

Emodin enhances antitumor effect of paclitaxel on human non-small-cell lung cancer cells in vitro and in vivo

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Background: Non-small-cell lung cancer (NSCLC) was known as the most malignant tumor. Paclitaxel (PTX) is the effective drug used for the treatment of NSCLC; however, it also exhibits severe side effects. Emodin could induce apoptosis of NSCLC cells and serve as a potential cancer therapeutic agent. However, the effects of combination of emodin with PTX on NSCLC remain unclear. Thus, this study aimed to investigate the effects of emodin in combination with PTX on A549 cells.

Materials and methods: The effects of combination treatment on the proliferation, apoptosis and invasion of NSCLC cells were evaluated by CCK-8, flow cytometric and TUNEL assays, respectively. In addition, Western blotting was used to detect the expressions of Bax, Bcl-2, active caspase 3, p-Akt and ERK in cells.

Results: Combination of emodin with PTX synergistically inhibited the proliferation of A549 cells in vitro. In addition, we found that emodin significantly enhanced PTX-induced apoptosis in A549 cells via increasing the expressions of Bax and active caspase 3 and decreasing the levels of Bcl-2, p-Akt and p-ERK. Moreover, emodin markedly enhanced antitumor effect of PTX on A549 xenograft without significant side effects in vivo.

Conclusion: Our findings indicated that emodin could significantly enhance antitumor effect of PTX in vitro and in vivo. Therefore, the combination of emodin with PTX may serve as a potential strategy for the treatment of patients with NSCLC.

Keywords: non-small-cell lung cancer, emodin, paclitaxel, apoptosis

Introduction

Lung cancer is the main cause of cancer-related death in worldwide,¹ which is characterized by high morbidity rates, high metastasis rates and high death rates.² Non-small-cell lung cancer (NSCLC) is the most common malignant tumor in the world and accounting for 80 percent of all lung tumors.³ A 60% of patients with NSCLC were diagnosed with advanced stage of disease.⁴ Patients with advanced NSCLC have less response to chemotherapy, because of the side effects and drug tolerance.^{5,6} Although surgery is the most commonly method to treat patients with NSCLC, the 5-year survival rate is poor.⁷ Therefore, novel effective therapies for the treatment of NSCLC are imminently needed.

Paclitaxel (PTX) is an effective drug used for the treatment of cancer, which is extensively applied to clinical therapeutic treatment of NSCLC.^{8,9} However, PTX also exhibits severe side effects including vomiting, diarrhea and myelosuppression.^{8,10} In recent years, drug combination is an effective method to reduce cell toxicity and

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improve the efficacy of therapy.¹¹ Recently, several traditional Chinese medicine (TCM) monomers, such as andrographolide, tomatine and piperlongumine, have been found to exhibit synergistically antitumor effects against various cancers when combined with PTX.^{8,11,12} Therefore, combination of TCM with PTX may be a potential therapeutic method for the treatment of NSCLC.

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), a natural active anthraquinone compound, was extracted from the rhizome of *Rheum palmatum* (a Chinese medicinal herb). Emodin has been indicated to have a multiple of pharmacological and biological functions, including anti-inflammation, anti-neuro and anti-renal protection effects.^{13–15} In addition, emodin was reported to inhibited A549 cell invasion and migration via inducing apoptosis.^{16,17} Therefore, the present study aimed to explore the antitumor effects of combination of PTX with emodin on human NSCLC cells A549 in vitro and in vivo.

Materials and methods

Cell culture

A549 human NSCLC cell line was purchased from American Type Culture Collection (Rockville, MD, USA). A549 cells were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific) in a 5% CO₂ humidified incubator at 37 °C.

CCK-8 assay

The cell viability value was measured by cell counting kit-8 (CCK8, Beyotime, Shanghai, China) according to the manufacturer's protocols. Briefly, A549 cells (5×10^3 cells/well) were plated into each well of 96-well plate at 37°C overnight. Then, the cells were treated with emodin (0, 10, 20, 40, 80, 120 µM) and/or PTX (0, 2, 4, 8, 16, 32 µM) for 72 h. After that, 10 µL of CCK-8 reagent was added to each well for another 1 hr. Next, the absorbance at 450 nm was measured with a microplate reader (Bio-Rad Laboratories, Benicia, CA, USA). Emodin and PTX standard product were purchased from Sigma (St. Louis, MO, USA, #30,269; #580,555).

