Emodin enhances cisplatin sensitivity in non-small cell lung cancer through Pgp downregulation

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Abstract. Cisplatin resistance is one of the main causes of chemotherapy failure and tumor progression in non-small cell lung cancer (NSCLC). Emodin has been demonstrated to induce NSCLC cell apoptosis and act as a potential cancer therapeutic agent. However, whether emodin could affect NSCLC cell sensitivity toward cisplatin remains unclear. The present study aimed to determine the effect of emodin and cisplatin combination on the chemosensitivity of NSCLC cells. A549 and H460 cells were treated with different concentrations of cisplatin and/or emodin. Cell Counting Kit-8, fluorescence microscopy, immunofluorescence assays and flow cytometry were used to determine cell proliferation, drug efflux, DNA damage level and cell apoptosis, respectively. P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1) expression was detected by western blotting. The results demonstrated that emodin and cisplatin inhibited the proliferation of A549 and H460 cells. Furthermore, emodin inhibited the drug efflux in A549 and H460 cells in a dose-dependent manner. In addition, emodin enhanced cisplatin-induced apoptosis and DNA damage in A549 and H460 cells. Emodin also decreased Pgp expression in A549 and H460 cells in a dose-dependent manner; however, it had no effect on MRP1 expression. Taken together, the results from the present study demonstrated that emodin can increase A549 and H460 cell sensitivity to cisplatin by inhibiting Pgp expression. Emodin may therefore be considered as an effective adjuvant for cisplatin treatment.

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Introduction

Lung cancer is a common malignant tumor and a leading cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer worldwide, accounting for 80% of all lung cancer cases (2). Furthermore, 60% of patients with NSCLC are diagnosed with advanced-stage tumors (1). At present, patients with advanced or metastatic NSCLC are usually treated with platinum-based chemotherapy (3). Due to adverse effects of chemotherapy, such as neutropenia, stomatitis, mucositis, diarrhea, emesis and chemoresistance, patients with advanced NSCLC become less sensitive to chemotherapy (4,5). It is therefore essential to improve the specificity of platinum-based chemotherapy and decrease its side effects in order to improve its efficiency. Numerous natural extracts, such as glycyrrhizin, 18β-glycyrrhetinic acid and glabrin A and B, have demonstrated extensive biological activity and low toxicity in animal models of NSCLC and might therefore be considered as potential adjuvant drugs for the treatment of NSCLC (6).

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a natural anthraquinone derivative extracted from the roots of Chinese rhubarb and other plants, such as buckthorn and cassia tora (7,8). Emodin displays a variety of pharmacological and biological functions, including some anti-inflammatory, antibacterial and chemoprophylactic effects (9-11). In addition, previous studies have demonstrated that emodin exhibits some anticancer effects in breast, pancreatic and cervical cancers by inhibiting cancer cell proliferation and increasing cancer cell apoptosis and chemosensitization (12-14). Other studies have reported that emodin can reverse the chemoresistance in certain types of cancer, including leukemia, NSCLC and gallbladder cancer (15-17). Although certain studies have reported the effect of emodin on NSCLC chemosensitivity toward paclitaxel (17),

the effect of emodin on NSCLC chemosensitivity toward other chemical drugs and the underlying mechanisms remain unclear.

Multidrug resistance proteins are the most important factors that cause chemoresistance, which leads to a decrease in chemotherapy efficacy and survival rate of patients with cervical, liver, breast and lung cancers (18). Members of the ATP binding cassette (ABC) family are associated with multidrug resistance (MDR), and include P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1) and MRP2 (19-21). MDR often occurs during the treatment of NSCLC, which leads most patients to eventually relapse or to the disease to progress (22). Therefore, determining adjuvant drugs that could inhibit the expression of multidrug resistance protein may improve NSCLC sensitivity to chemotherapy.

The present study investigated the effect of emodin on the chemosensitivity of A549 and H460 cells and on the expression of Pgp and MRP1, which are key proteins involved in MDR.

