

# Emodin sensitizes the gemcitabine-resistant cell line Bxpc-3/Gem to gemcitabine via downregulation of NF- $\kappa$ B and its regulated targets

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**Abstract.** The aim of this study was to evaluate whether emodin can overcome the chemoresistance of the gemcitabine-resistant cancer cell line (Bxpc-3/Gem) *in vitro*. The cell line Bxpc-3/Gem was derived from the human pancreatic cancer cell line Bxpc-3. We found that Bxpc-3/Gem cells were characterized by a series of morphological changes with a resistance index of 43.51 comparing with the parental cell line. Emodin reduced Bxpc-3/Gem cell proliferation in a dose-dependent manner. Emodin and gemcitabine combination treatments resulted in decreased cell proliferation and increased apoptosis in Bxpc-3/Gem cells. In addition, combination treatments resulted in downregulation of gene and protein expression of MDR-1 (P-gp), NF- $\kappa$ B, XIAP, survivin, as well as inhibition of NF- $\kappa$ B activity and P-gp function. These observations suggest that emodin may sensitize the pancreatic cancer gemcitabine-resistant cell line Bxpc-3/Gem to gemcitabine therapy via inhibition of survival signaling.

## Introduction

Pancreatic cancer is characterized by late diagnosis, an aggressive rate of invasion to surrounding tissues and liver and lymph node metastases (1). In the United States, pancreatic cancer is the fourth major cause of cancer-related deaths, with 43,140 new cases of pancreatic cancer diagnosed yearly (2). In western countries, pancreatic cancer has increased as the fourth ranked in cancer mortality (3). Besides surgery, chemotherapy is an important treatment for advanced pancreatic cancer minimizing post-surgical recurrence to prolong survival times and to improve quality of life. Gemcitabine has

been the standard treatment for advanced pancreatic cancer (4), but due to intrinsic and acquired drug resistance, gemcitabine alone has limited efficacy (5,6), with a response rate of <10% (7). In addition, dose-limiting toxicities often prevent patients from receiving optimal clinical benefits. Thus, we have to search for a drug that can reverse chemo-resistance and sensitize pancreatic cancer to the antitumor effects of gemcitabine.

Emodin (1,3,8-trihydroxy-6-methylantraquinone) is the main active monomer isolated from Rheum, Polygonum, Buckthorn and Senna. Emodin functions as a tyrosine kinase II inhibitor and has potent antimicrobial activity (8,9), anti-inflammatory properties (10,11), functions as an immunosuppressor (12), antitumor properties (13-15) and functions to protect liver cells (16). Studies have shown that emodin interacts structurally with cancer cells to amplify pro-apoptotic signal transduction pathways. Other studies demonstrate that normal cells are resistant to emodin (15). Additionally, it has been shown that low concentrations of emodin can potentiate the antitumor effects of paclitaxel in ovarian cancer cells (17). In previous studies, we demonstrated that emodin induced apoptosis in pancreatic cancer cells (18). However, the mechanism of emodin altering chemo-resistance of pancreatic cancer cells to gemcitabine remains unclear. This study focuses on the ability of emodin to sensitize gemcitabine-resistant pancreatic cancer to gemcitabine and provides insight into the possible mechanisms involved.

## Materials and methods

**Reagents and drugs.** Emodin was purchased from Sigma (St. Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO) at 0.2 mmol/l in stock and stored at -20°C. The final concentration of DMSO was <0.1%. Gemcitabine was purchased from Ely Lilly (Bad Homburg, Germany) and dissolved in sterile saline at 50 g/l in stock. RPMI-1640, fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA).

**Cell culture.** The human pancreatic cancer cell line Bxpc-3 (ATCC, Rockville, MD, USA) was cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (FBS) (Sijiqing, Hangzhou, China), in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C.

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**Drug preparation.** Emodin (purity >98%, Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich) as a 0.2-mM stock solution and stored at -20°C. DMSO concentration <0.1% was used, as it has no effect on cell proliferation at this concentration. Gemcitabine was reconstituted as a 0.02-mM stock solution in sterile saline.

**Establishment of the resistant cell line Bxpc-3/Gem.** Gemcitabine-resistant cell line Bxpc-3/Gem was derived from the human pancreatic cancer cell line Bxpc-3 by exposing the cells to intermittently increasing concentrations of gemcitabine. After cultivating Bxpc-3 cells with different concentrations of gemcitabine for 1 week, we observed cell death conditions and chose the concentration with median lethal dose (LD80) as the initial concentration to cultivate the resistant cell line. The cells were incubated in RPMI-1640 medium without drugs after cultivating the Bxpc-3 cells in this medium for 72 h. When cells entered the logarithmic growth phase, they were passaged twice and cultivated with a double LD80 concentration of gemcitabine, which increased through nine concentration gradients over a ten-month period. The cells were then cultivated in RPMI-1640 medium without gemcitabine for 2 months.

**Morphological assay of drug resistant cell line Bxpc-3/Gem.** Bxpc-3/Gem and Bxpc-3 cell lines were seeded into 6-well plates ( $1 \times 10^5$ /well) and cultured two days later. Cells were collected, labelled and fixed for electron microscopic observation of cell ultra-structures.

