 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml aprotinin] on ice for 20 min. The lysates were centrifuged at 12000 g at 4°C for 10 min, and the protein concentrations of

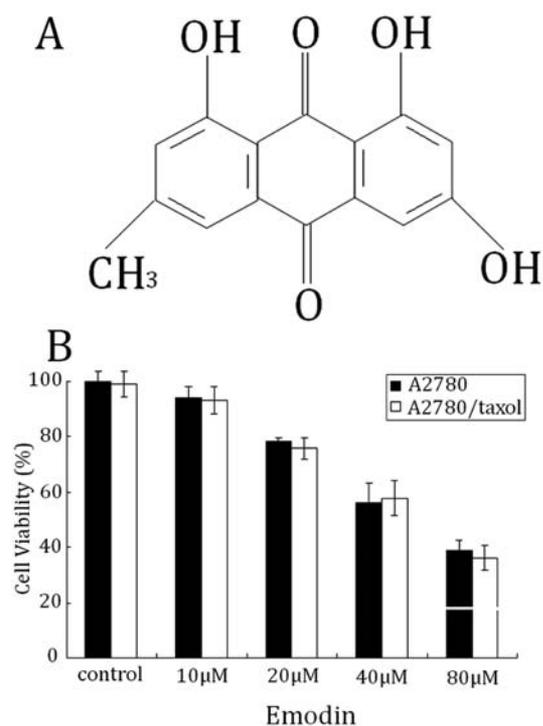


Figure 1. Effect of emodin on cell viability. (A) Molecular structure of emodin (1,3,8-trihydroxy-6-methylanthraquinone). (B). The effect of emodin on growth of A2780 and A2780/taxol cell lines. Cells were incubated with different concentration of emodin for 24 h. Cell viability was assessed by MTT assay. Emodin (10  $\mu$ M) treated cells displayed no significant growth inhibition as compared to that of control treated group ( $P>0.05$  for both cell lines), but 80  $\mu$ M of emodin showed significant growth inhibition in both cell lines ( $P<0.01$ ). Data represent mean  $\pm$  SD of triplicates, similar results were obtained in three independent experiments.

the supernatants were determined using the BCA method. For Western blot analysis, equal amounts of total protein were loaded onto 8 or 10% SDS-polyacrylamide gels and the proteins electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 2 h at 37°C, and then incubated with an anti-XIAP, anti-survivin, anti-P-gp, anti-caspase 3 or anti- $\beta$ -actin primary antibody overnight at 4°C, respectively. Horseradish peroxidase (HRP)-conjugated secondary antibody was added for 2 h at room temperature. Detection was performed by enhanced chemiluminescence (ECL, Pierce, USA).

**Statistical analysis.** Data were expressed as means  $\pm$  SD, and analyzed by the Student's t-test on SPSS 11.0. P-values below 0.05 were regarded as statistically significant.

## Results

**Emodin inhibits the viability of ovarian cancer cells.** The chemical structure of emodin is shown in Fig. 1A. To determine the effect of emodin on ovarian cancer cell viability alone, we treated ovarian cancer cell lines A2780 and A2780/taxol with different concentrations (10, 20, 40 and 80  $\mu$ M) of emodin for 24 h. Cell viability was measured by MTT assay. As shown in Fig. 1B, a dose-dependent decrease of cell viability was observed in both cases. cells treated with 10  $\mu$ M of emodin displayed no significant growth inhibition as