Materials and methods

Cell culture. The NSCLC cell lines A549 and H460 were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biological Industries), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and placed at 37˚C in a humidified atmosphere with 5% CO_2. Trypsin (0.25%; Gibco; Thermo Fisher Scientific, Inc.) was used to passage cells once they reached 70-90% confluence.

Cell proliferation assay. Once cells reached 70-90% confluence, they were harvested and seeded into 96-well plates at the density of 2,500 cells/well and cultured for 12-24 h at 37˚C. Subsequently, cells were treated with emodin (0, 1, 2.5, 5, 10, 20, 50, 100, 200 and 300 μM) (17) and/or cisplatin (0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 μM) (23) for 48 h, the blank control (0 μM) was treated with equal amounts of vehicle (DMSO). Cell Counting Kit-8 (CCK-8; 10 μl) reagent (Nanjing KeyGen Biotech Co., Ltd.) was added to each well and incubated for 2-4 h at 37˚C. Absorbance was read at 450 nm using a Multilabel Plate Reader (Monobind, Inc.) (24).

Western blotting. Cells were lysed with RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1% (v/v) Nonidet P40 (NP40), 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor; pH 7.4) on ice and samples were centrifuged at 10,000 x g for 5 min at 4˚C. Protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific, Inc.). Proteins were mixed with 5X loading buffer (0.5 M Tris-HCl pH 6.8, 2% SDS, 0.05% bromphenol-blue, 20% 2-mercaptoethanol and 10% glycerol) and boiled for 5 min. Proteins were separated via SDS-PAGE (10% gel) as previously described and transferred onto PVDF membranes (25). After blocking for 2 h in TBST containing 5% non-fat milk, membranes were incubated with primary antibodies against Pgp (1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. P7965), MRP1 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 72202) and GAPDH (1:5,000; ProteinTech Group, Inc.; cat. no. 60004-1-Ig) for overnight at 4˚C. Membranes were then incubated with the secondary antibodies, HRP-conjugated anti-rabbit IgG (1:5,000; ProteinTech Group, Inc.; cat. no. 51832-2) or HRP-conjugated anti-mouse IgG (1:5,000; ProteinTech Group, Inc.; cat. no. 51866-5) for 2 h at 37˚C. The signal on the membrane was detected using enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.). Relative expression levels were normalized to endogenous control GAPDH using ImageJ software (version 1.32; National Institutes of Health).

Cell apoptosis assay. Following treatment with emodin and/or cisplatin for 48 h, cells were harvested and $4x10^5$ cells were double-stained with 5 μl Annexin V-FITC and PI solution for 10 min at room temperature (Absin Technologies, Inc.; cat. no. abs50001). Apoptotic cells were subsequently analyzed using a CytoFlex flow cytometer (Beckman Coulter, Inc.) the apoptotic rate was determined using CytExpert 2.3 software (Beckman Coulter, Inc.) (26).

Immunocytochemical analysis of γ-H2A.X Foci. Double-stranded DNA breaks (DSBs) induce serine phosphorylation of histone H2A.X, producing γ-H2A.X foci that are then recognized by DNA damage response pathway proteins. γ-H2A.X foci are hallmark of DSBs and are markedly enhanced in irradiated cells (27). Following cell treatment with emodin and/or cisplatin for 48 h, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 for 5 min at room temperature and blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. Subsequently, cells were incubated with antibodies against phospho-histone H2A.X (Ser139; 1:400; Cell Signaling Technology, Inc.; cat. no. 9718) overnight at 4˚C. Cells were then incubated with CL488-conjugated anti-rabbit IgG (1:200; ProteinTech Group, Inc.; cat. no. SA00013-2) antibody for 1 h at 37˚C and were washed three times with PBS. Cells were eventually stained with DAPI (1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. D9542) for 3 min at room temperature and washed with PBS three times. Cells were imaged using a fluorescence microscope (magnification, x20; Leica Microsystems GmbH).