Combination studies

The combination index (CI) by Chou was used to analyze the drug combination studies.¹⁸ Combinations of PTX with emodin were utilized in the cell treatments. A549

cells were exposed to solutions containing 0, 5, 10, 20, 40 µM emodin combined with PTX (range 0 from 32 µM). The CI for the combination of PTX and emodin in NSCLC can be described as $CI = DA / IC_{x,A} + DB/IC_{x,B}$. (DA and DB represent the concentrations of samples A and B in combination to reach x% inhibition; IC_{x,A} and IC_{x,B} represent concentrations of samples A and B to reach x% inhibition when used alone, respectively.) The above parameters can be automatically determined from the median-effect equation using CalcuSyn software (Biososoftware, Ferguson, MO, USA). $0.9 \leq CI \leq 1.1$ indicates additive; $0.8 \leq CI \leq 0.9$ indicates slight synergism; $0.6 \leq CI \leq 0.8$ indicates moderate synergism; $0.4 \leq CI \leq 0.6$ indicates synergism, $0.2 \leq CI \leq 0.4$ indicates strong synergism. The dose reduction index (DRI) was used to evaluate the extent of dose reduction in the combination treatment compared with the dose of single treatment. DRI can be described as $DRI = IC_{x,A}/DA$.

Immunofluorescence

A549 cells (4×10^4 cells/well) were plated into each well of 24-well plate at 37°C overnight, and then cells were treated with emodin (10 µM) and/or PTX (4 µM) for 72 hr. After that, cells were washed in PBS three times and fixed in pre-cold methanol for 10 min at -20°C. Next, cells were incubated with primary antibodies for anti-Ki67 (Abcam; ab15580) (1:1,000), DAPI (ab104139), at 4°C overnight. Subsequently, cells were incubated with goat anti-rabbit IgG second antibody (Abcam; ab150077) (1:5,000) at 37°C for 1 hr. The samples were observed by fluorescence microscope (Olympus CX23 Tokyo, Japan).

Flow cytometric analysis of cell apoptosis

A549 cells (5×10^4 cells/well) were plated into each well of 6-well plate at 37°C overnight, and then cells were treated with emodin (10 µM) and/or PTX (4 µM) for 72 hr. Later on, cells were washed in cold PBS three times and re-suspended with Annexin V binding buffer. Then, cell suspension was cultured with 5 µL Annexin V-FITC and 5 µL propidium for 15 min at room temperature in the dark, and apoptotic cells were measured by flow cytometer (BD, Franklin Lake, NJ, USA).

Western blot analysis

The cells were collected and protein concentrations were measured using BCA protein assay kit (Beyotime). Equal amounts of protein extracts (30 µg) were loaded in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) with 10% SDS polyacrylamide gel. Then, the proteins were transferred to polyvinylidene fluoride membranes (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) for 2 hr and blocked with 5% defatted milk in TBST for another 1 hr. After that, the membranes were incubated with primary antibodies overnight at 4°C: Bax (Abcam; cat. no. ab32503) (1:1,000), Bcl-2 (Abcam; cat. no. ab32124) (1:1,000), active caspase 3 (Abcam; cat. no. ab2302) (1:1,000), Akt (Abcam; cat. no. ab8805) (1:1,000), p-Akt (Abcam; cat. no. ab38449) (1:1,000), ERK (Abcam; cat. no. ab54230) (1:1,000), p-ERK (Abcam; cat. no. ab50011) (1:1,000), and anti-GAPDH (Abcam; cat. no. ab8245) (1:1,000). After washing with PBS, the PVDF membrane was incubated with goat anti-rabbit IgG second antibody (Abcam; ab150077) (1:5,000) at room temperature for 1 hr. Finally, the immunoreactivity was detected using the ECL reagent (Santa Cruz Biotechnology).

Animal study

To investigate the anti-tumor effects of combination of PTX with emodin on NSCLC tumor-burdened animals, 16 male BALB/nude mice (aged 4–6 weeks) were purchased from Shanghai Slac Animal Center (Shanghai, China) and housed within a constant temperature of 18–23°C and 55–65% humidity. Sixteen nude mice were randomly divided into four groups (4 mice/group): vehicle, PTX (10 mg/kg), emodin (50 mg/kg), or PTX + emodin. Aliquots of A549 cells (5×10^6 cells, in 100 μ L of PBS) were injected subcutaneously into the right armpit area of the mice. Tumor volume was measured by caliper according to the formula: $1/2 \times (\text{length} \times \text{width}^2)$ weekly for three weeks until mice were sacrificed under anesthesia. The body weight and tumor weight were recorded when nude mice were sacrificed on day 21. Parts of each tumor tissue were stored at –20°C for immunohistochemical staining. All animal experiments were performed in accordance with institutional guidelines, following a protocol approved by the Ethics Committees of Zhejiang University. National Institutes of Health guide for the care and use of laboratory animals was strictly followed by us.