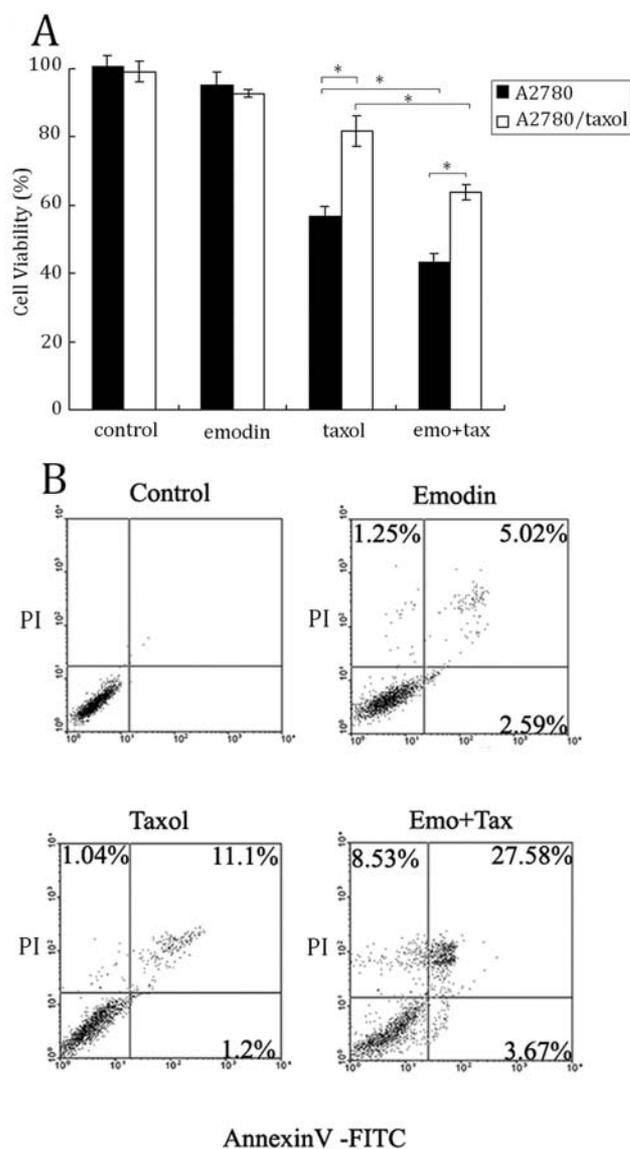


Figure 2. Effect of emodin and/or paclitaxel on the viability of A2780 and A2780/taxol cell lines. Cells were incubated with 10  $\mu$ M of emodin and 1  $\mu$ M of paclitaxel for 24 h. (A) Cell viability was evaluated using MTT assay. (B) Apoptotic rate of A2780/taxol cells treated for 24 h. Density plots of flow cytometry. The experiment were repeated three times.

compared to control treated cells (0.1% DMSO) ( $P>0.05$  for both cell lines), but 80  $\mu$ M of emodin showed significant growth inhibition in both cell lines ( $P<0.01$ ). The cytotoxic effect of emodin against paclitaxel-sensitive and -resistant ovarian cancer cells shows no significant difference in each group. This result indicates the anticancer potential of emodin is not inhibited in paclitaxel-resistant cells.

**Low concentration of emodin enhances the cytotoxicity of paclitaxel in resistant ovarian cancer cell lines.** To investigate the possibility that emodin enhance the cytotoxicity of paclitaxel, we examined the combined effects of emodin and paclitaxel in both paclitaxel sensitive and resistant ovarian cancer cells. Cells were treated with low concentration of emodin (10  $\mu$ M), paclitaxel (1  $\mu$ M), or combined for 24 h, respectively. Cytotoxicity was assessed using MTT viability assay. The results (Fig. 2A) indicated that 10  $\mu$ M emodin alone showed no significant growth inhibition as compared