**Sensitivity analysis of resistant cell line Bxpc-3/Gem to gemcitabine.** The logarithmic phase cells were grown in 96-well plates ( $4 \times 10^3$ /well). After adherence, cells were then cultured in different concentrations (10, 20, 40 and 80  $\mu$ M) of gemcitabine for 72 h, with 6-wells per concentration. After 72 h, the media was removed and 180  $\mu$ l of media and 20  $\mu$ l of MTT (5 mg/ml, Sigma-Aldrich) was added to each well. The media was removed and 150  $\mu$ l DMSO was added to each well 4 h later. The cells were agitated on a microplate shaker for 10 min. Then, absorbance (A) was read at 490 nm on a microplate reader. This experiment was repeated three times. The cell inhibition of each drug was calculated using the following formula: Inhibition =  $1 - \text{dosing group A} / \text{control group A} \times 100\%$ . Data were graphed on a semi-logarithmic curve with drug concentrations plotted on the X-axis and cell inhibitions on the Y-axis. SPSS software was used to calculate the 50% inhibitory inhibition ( $IC_{50}$ ) and the resistance index (RI). The formula used to calculate RI was as follows:  $RI = IC_{50} \text{ of resistance cell line} / IC_{50} \text{ of sensitive cell line}$ .

**Inhibition effect of different concentrations of emodin on Bxpc-3/Gem and Bxpc-3 cell proliferation.** The logarithmic phase cells were cultured in 96-well plates ( $4 \times 10^3$ /well) in 5%  $CO_2$  at 37°C overnight. Cells were then treated with various concentrations of emodin (10, 20, 40, 80 and 160  $\mu$ M) for 72 h (replicates of six) or treated with emodin (40  $\mu$ M) and gemcitabine (20  $\mu$ M) both alone and in combination for 72 h (6-wells per treatment group). Positive and negative control groups were also included, the positive control group was

treated with the same volume of normal saline solution, the negative control group was treated with the same volume of DMSO. The media was then removed and 180  $\mu$ l of media and 20  $\mu$ l of MTT (5mg/ml) were added to each well. The media was removed and 150  $\mu$ l DMSO was added to each well 4 h later. Cells were agitated on a microplate shaker for 10 min and the absorbances (A) were read at 490 nm on a microplate reader. This experiment was repeated three times. Cell viability was calculated as follows: Cell viability = test group A / control group A  $\times 100\%$ .

**Annexin V-FITC/PI double staining flow cytometric analysis of cell apoptosis in Bxpc-3/Gem and Bxpc-3 cells treated with emodin and gemcitabine.** Bxpc-3/Gem and Bxpc-3 cells were incubated with emodin (40  $\mu$ M) and gemcitabine (20  $\mu$ M) both alone and in combination for 72 h. The cells were then collected and digested with trypsin. Cells were centrifuged at 1000 r/min for 5 min. Cells were then washed 3 times with cold phosphate buffered saline (PBS) and resuspended with 500  $\mu$ l of binding buffer. Then, 5  $\mu$ l of Annexin V-FITC (Keygen Biological Co., Nanjing, China) was added and the mix was cultured in the dark for 5 min. Finally, 10  $\mu$ l propidium iodide (PI, Keygen Biological Co.) was added and the mix was allowed to undergo dark reaction for 5 min. Fluorescence of the cells was measured by flow cytometry at 488/530 nm. This experiment was repeated three times.

**Western blot analysis of the protein expression of P-gp, NF- $\kappa$ B, XIAP, survivin, caspase-9 and caspase-3 on Bxpc-3/Gem and Bxpc-3 cells.** Bxpc-3/Gem and Bxpc-3 cells untreated and Bxpc-3/Gem cells treated with normal saline, emodin (40  $\mu$ M), gemcitabine (20  $\mu$ M) or their combination for 72 h were collected and lysed with RIPA (Beytime, Shanghai, China) lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate 0.02% sodium azide, 100  $\mu$ g/ml PMSF, 1  $\mu$ g/ml aprotinin) for 30 min on ice. Cells were then centrifuged at 12,000 r/min for 15 min. The media was collected and the concentration of total proteins was detected with a bicinchoninic acid (BCA, Beyotime Institute of Biotechnology, Shanghai, China) assay. Proteins were electrophoresed on 8% (for P-gp), 10% (NF- $\kappa$ B, XIAP, caspase-9,  $\beta$ -actin), 12% (survivin and caspase-3), SDS-PAGE and then electrotransferred onto polyvinylidene fluoride membranes (PVDF, Millipore, Bedford, MA, USA) using a constant current of 30 mA overnight at 4°C. The PVDF membranes were blocked with 5% non-fat milk for 2 h and then incubated with anti-P-gp (Abbiotec, San Diego, CA, USA), anti-NF- $\kappa$ B (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-XIAP (Santa Cruz), anti-survivin (Santa Cruz), anti-caspase-9 (Santa Cruz) and anti-caspase-3 (Santa Cruz) primary antibodies overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibody (Beijing Zhongshan Biotechnology, Beijing, China) was added for 2 h at room temperature. After washing with TBST (1X Tris-buffered saline, 0.1% Tween-20), the bound antibody complexes were detected with enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to X-ray. This experiment was repeated three times.

Table I. Sequences of primers and the size of the sequences used for RT-PCR.