TUNEL staining

Tumor tissues were stained using an APO-BrdUTM TUNEL Assay Kit (Thermo Fisher Scientific, #A23210), according to the manufacturer's instructions.

Statistical analysis

Each group were performed at least three independent experiments and all values were expressed as the mean \pm standard deviation. The comparison between two groups was analyzed by Student's *t*-test. The comparisons among multiple groups were made with one-way analysis of variance followed by Dunnett's test. For all tests, values of $P < 0.05$ or $P < 0.01$ were considered to indicate a statistically significant difference.

Results

Combination of emodin with PTX synergistically inhibited the proliferation of A549 cells

The chemical structure of emodin was indicated in Figure 1A. CCK-8 assay was used to evaluate the effects of emodin or/and PTX on the viability of A549 cells. The result indicated both emodin (range from 0 to 120 μ M) and PTX (range from 0 to 32 μ M) inhibited the proliferation of A549 cells in a dose-dependent manner (Figure 1A and B). In addition, 4 μ M PTX significantly inhibited the proliferation of A549 cells and exhibited about 60% growth inhibition (Figure 1C). Therefore, 4 μ M PTX were utilized in the following experiments. Moreover, emodin (10, 20 or 40 μ M) markedly enhanced the antiproliferative effect of PTX on A549 cells (Figure 1D).

As shown in Table 1, IC_{50} value of PTX (alone treatment) was 10.08 μ M. However, when PTX was combined with emodin (5, 10, 20 or 40 μ M), the IC_{50} value of PTX was decreased in the range of 1.33–8.65 μ M (Table 1). In addition, it was noticed that the CI values of emodin (10 or 20 μ M) combined with PTX were less than 0.8, which indicated the moderate synergism (Table 1). Meanwhile, the CI value of 10 μ M emodin combined with PTX treatment group was lower than other groups. Therefore, 10 μ M emodin combined with 4 μ M PTX was utilized in the subsequent experiments. Meanwhile, the value of DRI was dose-dependent (Table 1). These results suggested that combination of emodin with PTX synergistically inhibited the proliferation of A549 cells.

Emodin could enhance PTX-induced apoptosis in A549 cells

To further demonstrate the effects of combination of emodin with PTX on A549 cells, immunofluorescence and flow cytometry were applied. Ki67 is normally used as a detector for cells in a proliferative state. In immunofluorescence assay, 4 μ M PTX significantly inhibited the proliferation