Fluorescence microscopy to analyze intracellular rhodamine 123 accumulation. A549 and H460 cells were cultured for 12-24 h and treated with 0, 1, 2.5, 5, 10 and 20 μM emodin for 12 h at 37˚C. Cells were harvested, resuspended in fresh medium, and stained with 5 μM rhodamine 123 (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. Cells were washed three times with PBS, and drug accumulation levels were determined by fluorescence microscopy (Leica Microsystems GmbH) (28). The fluorescence intensity was determined using ImageJ software (version 1.32; National Institutes of Health).

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics 22 (SPSS, Inc.) and GraphPad Prism 5 (GraphPad Software, Inc.) software. All data are expressed as the mean ± standard of three independent experiments. Student's t-test was used to evaluate differences between two groups. Differences between multiple groups were analyzed using two-way ANOVA followed by Tukey's post hoc test.

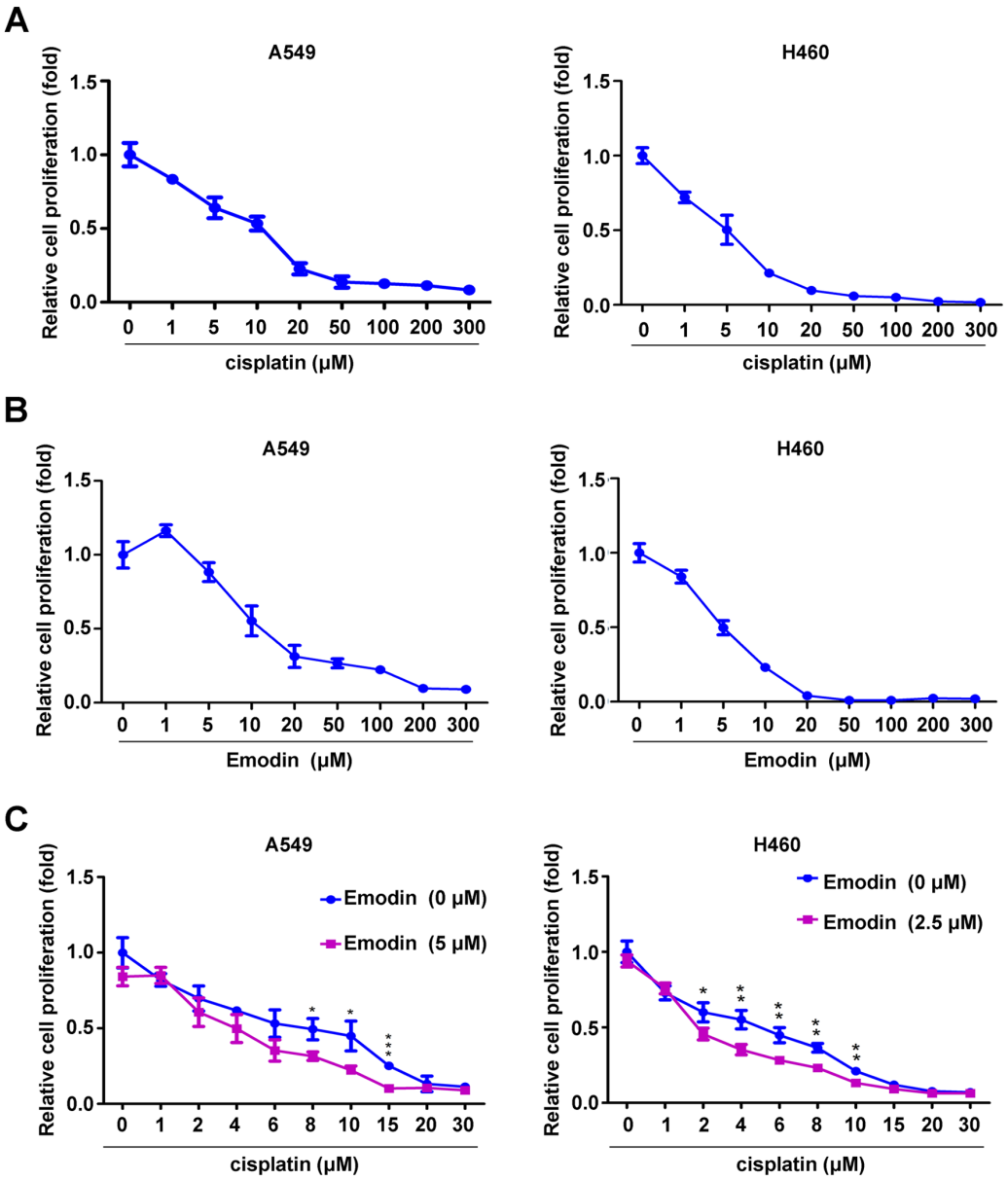

Figure 1. Effects of emodin and cisplatin on A549 and H460 cell proliferation. Cell Counting Kit-8 was used to detect A549 and H460 cell proliferation following treatment with different doses of (A) cisplatin, (B) emodin or (C) cisplatin combined with emodin for 48 h. The data are presented as the means ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001.

P<0.05 was considered to indicate a statistically significant difference.

Results