Gene	Sense primer	Antisense primer	PCR product (bp)
MDR-1	GAATCTGGAGGAAGACATGACC	TCCAATTTTGTACCAATTCC	259
NF- $\kappa$ B	AGCACAGATAACCACCAAGACCC	CCCACGCTGCTCTTCTATAGGAAC	300
Survivin	GGCATGGGTGCCCCGACGTTG	CAGAGGCCTCAATCCATGGCA	440
XIAP	TTCCTCGGGTATATGGTGTCTGAT	CCGTGCGGTGCTTTAGTTGT	292
Caspase-9	GGTTCTGGAGGATTTGGTGA	GACAGCCGTGAGAGAGAATGA	325
Caspase-3	AGCAAACCTCAGGGAAACATT	GTCTCAATGCCACAGTCCAGT	309
GAPDH	AACGGATTGGTCGTATTGGG	TCGCTCCTGGAAGATGGTGAT	216

**Reverse transcription-PCR (RT-PCR) analysis of gene expression of MDR-1, NF- $\kappa$ B, XIAP, survivin, caspase-9 and caspase-3.** Bxpc-3/Gem cells were treated with emodin (40  $\mu$ M) and gemcitabine (20  $\mu$ M) both alone and in combination for 72 h. Total RNA was extracted from cultured cells with TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The content of RNA was measured by UV spectrophotometry at 260 nm. The sequences of primers and the size of the sequences are shown in Table I. cDNA was synthesized according to the directions of the first cDNA strand synthesis kit (Fermentas, Carlsbad, CA, USA). PCR amplification was performed according to standard protocols (2X Taq PCR MasterMix, Tiangen, Beijing, China). The amplification cycling conditions were: MDR-1: 94°C 30 sec, 57°C 30 sec, 72°C 30 sec, 30 cycles; NF- $\kappa$ B: 94°C 30 sec, 54°C 30 sec, 72°C 20 sec, 30 cycles; survivin: 94°C 30 sec, 62°C 30 sec, 72°C 30 sec, 30 cycles; XIAP: 94°C 30 sec, 57°C 30 sec, 72°C 30 sec, 30 cycles; caspase-9: 94°C 30 sec, 56°C 30 sec, 72°C 30 sec, 35 cycles; caspase-3: 94°C 30 sec, 57°C 30 sec, 72°C 30 sec, 35 cycles; GAPDH: 94°C 30 sec, 54°C 30 sec, 72°C 20 sec, 25 cycles. GAPDH was used as an internal control. PCR products were electrophoresed on a 1.5% agarose gel.

**Electrophoretic mobility shift assay (EMSA) experiment.** Bxpc-3/Gem cells were treated with emodin and gemcitabine both alone and in combination for 72 h. NF- $\kappa$ B binding activity was evaluated by EMSA analysis. Nucleoproteins were extracted according to the manufacturer's instructions (Pierce nuclear protein extraction kit, Pierce, Rockford, IL, USA). The NF- $\kappa$ B consensus binding sequence was: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; 5'-GCC TGG GAA AGT CCC CTC AAC T-3' end-labeled with biotin. The binding reaction contained 2  $\mu$ l biotin end-labeled target DNA in a final volume of 20  $\mu$ l. After the reaction mixture was incubated for 20 min at room temperature, complexes were separated from the free probe on a 6.5% non-denaturing polyacrylamide gel in 0.5X TBE buffer at 120 V for 1 h and then transferred to a nylon membrane (Biodyne B membrane, Pierce) using 380 mA for 40 min. After transfer, the nylon membrane was exposed to 254 nm UV light crosslinking for 10 min and then blocked at room temperature for 20 min in blocking solution. The membrane was then incubated for 3 min at room temperature in avidin-streptavidin-horseradish peroxidase conjugate dissolved in blocking buffer (1:300 dilution). The membrane was then washed 4 times using 1X

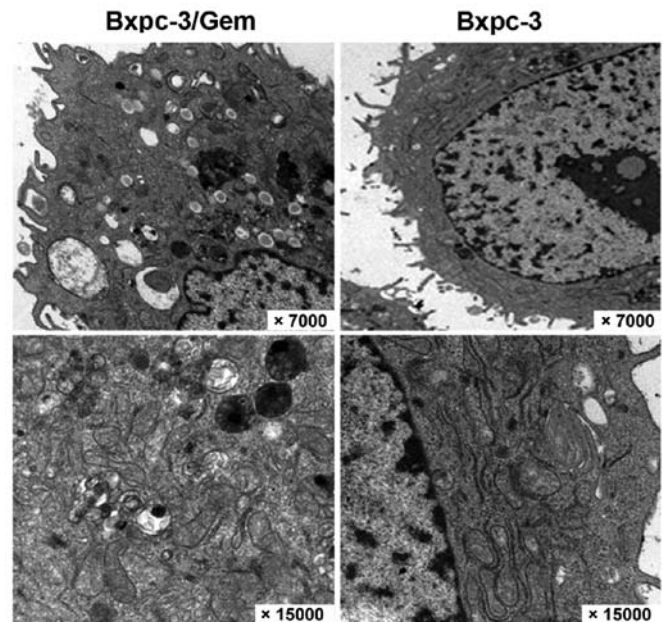


Figure 1. Electron microscopy image of Bxpc-3 and Bxpc-3/Gem. Under electron microscopy, the Bxpc-3/Gem cell line displayed significant changes in morphology compared to the parental line. Note the increase in microvilli at the cell membrane, the increase in cell surface area, the increase in cell organelles including endoplasmic reticula and vacuoles, as well as disorder of the mitochondrial cristae.

washing solution each for 5 min. Finally, the membrane was placed into the balance solution for 10 min and then exposed to film for 2-5 min before developing. The bands were scanned with an Epson Expression 1600 Pro and relative intensity was analyzed using an NIH Image 1.62 package.