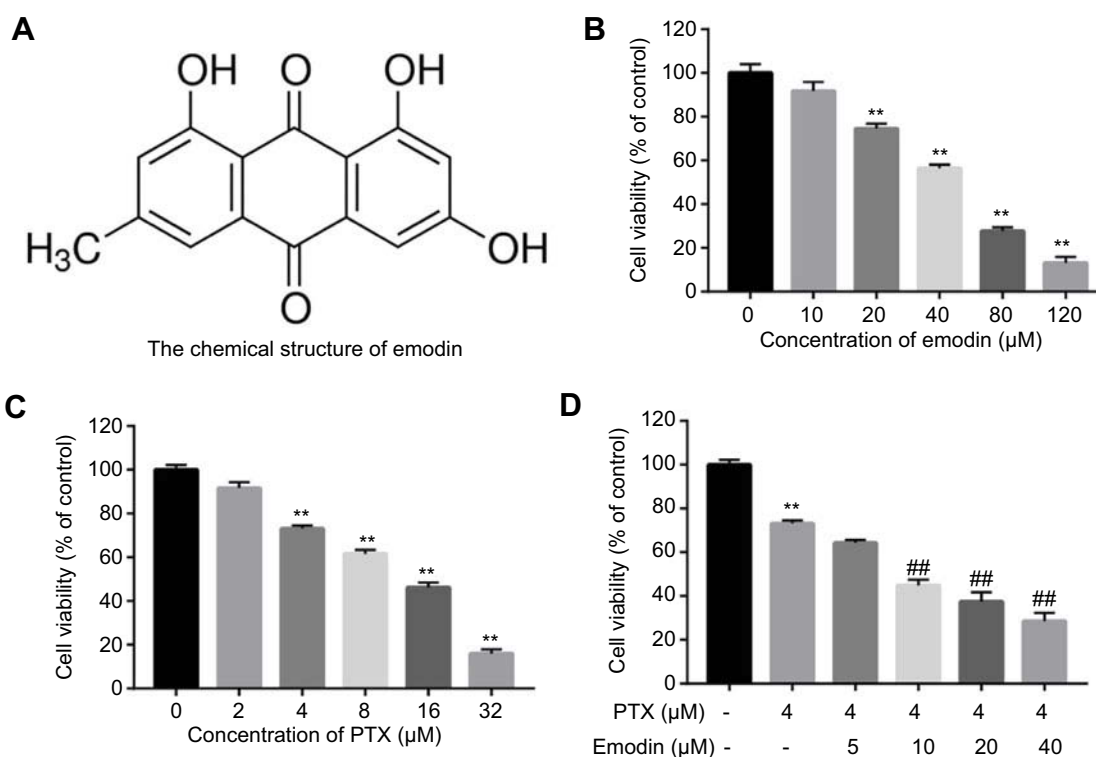


Figure 1 Combination of emodin with PTX synergistically inhibited the proliferation of A549 cells. **(A)** The chemical structure of emodin. **(B)** Cell viability was determined using CCK-8 assay in A549 cells treated with emodin (0, 10, 20, 40, 80, 120 μM) for 72 hr. **(C)** Cell viability was determined using CCK-8 assay in A549 cells treated with PTX (0, 2, 4, 6, 8, 16, 32 μM) for 72 hr. **(D)** Cell viability was determined using CCK-8 assay in A549 cells treated with PTX (4 μM) and emodin (0, 5, 10, 20, 40 μM) for 72 h. ** $P < 0.01$ compared with 0 μM group; ## $P < 0.01$ compared with 4 μM PTX alone group. Each group performed at least three independent experiments.

Abbreviation: PTX, paclitaxel.

Table 1 Evaluation of combination of PTX with emodin in NSCLC (72 hr treatment)

Combination strategy	IC ₅₀ value of PTX	95% CI values	DRI
PTX (range 0 from 32 μM)	IC ₅₀ = 10.08 \pm 1.04 μM	—	—
PTX +5 μM emodin	IC ₅₀ = 8.65 \pm 1.01 μM	0.980	1.165
PTX +10 μM emodin	IC ₅₀ = 4.68 \pm 0.71 μM	0.709	2.154
PTX +20 μM emodin	IC ₅₀ = 2.33 \pm 0.52 μM	0.720	4.326
PTX +40 μM emodin	IC ₅₀ = 1.33 \pm 0.33 μM	1.109	7.579

Abbreviations: DRI, dose reduction index; NSCLC, non-small-cell; PTX, paclitaxel.

of A549 cells, while 10 μM emodin had very limited effect on cell growth (Figure 2A). However, the antiproliferation effect of PTX was notably increased in the presence of emodin, compared with PTX alone (Figure 2A). In addition, PTX-induced cell apoptosis was markedly enhanced by treatment of emodin (Figure 2B).

Next, Western blotting was used to detect the apoptosis-related proteins Bax, Bcl-2 and active caspase-3. As shown in Figure 2C–F, PTX markedly increased the expressions of Bax and active caspase 3 and decreased the expression of Bcl-2, compared with the control group. As expected, the levels of Bax and active caspase 3 in cells

were further increased by emodin, while the expression of Bcl-2 was further decreased, compared with the PTX alone (Figure 2C–D). All these data suggested that emodin enhanced PTX-induced apoptosis in A549 cells.

Combination of emodin with PTX inhibited Akt and ERK pathways in A549 cells

To explore the mechanisms by which the combination inhibited the proliferation of A549 cells, Akt and ERK pathways were investigated. As indicated in Figure 3A–C, combination of emodin with PTX significantly downregulated the