Emodin and cisplatin inhibit A549 and H460 cell proliferation. CCK-8 assay was used to evaluate the effect of emodin and/or cisplatin on the proliferation of A549 and H460 cells. The results indicated that cisplatin and emodin at concentrations ranging from 0 to 300 μM inhibited A549 and H460 cell proliferation in a dose-dependent manner. Notably, low dose emodin (1 μM) slightly enhanced the proliferation of A549 cells, but not of H460 cells (Fig. 1A and B). The IC50 of cisplatin and emodin for A549 cells (29) was 5.25 and 13.65 μM, respectively, whereas the IC_{50} of cisplatin and emodin for H460 cells was 4.83 and 5.17 μM, respectively. To investigate whether emodin could be used as a cosensitizer for cisplatin, a low dose of emodin (A549 cells, 5 μM; H460 cells, 2.5 μM) was selected to determine its effect on cisplatin sensitization. Compared with cisplatin treatment alone, treatment with 5 μM emodin significantly enhanced the anti-proliferative effect of 8, 10 and 15 μM cisplatin on A549 cells, whereas 2.5 μM emodin significantly enhanced the anti-proliferative effect of 2, 4, 6, 8 and 10 μM cisplatin on H460 cells (Fig. 1C). These results indicated that emodin and cisplatin may synergistically inhibit the proliferation of A549 and H460 cells.

Emodin inhibits drug efflux in A549 and H460 cells. In order to investigate the effect of emodin on the chemosensitivity of A549 and H460 cells toward cisplatin, a drug efflux experiment was performed. Briefly, A549 and H460 cells were treated with various concentrations of emodin and stained with rhodamine 123, and immunofluorescence was used to detect the intracellular accumulation of rhodamine 123.