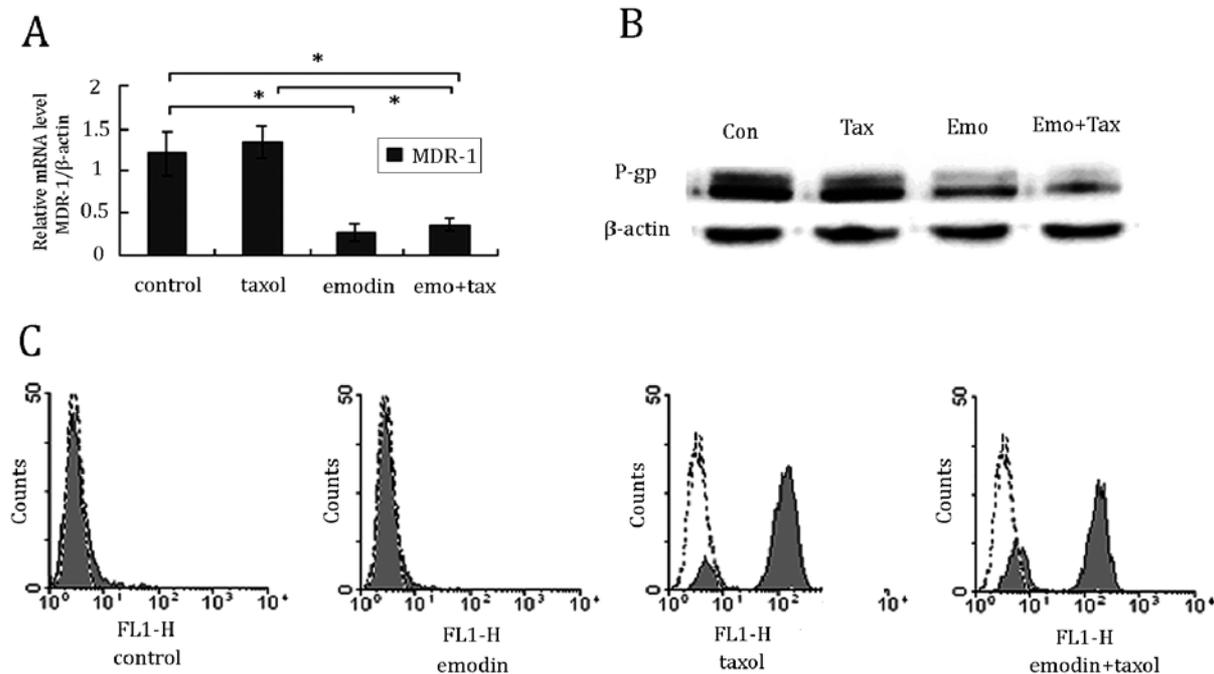


Figure 3. Effect of emodin on P-gp expression and function in A2780/taxol cells. Cells were pretreated with 10  $\mu$ M of emodin or/and 1  $\mu$ M paclitaxel for 24 h. (A) The total RNA was detected as described before. As shown in (A), there was a significantly decrease of MDR-1 mRNA expression in cells treated with emodin alone or combined with paclitaxel ( $P < 0.01$ ). (B) The expression of P-gp was detected by Western blotting with mouse anti-human monoclonal antibody against P-gp. The expression of P-gp was attenuated with treatment of emodin or combined with paclitaxel. (C) After cultured with emodin or paclitaxel, the cells were treated and analysed as described before. A2780/taxol cells in the absence of emodin exhibited a significant decrease of Rh123 compared to A2780 cells, while a notable increase was seen in A2780/taxol cells in the presence of emodin (10  $\mu$ M).

with that of control group ( $P > 0.05$  for both cell lines). Treatment of A2780 cells with paclitaxel or combined with emodin inhibited cell viability by 44 and 57%, respectively ( $P < 0.01$ ). In A2780/taxol cells, the results also showed a significant inhibition of cell growth by paclitaxel (18%) alone or combined with emodin (36%) ( $P < 0.01$ ). The effect of paclitaxel is lower in resistant cells compared with sensitive cells ( $P < 0.01$ ). The results of flow cytometry showed that emodin at 10  $\mu$ M facilitated paclitaxel-induced apoptosis, but it had little cytotoxicity when used alone at this low concentration (Fig. 2B).

*Emodin down-regulates MDR-1 expression and function.* We found that emodin enhanced paclitaxel cytotoxicity in paclitaxel-resistant cells, so we investigated further to find the possible mechanisms. Expression of MDR-1 was first investigated. The result of QRT-PCR showed that the gene expression of MDR-1 was significantly attenuated when the cells were treated with emodin alone or combination with paclitaxel ( $P < 0.01$ ), whereas no change was seen with paclitaxel alone ( $P > 0.05$ ) (Fig. 3A). These results were also confirmed by Western blot analysis (Fig. 3B).

Rhodamine 123 is a well-established P-gp substrate. The activity of the P-gp drug pump can be gauged by the degree of intracellular efflux of rhodamine 123, which can in turn be determined by the measurement of intracellular fluorescence. In this assay, we further investigated the effect of emodin on the P-gp activity by detecting efflux of Rh123 in A2780/taxol cells (Fig. 3C). Cells treated with emodin at the concentrations of 10  $\mu$ M significantly decreased the efflux of Rh123 ( $P < 0.01$ )

Table II. Rh123 efflux assay.

Group	Efflux activity (%)
Control	100
Taxol	106.24 $\pm$ 3.2
Emodin	32.11 $\pm$ 8.8 <sup>a</sup>
Emodin+Taxol	39.24 $\pm$ 5.9 <sup>a</sup>

<sup>a</sup> $P < 0.01$  compared with the control group MDR1-mediated rhodamine efflux assay as measured by flow cytometry. Cultured for 72 h, they were treated with 200 ng/ml Rh123 for 1 h as described. The value of efflux activity of A2780/taxol cells treated with 0.1% DMSO was set at 100%. Results show the mean  $\pm$  SD of three independent experiments.