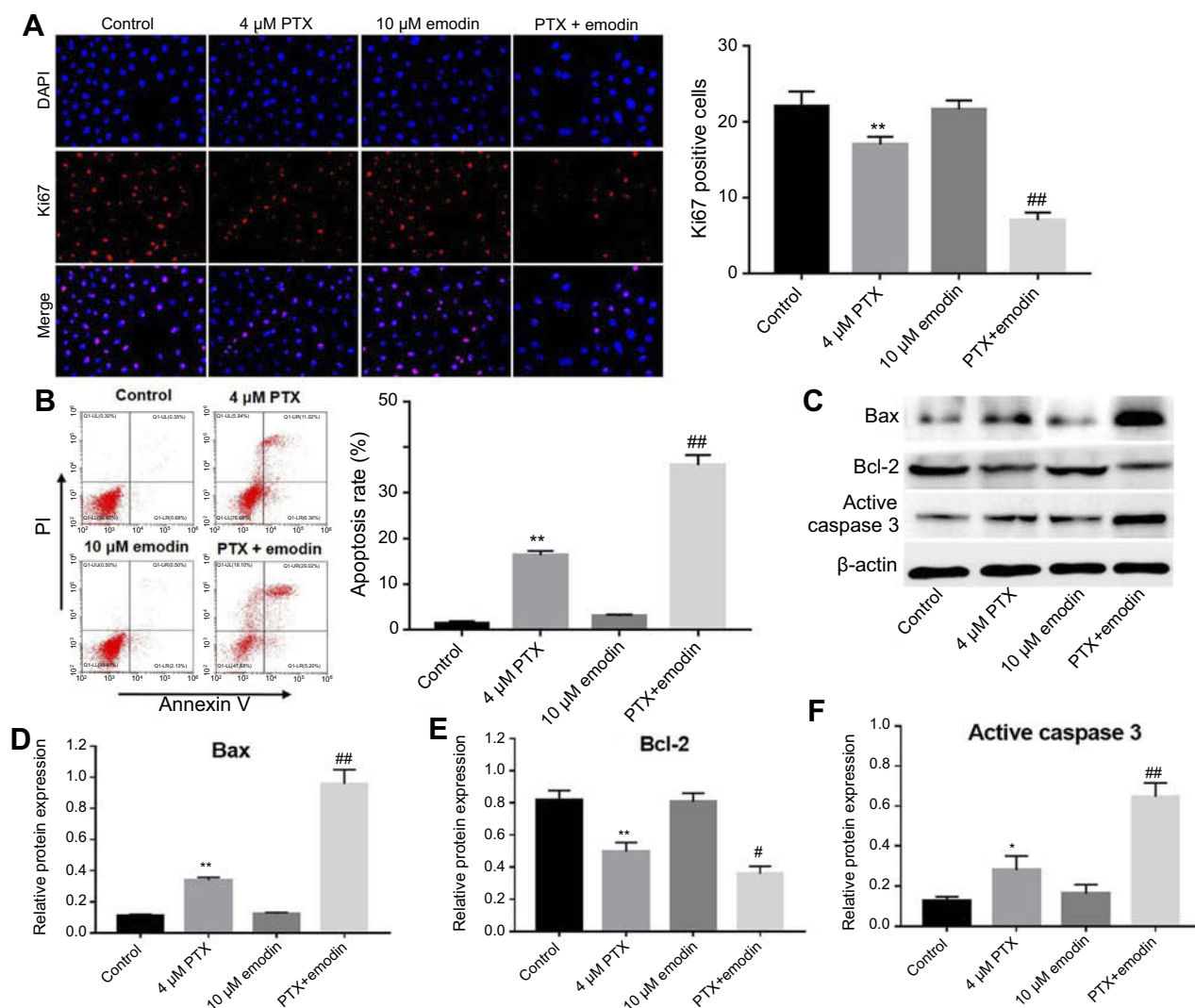


Figure 2 Emodin could enhance PTX-induced apoptosis in A549 cells. A549 cells were treated with 4 μ M PTX or/and 10 μ M emodin for 72 h.r (A) Relative fluorescence expression levels were quantified by Ki67 and DAPI staining. The number of Ki67 positive cells were counted. (B) Apoptotic cells were detected with Annexin V and PI double staining. The apoptosis cell rates were calculated. (C) The expressions of Bax, Bcl-2 and active caspase 3 were analyzed by Western blotting in A549 cells. (D) The relative expression of Bax was quantified by normalizing to β -actin. (E) The relative expression of Bcl-2 was quantified by normalizing to β -actin. (F) The relative expression of active caspase 3 was quantified by normalizing to β -actin. * P < 0.05, ** P < 0.01 compared with control group; # P < 0.05, ## P < 0.01 compared with 4 μ M PTX alone group. Each group performed at least three independent experiments.

Abbreviation: PTX, paclitaxel.

expressions of phosphorylated Akt (p-Akt) and phosphorylated ERK (p-ERK), compared with PTX alone or control group. This data suggested that combination of emodin with PTX inhibited Akt and ERK pathways in A549 cells.

Emodin could enhance the antitumor effects of the PTX on NSCLC in vivo

To further confirm the effects of the combination on tumor growth in vivo, a xenograft mouse model was used. As shown in Figure 4A and B, 10 mg/kg PTX decreased the

tumor volume, while 50 mg/kg emodin had very limited effect on the tumor volume on day 21. Similar to in vitro data, the volumes of the tumors were further decreased by the combination treatment compared with the PTX group. In addition, the tumor weights in combination treatment group were notably decreased, compared with 10 mg/kg PTX or control group (Figure 4C). Meanwhile, the combined treatment had no effect on the body weight changes of mice, which indicated this treatment strategy was tolerable (Figure 4D). These results illustrated that emodin could enhance the antitumor effects of PTX on NSCLC in vivo.