**Rhodamine123 efflux experiment assay of P-gp function in Bxpc-3/Gem cells (flow cytometry).** Bxpc-3/Gem cells were treated with emodin (40  $\mu$ M) and gemcitabine (20  $\mu$ M) both alone and in combination for 72 h. The cells were resuspended in media (1x10<sup>6</sup>/ml), 10  $\mu$ l/ml rhodamine 123 staining solution (Keygen Biological Co.) was added and the cells were then cultured at 37°C in 5% CO<sub>2</sub> for 30 min. Cells were centrifuged at 2000 r/min for 5 min and washed twice with media, resuspended and incubated for 120 min. The cells were centrifuged again, washed twice with PBS and measured by flow cytometry at 488/530 nm. This experiment was repeated three times.

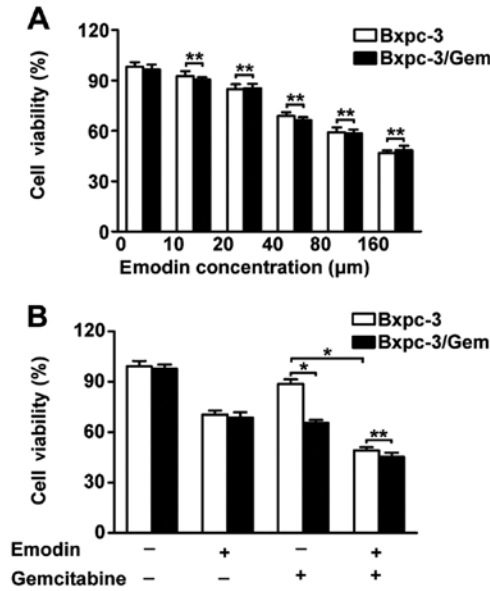


Figure 2. Effect of emodin on Bxpc-3/Gem and Bxpc-3 cell proliferation. (A) Bxpc-3 and Bxpc-3/Gem cells were treated with different concentrations of emodin (10, 20, 40, 80 and 160  $\mu\text{M}$ ) for 72 h and underwent cell proliferation analysis using MTT. Emodin was able to inhibit both Bxpc-3 and Bxpc-3/Gem cell proliferation in a dose-dependent manner. (B) Bxpc-3/Gem and Bxpc-3 cells were treated with emodin (40  $\mu\text{M}$ ) and gemcitabine (20  $\mu\text{M}$ ) alone or together for 72 h. Cell proliferation was analyzed by MTT. Quantification was performed by assigning a value of 100% to the untreated group of Bxpc-3/Gem cells. Positive control, same volume of normal saline solution; negative control, same volume of DMSO. \* $P < 0.05$ ; \*\* $P > 0.05$ .

*Statistical analysis.* All data were expressed as mean values  $\pm$  standard deviation. ANOVA analysis was performed with SPSS 10.0.  $P < 0.05$  was considered as statistically significant.

Results

*Biological properties of the drug resistant cell line Bxpc-3/Gem.* A stable gemcitabine-resistant cell line (Bxpc-3/Gem) was generated from the human pancreatic cancer cell line Bxpc-3 by exposing the cells to intermittently increasing concentrations of gemcitabine for 10 months. Compared with the parental Bxpc-3 cell line, the Bxpc-3/Gem cells changed significantly in morphology. Under electron microscopy (Fig. 1), there was an increase in the number of microvilli at the cell membrane, cell surface area, as well as the number of cell organelles including the endoplasmic reticulum and vacuoles. Furthermore, the mitochondrial cristae were disordered in the Bxpc-e/Gem cells.  $\text{IC}_{50}$  of the Bxpc-3/Gem cells was 1107.53  $\mu\text{M}$  and the resistance RI was 43.51, indicating that the Bxpc-3/Gem cell line had significant resistance to gemcitabine.

*Inhibitory effect of emodin on Bxpc-3/Gem and Bxpc-3 cell proliferation.* Emodin was able to inhibit both Bxpc-3 and Bxpc-3/Gem cell proliferation in a dose-dependent fashion as shown in Fig. 2A. Gemcitabine alone inhibited growth of Bxpc-3/Gem and Bxpc-3 cells by 11.24 and 34.53%, respectively. Inhibition of growth of Bxpc-3/Gem and Bxpc-3 cells