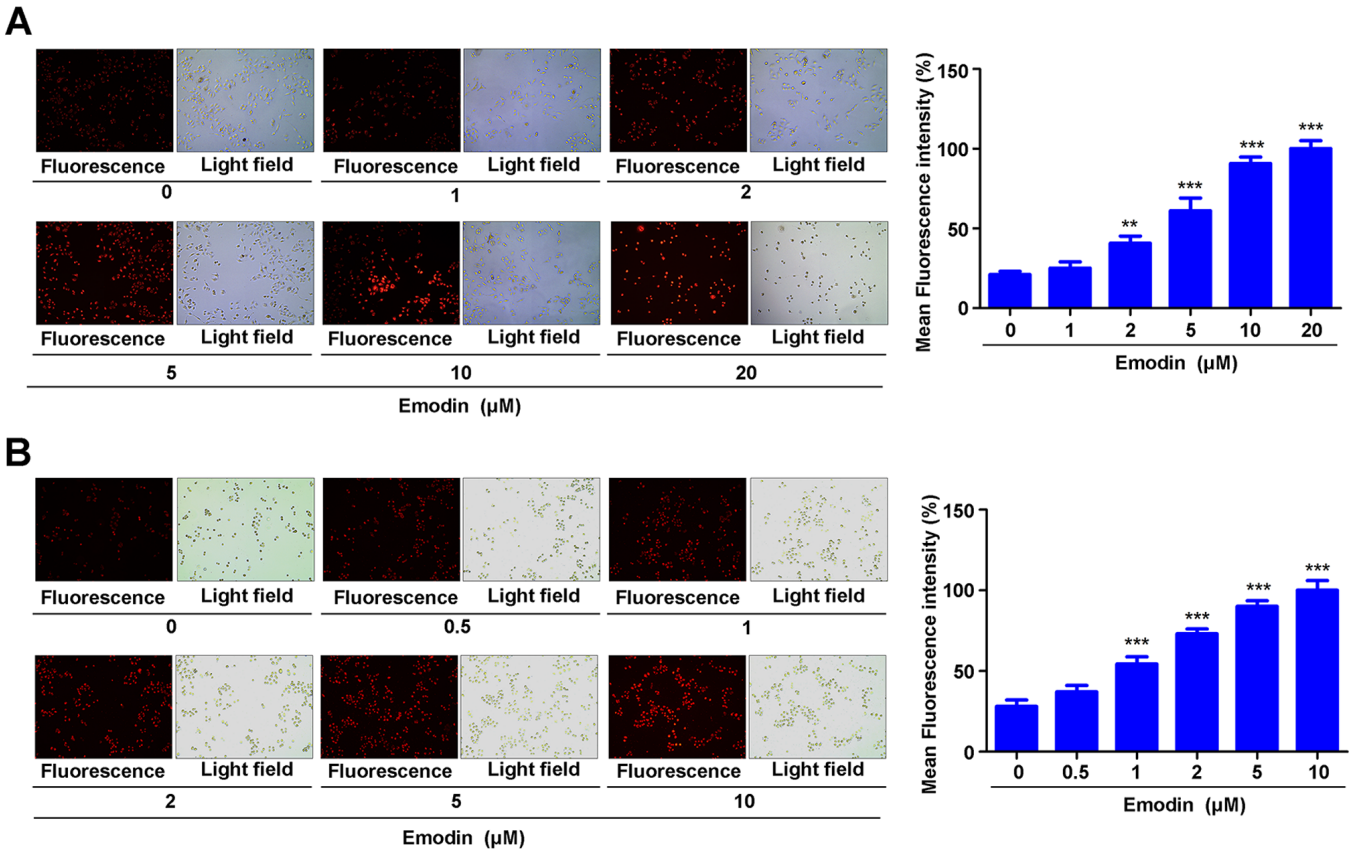

Figure 2. Effect of emodin on the drug efflux of A549 and H460 cells. Following A549 and H460 cell treatment with different concentrations of emodin for 12 h, cells were stained with 5 μM rhodamine 123 for 30 min and fluorescence microscopy was used to detect drug accumulation levels in (A) A549 and (B) H460 cells. **P<0.01 and ***P<0.001.

The results demonstrated that 2, 5, 10 and 20 μM emodin significantly enhanced the accumulation of rhodamine 123 in A549 cells (Fig. 2A). Emodin (1, 2, 5 and 10 μM) also significantly enhanced rhodamine 123 accumulation in H460 cells (Fig. 2B). These data indicated that emodin may inhibit the efflux of drugs from A549 and H460 cells.