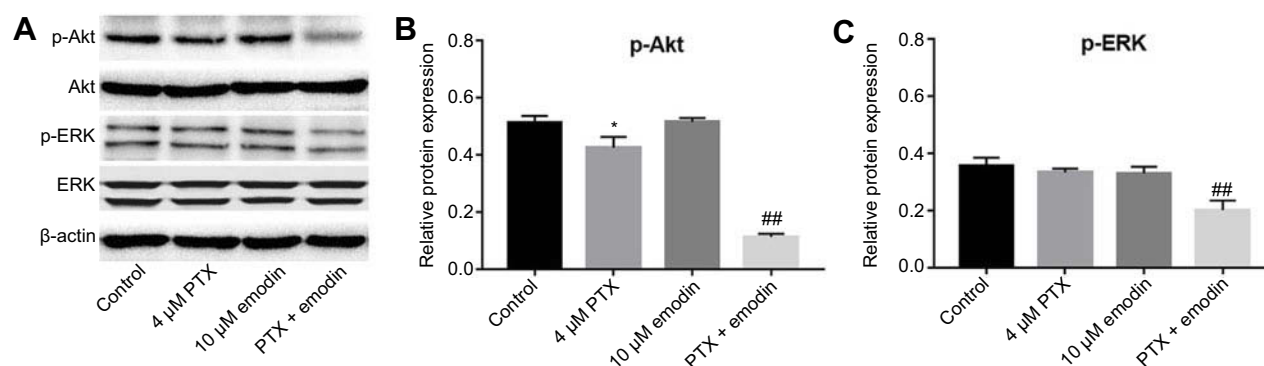


Figure 3 Combination of emodin with PTX inhibited Akt and ERK pathways in A549 cells. A549 cells were treated with 4 μ M PTX or/and 10 μ M emodin for 72 hr. **(A)** The expressions of p-Akt and p-ERK in A549 cells were analyzed by Western blotting. **(B)** The relative expression of p-Akt was quantified by normalizing to Akt. **(C)** The relative expression of p-ERK was quantified by normalizing to ERK. * P < 0.05 compared with control group. ## P < 0.01 compared with 4 μ M PTX group. Each group performed at least three independent experiments.

Abbreviation: PTX, paclitaxel.