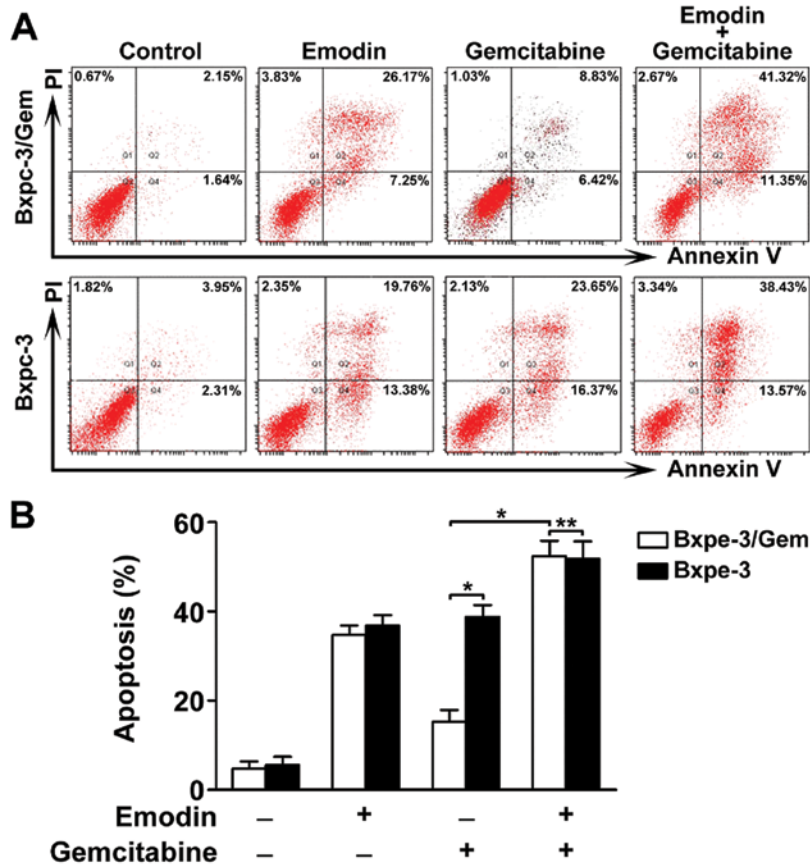


Figure 3. Effect of emodin on the apoptosis of Bxpc-3/Gem and Bxpc-3 cells. (A) Representative dot-plots illustrating the apoptotic status of SW1990 cells and Panc-1 cells. (B) The percentage of apoptotic Bxpc-3/Gem and Bxpc-3 cells. Bxpc-3/Gem and Bxpc-3 cells were treated with emodin (40  $\mu\text{M}$ ) and gemcitabine (20  $\mu\text{M}$ ) and the combination for 72 h. Apoptosis was detected by Annexin V-FITC/PI analysis by FCM. \* $P < 0.05$ ; \*\* $P > 0.05$ .

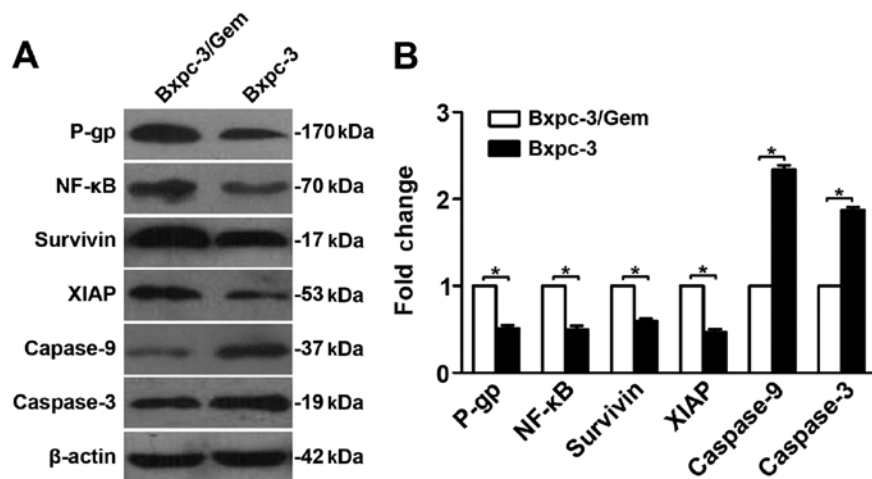


Figure 4. Expression differences of protein P-gp, NF-κB, XIAP, survivin, caspase-9 and caspase-3 in Bxpc-3/Gem and Bxpc-3 cells detected by western blot analysis. (A) Compared with the parental cell line (Bxpc-3 cells), protein expression of P-gp, NF-κB, survivin and XIAP were significantly increased, protein expression of caspase-9 and caspase-3 were significantly reduced. (B) Quantified data are presented. Quantification was performed by assigning a value of 1 to the Bxpc-3/Gem cells group. \*P<0.05.