Emodin enhances cisplatin-induced apoptosis in A549 and H460 cells. To investigate the effect of emodin on the chemosensitivity of A549 and H460 cells, apoptosis experiments were conducted. A549 and H460 cells were treated with emodin and/or cisplatin, and the apoptotic rate was detected by flow cytometry. The results demonstrated that 5 μM emodin did not induce A549 cell apoptosis, but significantly enhanced A549 cell apoptosis induced by 5 and 10 μM cisplatin (Fig. 3A). Similarly, 2.5 μM emodin did not induce H460 cell apoptosis, but significantly enhanced the apoptosis of H460 cells induced by 5 and 10 μM cisplatin (Fig. 3B). These results suggested that emodin may enhance cisplatin-induced apoptosis in A549 and H460 cells.

Emodin enhances cisplatin-induced DNA damage in A549 and H460 cells. Cisplatin mainly kills tumor cells by inducing DNA damage (30). The effect of emodin on the chemosensitivity of A549 and H460 cells toward DNA damage was therefore determined through immunocytochemical analysis of γ-H2A.X foci. The results demonstrated that 5 μM emodin did not induce γ-H2A.X foci formation in A549 cells but significantly enhanced γ-H2A.X foci formation in A549 cells induced by 5 and 10 μM cisplatin (Fig. 4A). Similarly, 2.5 μM emodin did not induce γ-H2A.X foci formation in H460 cells but significantly enhanced 5 and 10 μM cisplatin-induced γ-H2A.X foci formation in H460 cells (Fig. 4B). These data indicated that emodin may increase cisplatin-induced DNA damage in A549 and H460 cells.

Emodin decreases Pgp expression in A549 and H460 cells. In order to investigate the molecular mechanism by which emodin enhances cisplatin sensitivity in A549 and H460 cells, the effect of different concentrations of emodin on the expression of Pgp and MRP1 were analyzed. The results from western blotting demonstrated that 2, 5, 10 and 20 μM emodin significantly decreased the expression of Pgp in A549 cells but did not affect the expression of MRP1 (Fig. 5A). Emodin (0.5, 1, 2, 5 and 10 μM) significantly inhibited the expression of Pgp in H460 cells but did not affect the expression of MRP1 (Fig. 5B). These results suggested that emodin may inhibit MDR related protein-Pgp expression.

Discussion

Cisplatin is a common chemotherapy drug used in the treatment of various types of cancer; however, cisplatin also exhibits serious adverse effects, particularly nephrotoxicity and oxidative injury (23,31). Previous studies have reported that the therapeutic efficacy of emodin combined