(Table II). Emodin induced the increase of Rh123 in A2780/taxol cells, suggesting that emodin had the ability to inhibit the drug-transport activity of P-gp, which is a major reason for inducing elevation of intracellular accumulation of paclitaxel in A2780/taxol cells.

*Emodin reduces the expression of anti-apoptotic molecules.* The cells were co-treated with emodin and paclitaxel for 72 h, then the levels of survivin and XIAP were detected by QRT-PCR and Western blotting. As shown in Fig. 4A, we found when co-treated with emodin, the expression of XIAP and

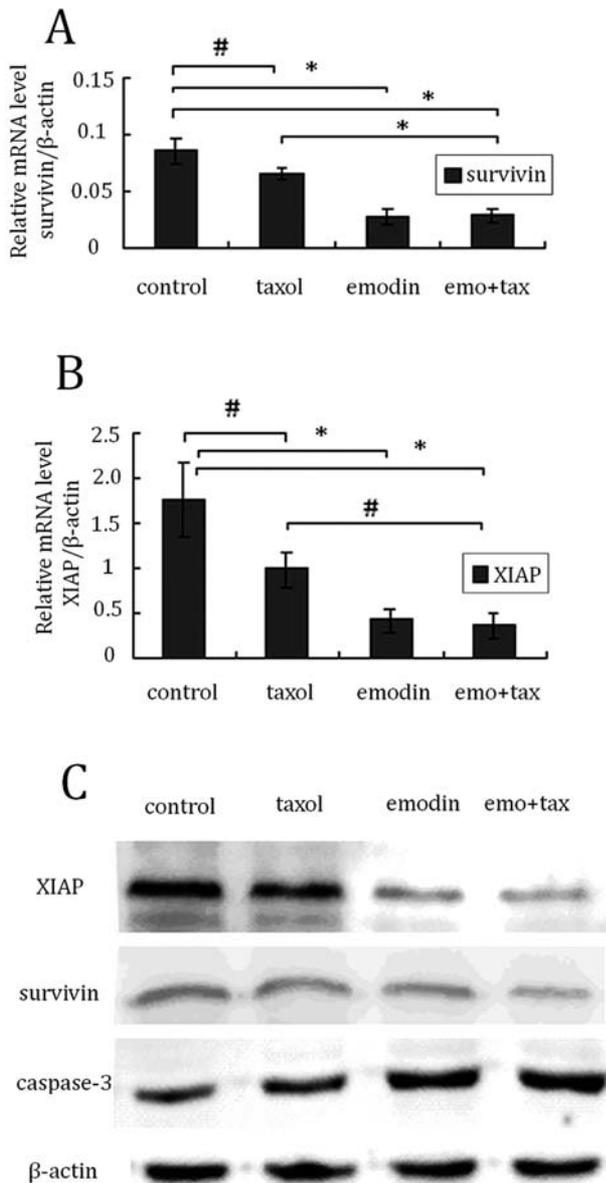


Figure 4. Effect of emodin on anti-apoptotic molecule expression in A2780/taxol cells. QRT-PCR showed transcript expression of survivin (A) and XIAP (B) was decreased in cells treated with emodin alone or combined with paclitaxel ( $P < 0.01$  and  $< 0.05$ , respectively). The expression of XIAP and survivin protein were detected by Western blotting. The expressions of XIAP and survivin were reduced with treatment of emodin or combined with paclitaxel. Furthermore, the expressions of cleaved fragments of caspase-3 were significantly enhanced (C). (\* $P < 0.01$ , # $P < 0.05$ ).

survivin was decreased compared to treatment with paclitaxel alone (Fig. 4A and B). Furthermore, treatment with emodin enhanced the activation of caspase-3 as evidenced by significantly enhancing expression of cleaved fragments of caspase-3.

## Discussion

Drug resistance is the main cause of treatment failure and mortality in cancer patients. Paclitaxel is a widely used chemotherapeutic agent against a number of malignancies, including ovarian cancer (18). However, development of drug-resistance following prolonged treatment or upon

relapse of the disease is an undesirable effect (19). Previous studies have suggested several possible reasons for paclitaxel resistance, including increased drug cellular efflux by overexpressed P-gp encoded by multidrug resistance gene-1 (MDR-1) and deregulated expression of anti-apoptotic or pro-apoptotic molecules (20,21).