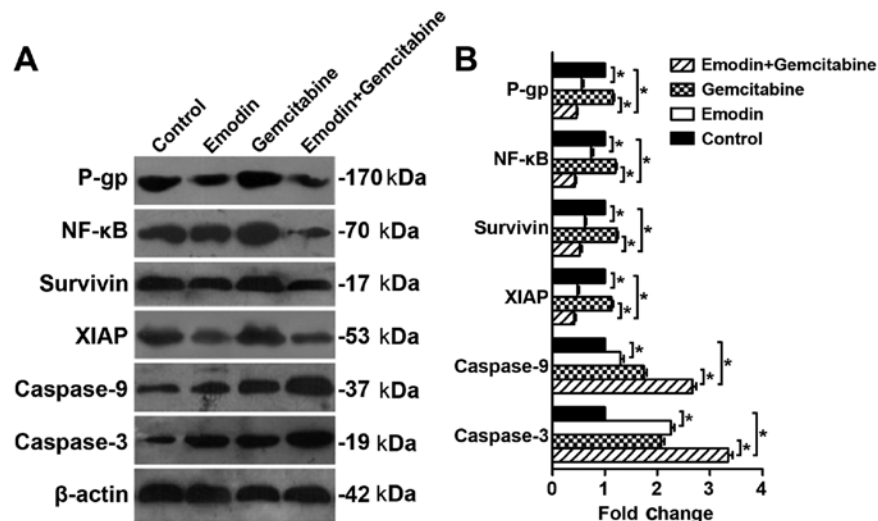


Figure 5. Effect of emodin on NF-κB and its downstream signalling proteins expression in Bxpc-3/Gem cells detected by western blot analysis. (A) The combination of emodin and gemcitabine resulted in the greatest decrease in NF-κB, P-gp, survivin and XIAP, while increasing caspase-9 and caspase-3 levels in Bxpc-3/Gem cells. (B) Quantified data are presented. Quantification was performed by assigning a value of 1 to the control group. Bxpc-3/Gem cells were treated with emodin (40 μM) and gemcitabine (20 μM) and the combination for 72 h. \*P<0.05.

by gemcitabine combined with emodin were 50.74 and 54.72%, respectively (Fig. 2B).

**Effect of emodin on apoptosis: role of gemcitabine on the drug-resistant cell line Bxpc-3/Gem.** Apoptosis in Bxpc-3/Gem cells in each group were: control group  $4.73 \pm 1.63\%$ ; emodin group  $34.72 \pm 2.14\%$ ; gemcitabine group  $15.26 \pm 2.45\%$ ; combined group  $52.44 \pm 2.56\%$ ; while cell apoptosis rate of Bxpc-3 as following: control group  $5.57 \pm 1.87\%$ ; emodin  $36.86 \pm 2.33\%$ ; gemcitabine group  $38.81 \pm 2.32\%$ ; combined group  $51.87 \pm 2.26\%$ . Strikingly, the combination of emodin and gemcitabine resulted in significant apoptosis of Bxpc-3/Gem cells (Fig. 3).

**Expression differences of protein P-gp, NF-κB, XIAP, survivin, caspase-9 and caspase-3 in Bxpc-3/Gem and Bxpc-3 cells.** Western blot analysis was performed to determine the protein

expression of P-gp, NF-κB, XIAP, survivin, caspase-9 and caspase-3 in Bxpc-3/Gem and Bxpc-3 cells (Fig. 4). Compared to the parental cell line Bxpc-3, the expression of P-gp, XIAP, survivin and NF-κB in Bxpc-3/Gem cells were significantly increased, while the expression of caspase-9 and caspase-3 were significantly reduced in Bxpc-3/Gem cells.

**Effect of emodin on NF-κB and its downstream signalling proteins in Bxpc-3/Gem cells.** Bxpc-3/Gem cells were treated with normal saline, emodin (40 μM), gemcitabine (20 μM) or their combination for 72 h (Fig. 5). Changes in protein expression were evaluated by western blot analysis. The combination treatment of emodin and gemcitabine effectively downregulated NF-κB, P-gp, survivin and XIAP. In addition, combination treatment also resulted in an increase in caspase-9 and caspase-3 levels.

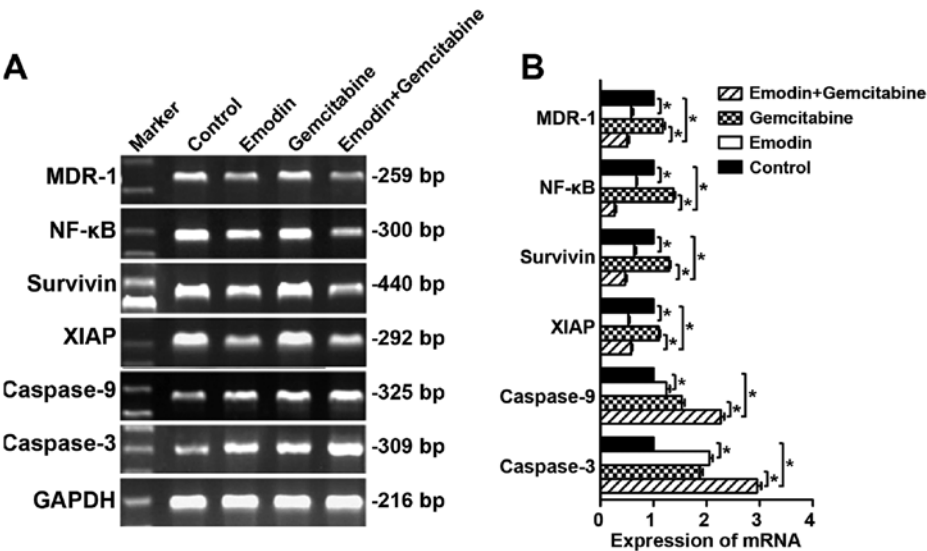


Figure 6. Effect of emodin on NF-κB and its downstream signalling proteins expression in Bxpc-3/Gem cells detected by western blot analysis. (A) The combination of emodin and gemcitabine resulted in the greatest decrease in NF-κB, P-gp, survivin and XIAP, while increasing caspase-9 and caspase-3 levels in Bxpc-3/Gem cells. (B) Quantified data are presented. Quantification was performed by assigning a value of 1 to the control group. Bxpc-3/Gem cells were treated with emodin (40 μM) and gemcitabine (20 μM) and the combination for 72 h. \*P<0.05.