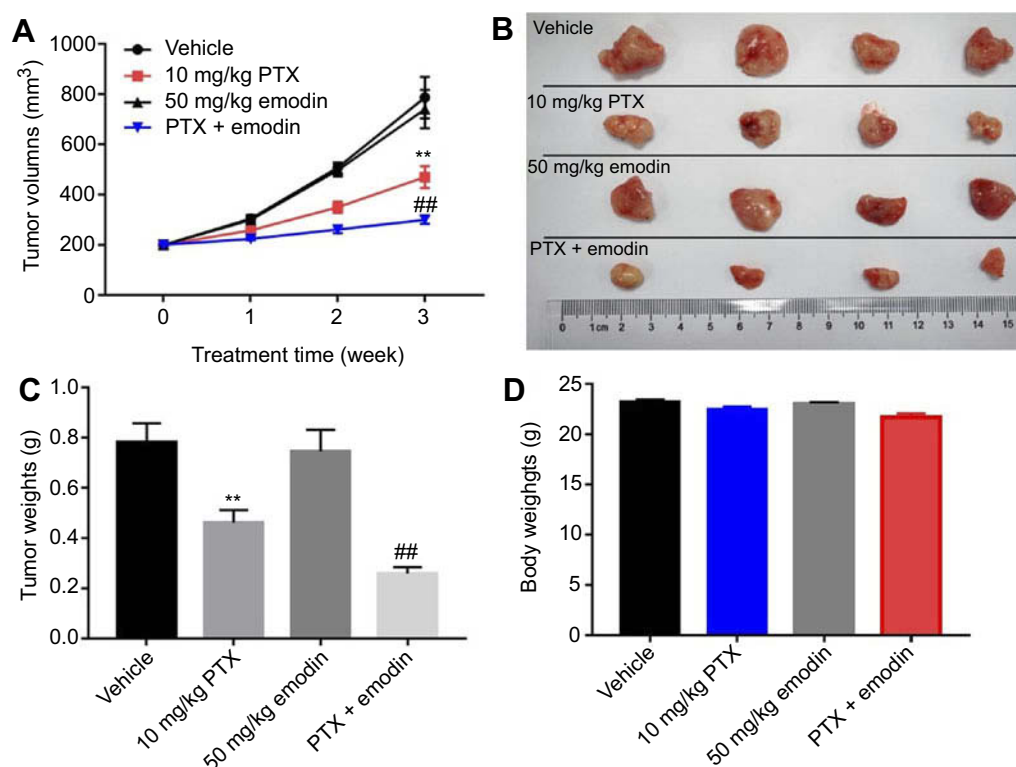


Figure 4 Emodin could enhance the antitumor effects of the PTX on NSCLC in vivo. **(A)** Tumor volumes of xenograft were measured weekly post-inoculation of A549 cells. **(B)** Tumors were isolated from xenografts and pictured after 3 weeks of treatment. **(C)** Tumor weights in each group were calculated. **(D)** Body weights of mice were calculated. $N = 4$, ** P < 0.01 compared with vehicle group; ## P < 0.01 compared with 10 mg/kg PTX group.

Abbreviations: NSCLC, non-small-cell; PTX, paclitaxel.

Combination of emodin with PTX-induced apoptosis in tumor tissues via inhibition of Akt signaling pathway in vivo

We next examined the effects of the combination on apoptosis and Akt signaling in A549 tumor tissues in vivo. The data of

TUNEL indicated that emodin markedly enhanced PTX-induced cell apoptosis in tumor tissues compared with the 10 mg/kg PTX group (Figure 5A). In addition, 10 mg/kg PTX significantly downregulated the expression of p-Akt and upregulated the expression of active caspase 3 in tumor tissues compared with vehicle group. Similar to in vitro data,