Emodin has been reported to possess anticancer effect on several human cancers *in vitro* (22,23). It has also been proved to enhance cytotoxic effects of chemotherapeutic agents in different cancer cells (14,15). The treatment of cancer cells with emodin alone or in combination with other chemotherapeutic agents has been shown to effectively counteract tumor progression, although the emodin-mediated molecular mechanism responsible for this effect remains to be fully elucidated.

Our report described that emodin inhibits cell proliferation in ovarian cancer cells A2780 and A2780/taxol, and the anticancer activity is based on apoptotic cell death. The cytotoxic effect of emodin against paclitaxel sensitive and resistant cells showed no significant difference, while comparable to that of paclitaxel under the conditions used; its effect was significantly higher in the resistant cells. This indicated that the anticancer potential of emodin was not inhibited in paclitaxel-resistant cells. The substantially higher cytotoxicity induced by the co-administration of emodin and paclitaxel seemed to suggest a possible synergistic effect. Therefore, we tried to find the possible mechanisms with further research.

MDR-1 encodes a 170-kDa transmembrane protein P-gp. P-gp functions as an efflux pump to decrease the intracellular accumulation of a variety of lipophilic drugs, including paclitaxel (25). Previous studies showed that P-gp contributed to paclitaxel resistance and MDR-1 gene expression might be a useful predictor for combination paclitaxel based chemotherapy in ovarian cancer cells (26). Cellular P-gp can be detected in protein levels by Western blot analysis with monoclonal antibodies against human P-gp. Its function is also evaluated by measuring Rh123 efflux from cells. Rh123 is a special substrate for P-gp. The uptake of Rh123 results from passive inward diffusion (27), while the efflux is known to be P-gp-dependent. Rh123 has been used extensively as an indicator of P-gp activity in drug-resistant cell lines with P-gp overexpression (28).

Paclitaxel-resistant cell line A2780/taxol was generated from cell line A2780 through exposure to increasing sublethal concentrations of paclitaxel (17). Analysis of cellular protein expression demonstrated the highly elevated expression of survivin, XIAP and P-gp, compared with its parental cell line A2780 (29). In our study, we found emodin has a dual function of inhibiting the P-gp drug-pump and expression. The reversal effect of emodin on paclitaxel-resistance of A2780/taxol cells might be via down-regulating P-gp.

Successful treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells; therefore, trying to decrease the overexpression of anti-apoptotic molecules may affect the proximal level of apoptotic threshold. As direct caspase inhibitors and participants in a variety of survival signaling pathways, the inhibitor of apoptosis protein (IAP) family are important to the control of cell proliferation and drug resistance in multiple cancer

types (30). As the important IAPs members, XIAP (X-linked inhibitor of apoptosis) and survivin are up-regulated in most human tumor cells and make the cancer cells escape from apoptosis. Evidence indicates that survivin and XIAP are associated with chemoresistance and decrease of these IAPs induced apoptosis in chemoresistant human ovarian cancer cells (31,32). In the present study, we have investigated the regulation and role of two anti-apoptotic factors, survivin and XIAP, in ovarian cancer cells, and tried to explain their relationship with cellular resistance to paclitaxel. We found emodin could increase the apoptosis induced by paclitaxel, and the expression of XIAP and survivin were reduced. Treatment with emodin enhanced the activation of caspase-3. It was suggested that emodin could down-regulate the expression of anti-apoptotic protein, survivin, in esophageal carcinoma cell line EC/CUHK1 (24), coinciding with our results. The enhanced apoptosis by emodin and paclitaxel may be partly due to the reduction of XIAP and survivin.

Collectively, we demonstrated for the first time that emodin induced apoptosis of ovarian cancer cells and enhanced the sensitivity of A2780/taxol cells to paclitaxel. The results demonstrated that emodin could induce apoptosis of resistant ovarian cancer cells by increasing the cellular concentration of paclitaxel and decreasing the expression of anti-apoptotic molecules. The substantially higher cytotoxicity induced by the co-administration of emodin and paclitaxel seems to suggest a possible synergistic effect presumably due to emodin-mediated reduction of the expression of P-gp, XIAP and survivin.

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