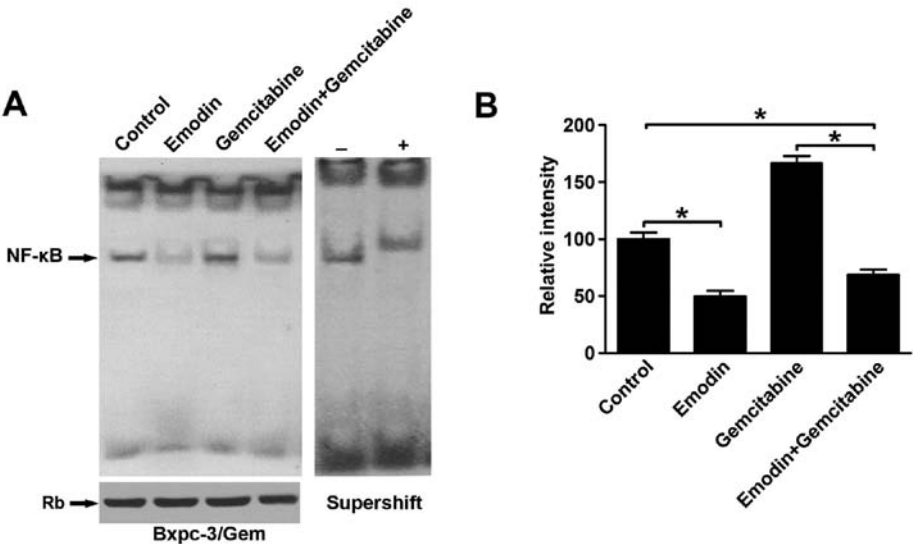


Figure 7. Effect of emodin on NF-κB and its downstream signalling proteins expression in Bxpc-3/Gem cells detected by western blot analysis. (A) The combination of emodin and gemcitabine resulted in the greatest decrease in NF-κB, P-gp, survivin and XIAP, while increasing caspase-9 and caspase-3 levels in Bxpc-3/Gem cells. (B) Quantified data are presented. Quantification was performed by assigning a value of 1 to the control group. Bxpc-3/Gem cells were treated with emodin (40 μM) and gemcitabine (20 μM) and the combination for 72 h. \*P<0.05.

*Effect of emodin on NF-κB mRNA expression and its downstream genes in Bxpc-3/Gem cells.* Bxpc-3/Gem cells were treated with emodin (40 μM) and gemcitabine (20 μM) alone or together for 72 h and then to detect changes in gene expression were detected by RT-PCR (Fig. 6). The combination of emodin and gemcitabine decreased NF-κB mRNA expression as well as MDR-1, survivin and XIAP. An upregulation of caspase-9 and caspase-3 gene expression was observed in the combination treatment of emodin and gemcitabine, which was greater than single agents and controls.

*Effect of emodin on DNA-binding activity of NF-κB in Bxpc-3/Gem cells.* Bxpc-3/Gem cells were treated with emodin

(40 μM) and gemcitabine (20 μM) alone or together for 72 h. NF-κB DNA binding activity of nuclear extracts were detected by EMSA (Fig. 7). Compared with the control group, emodin significantly reduced the expression of NF-κB activity. On the other hand, gemcitabine increased the expression of NF-κB activity. Additionally, emodin combined with gemcitabine significantly decreased NF-κB DNA-binding activity.

*Effect of emodin on P-gp function in Bxpc-3/Gem cells.* Flow cytometry was used to measure rhodamine 123 efflux in pancreatic cancer drug-resistance cell lines. Bxpc-3/Gem was treated with emodin and gemcitabine alone or together for 72 h. The control group intracellular fluorescence intensity

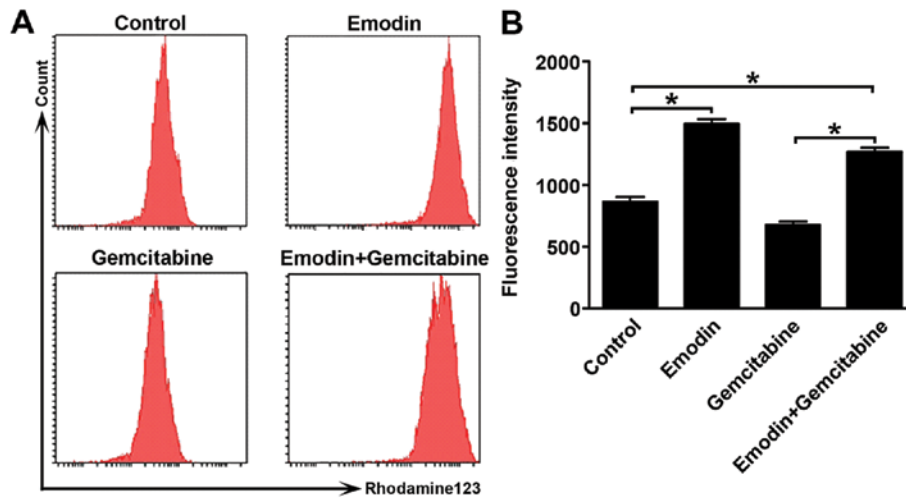


Figure 8. Effect of emodin on P-gp function in Bxpc-3/Gem cells. (A) Emodin combined with gemcitabine resulted in increasing intracellular fluorescence intensity in Bxpc-3/Gem cell. Representative charts are presented. (B) Quantified data are presented. Compared with the control group, emodin in combination with gemcitabine significantly reduced the function of P-gp. Bxpc-3/Gem cells were treated with emodin (40  $\mu$ M) and gemcitabine (20  $\mu$ M) and the combination for 72 h, \* $P < 0.05$ .

was  $863.75 \pm 29.54$ . Emodin treatment resulted in an intracellular fluorescence intensity of  $1495.68 \pm 38.35$ . Gemcitabine treatment resulted in an intracellular fluorescence intensity of  $675.13 \pm 18.5$ . The combination of emodin and gemcitabine resulted in the greatest amount of intracellular fluorescence intensity of  $1268.47 \pm 32.65$  (Fig. 8). Compared with the control group, emodin in combination with gemcitabine significantly reduced the function of P-gp.