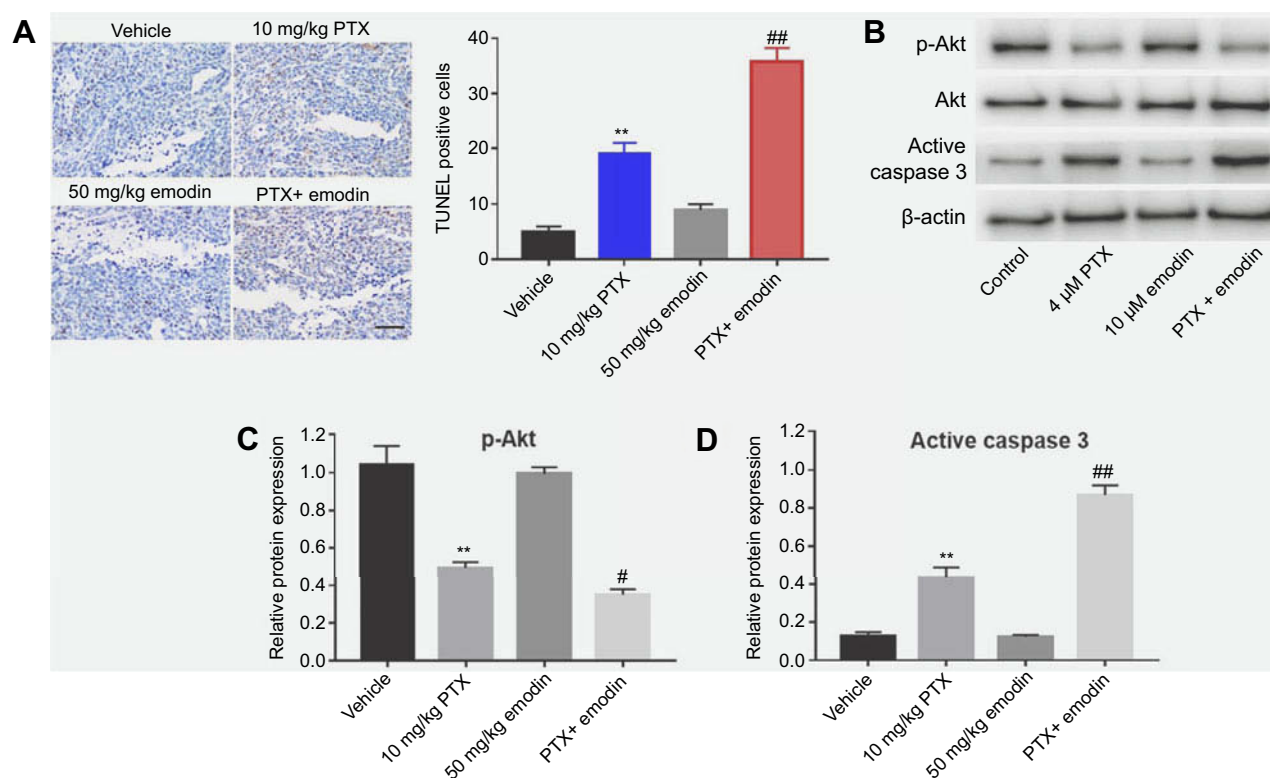


Figure 5 Combination of emodin with PTX-induced apoptosis in tumor tissues via inhibition of Akt signaling pathway in vivo. **(A)** TUNEL staining of tumor tissues in each group. Quantification of TUNEL positive cells in tissues. **(B)** Expressions of p-Akt and active caspase 3 were analyzed by Western blotting in A549 cells. **(C)** Relative expression of p-Akt was quantified by normalizing to Akt. **(D)** Relative expression of active caspase 3 was quantified by normalizing to β -actin. ** $P < 0.01$ compared with vehicle group; # $P < 0.05$, ## $P < 0.01$ compared with 10 mg/kg PTX group. Each group performed at least three independent experiments.

Abbreviation: PTX, paclitaxel.

the expressions of these proteins were further decreased (p-Akt) or increased (active caspase 3) in the presence of emodin (Figure 5B–D). All these results suggested that combination of emodin with PTX induced apoptosis in tumor tissues via inhibition of Akt signaling pathway in vivo.

Discussion

PTX is a common and an effective drug used for the treatment of cancer, while PTX also exhibits severe side effects.^{8,9} The use of TCM monomer and PTX in combination is a prospective method to reduce cytotoxicity induced by PTX and to increase the curative effect. In this study, we aimed to investigate the antitumor effect of emodin and PTX on A549 cells in vitro and in vivo. Our results indicated that emodin significantly enhanced the antiproliferative and proapoptotic effects of PTX in vitro and in vivo.

The results suggested that low-dose emodin markedly enhanced the PTX-induced antitumor activities on A549 cells in vitro and in vivo. This finding similar to previous reports that combination of carboplatin with

PTX or combined PTX with triptolide-co-loaded could synergistically inhibited tumor growth in lung cancer.^{19,20} In addition, our data suggested that 50 mg/kg emodin had very limited effect on the tumor weight or body weight of mice. Moreover, the combination treatment significantly reduced the tumor weight and had no effect on the body weight changes of mice, which indicated that this treatment strategy had no obvious systemic toxicity. Consistently, Li et al found that emodin increased cisplatin-induced cell apoptosis in xenograft tumors without obvious systemic toxicity.²¹ All these results demonstrated that combination treatments could inhibit A549 cell proliferation without systemic toxicity in vivo.

On the other hand, emodin enhanced the PTX-induced apoptosis in A549 cells via increasing the level of Bax and active caspase 3 and decreasing the level of Bcl-2. Lai et al found that emodin induced apoptosis in A549 cells via upregulating the expression of Bax in lung cancer.²² PTX in combination with 5-demethylnobiletin induced cell apoptosis through regulating caspase 3 pathway in lung

cancer.²³ Consistent with these results, the combination of emodin with PTX could induce apoptosis via upregulation of Bax and active caspase 3 and downregulation of Bcl-2 in A549 cells.

Next, the mechanisms underlying the antitumor effects of the combination on NSCLC were further investigated. Akt and ERK signaling pathways are key modulators of cell proliferation and apoptosis in NSCLC, which can promote tumor cell growth and inhibition of apoptosis.^{24,25} Previous evidence has indicated that activation of Akt/ERK could increase PTX-induced gastric cancer cell resistance.²⁶ Previous studies also suggested that inhibition of Akt could increase PTX sensitivity in human ovarian cancer cells.²⁷ In addition, Bartling et al also found that PTX induced lung cancer cell apoptosis via inhibition of ERK and Akt kinases.²⁸ Therefore, we detected the level of the Akt and ERK signaling pathway proteins in A549 cells treated with emodin or/and PTX in the present study. Our results demonstrated that the combination of emodin with PTX synergistically induced apoptosis via inhibition of Akt and ERK pathways in A549 cells. Consistent with these results, PTX combined with alpha-tomatine synergistically induced apoptosis via inhibition of Akt signaling pathway in human prostate cancer PC-3.¹¹ Meanwhile, emodin induced apoptosis via inhibition of ERK and Akt pathways in human lung adenocarcinoma cells.²⁹ These data clearly confirmed that the combination treatment induced apoptosis via inhibition of Akt and ERK pathways in A549 cells. However, the effect of combination of emodin with PTX on PTX-resistant A549 cells remained unclear and further investigations are needed.

Conclusion

In conclusion, emodin enhanced the antitumor effects of PTX on A549 cells in vitro and in vivo. The results suggested that the combination of emodin with PTX may serve as a potent strategy in the treatment of patients with NSCLC.

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Disclosure

The authors report no conflicts of interest in this work.

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