## Discussion

Chemo-resistance remains a major obstacle in improving responses in the treatment of pancreatic cancer. Emodin is a natural product that has been shown to inhibit the growth of a variety of cancer cells (19) and has been shown to potentiate the antitumor effects of gemcitabine on pancreatic cancer (18). In this study, we demonstrated the establishment of a drug-resistant cell line Bxpc-3/Gem to evaluate the ability of emodin to sensitize resistant pancreatic cancer cells to gemcitabine. Our studies demonstrated that as a single agent, emodin inhibited cell growth and induced apoptosis in Bxpc-3 pancreatic cancer cells, as well as in gemcitabine resistant cells, Bxpc-3/Gem. Interestingly, combination treatment of emodin and gemcitabine in Bxpc-3/Gem cells resulted in a significant decrease in cellular proliferation and increase in apoptotic positive cells. Next, we further explored the possible underlying mechanisms that would explain these results.

NF- $\kappa$ B is a critical mediator of cell survival that is activated by chemotherapy (20) and has been shown to contribute to chemo-resistance (21,22). Studies have shown that inhibitors of NF- $\kappa$ B potentiate the antitumor effects of chemotherapeutic agents (23). Results of this study demonstrated that emodin as a single agent and in combination with gemcitabine, significantly decreased the expression and activity of NF- $\kappa$ B compared to gemcitabine alone.

NF- $\kappa$ B plays a very important role in chemotherapy-resistance by a variety of mechanisms. Researchers have shown that NF- $\kappa$ B is involved in upregulation of MDR-1 mRNA

expression which contributes to chemo-resistance (24). NF- $\kappa$ B is involved in chemo-resistance and can increase the expression of survivin and other survival-related proteins. NF- $\kappa$ B is involved in pancreatic cancer chemo-resistance through the upregulation of XIAP, Bcl-xl and survivin (25). Therefore, we detected the effect of emodin on NF- $\kappa$ B-regulated chemo-resistance related gene products (MDR-1), XIAP and survivin.

Studies have shown that chemo-resistance may be attributed to the multi-drug resistance gene (MDR-1) and its encoded protein P-gp, which has been shown to reduce drug uptake (26). Studies have shown that chemo-resistance is also associated with the multi-drug resistance-associated protein (MRP) family (27,28). In this study, the expression of MDR-1(P-gp) significantly decreased when treated with emodin alone or with gemcitabine and emodin together. P-gp encoded by the MDR-1 gene is a transmembrane protein, which belongs to the ATP-binding cassette (ABC) transporter protein superfamily. It functions as an ATP-dependent drug efflux pump (29), which has been shown to decrease the intracellular concentration of chemotherapeutic agents (30). MDR-1/P-gp plays a very important role in tumor chemoresistance (24,26). In this study, the rhodamine 123 efflux assay demonstrated that the function of P-gp was significantly reduced in emodin single agent and combination treatments. Thus, emodin was able to inhibit one major cause of chemoresistance in cells when used in combination with gemcitabine, which suggests that emodin sensitizes Bxpc-3/Gem cells to gemcitabine via inhibition of the expression of NF- $\kappa$ B-regulated MDR-1 and function of MDR-1 encoded P-gp.

Another major cause of chemo-resistance is a result of multiple cycle chemotherapeutic regimens that can alter the balance between pro-apoptotic and anti-apoptotic (survivin) signaling mechanisms (31). Survival signaling proteins or apoptosis inhibitory proteins (inhibitor of apoptosis proteins, IAPs) are important regulators of apoptosis and if overexpressed can reduce the antitumor effects of chemotherapeutic agents. Survivin is a member of the IAPs family and has a dual function of inhibiting apoptosis as well as regulating cell



proliferation (32). Studies have shown that survivin expression is associated with chemo-resistance (33) and has been correlated with poor prognostic parameters in ovarian cancer (34). Another important IAPs member is XIAP (X-linked inhibitor of apoptosis), which can inhibit apoptosis via downregulation of caspase-9 and caspase-3 expression. XIAP expression has been linked to chemo-resistance in colon cancer cells (35). The results of this study demonstrated that emodin as a single agent and in combination treatment with gemcitabine, resulted in decreased survivin and XIAP expression compared to single agent gemcitabine and controls. Thus, emodin appears to sensitize Bxpc-3/Gem cells to gemcitabine therapy by inhibiting survival proteins (survivin and XIAP) in Bxpc-3/Gem cells.

Our data suggest that emodin may sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine therapy by inhibition of NF- $\kappa$ B activation, leading to downregulation of MDR-1 gene as well as P-gp function and expression of XIAP and survivin. The use of emodin in combination with first-line chemotherapy agents may help to improve response rates, decrease toxicity and reduce chemo-resistance.

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