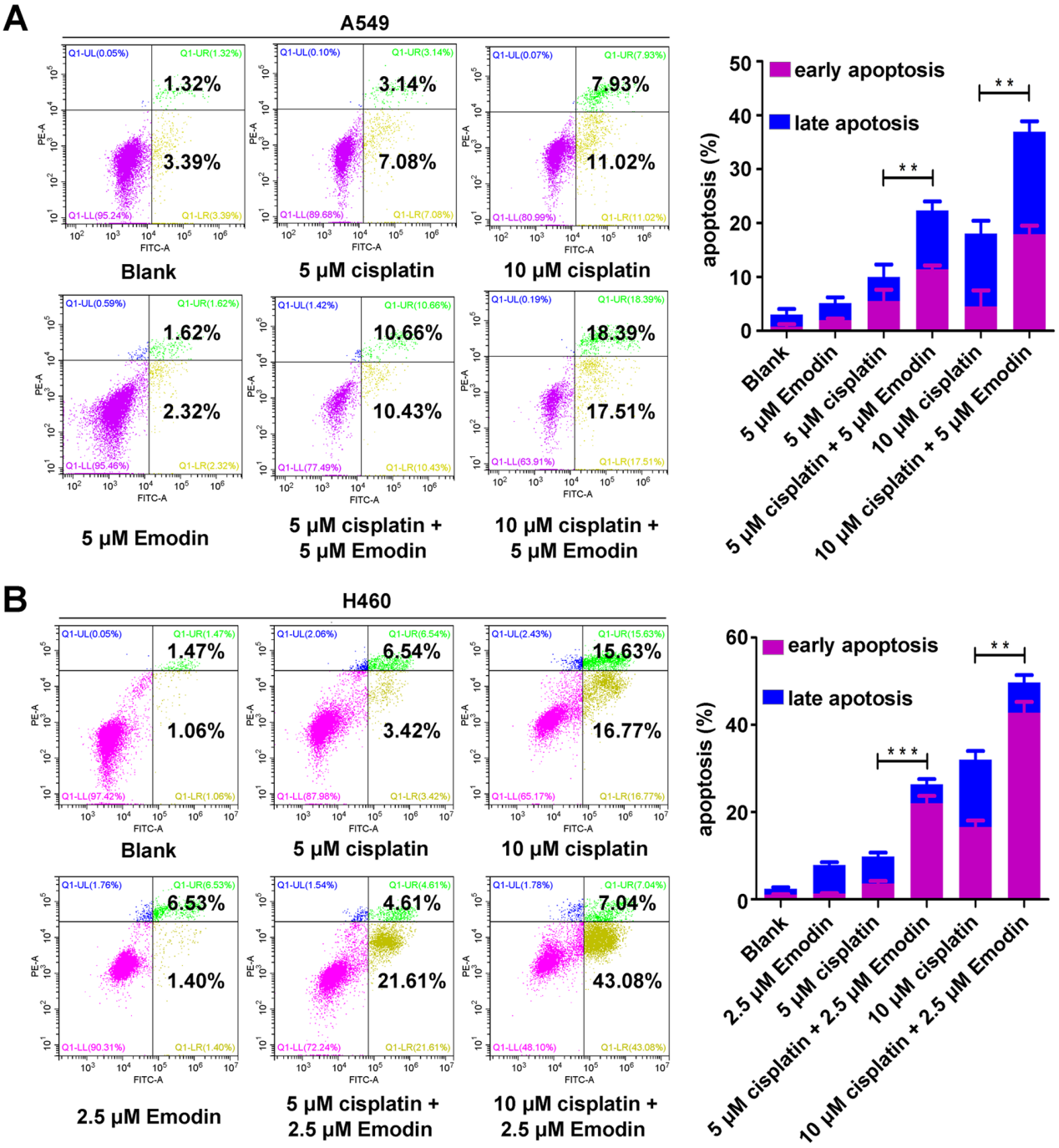

Figure 3. Effect of emodin on cisplatin-induced apoptosis in A549 and H460 cells. Following A549 and H460 cell treatment with different concentrations of emodin and/or cisplatin for 24 h, the blank control group was treated with equal amounts of vehicle (DMSO), and cells were stained with an Annexin V-FITC/PI apoptosis detection kit, and flow cytometry was used to detect the cell apoptosis rate in (A) A549 and (B) H460 cells. **P<0.01 and ***P<0.001.

with chemical drugs is higher than that of chemical drugs alone, and that combination treatment also results in fewer adverse effects (7,15,32). For example, emodin can enhance the therapeutic effect of gemcitabine on pancreatic cancer without other toxic effects (33). Other studies have demonstrated that emodin can increase the antitumor effect of gemcitabine even when gemcitabine is administered at a low dose (33,34). Compared with treatment with cisplatin, carboplatin or oxaliplatin alone, cotreatment of emodin with cisplatin, carboplatin or oxaliplatin effectively enhances the chemosensitivity of the gallbladder cancer cell line SGC996 via glutathione depletion and MRP1 downregulation both *in vitro* and *in vivo* (34,35). Therefore, the combination of emodin derived from traditional Chinese medicine and cisplatin may therefore represent a potential method to decrease the toxicity of cisplatin toward normal cells and increase its toxicity toward tumor cells.

In the present study, the effect of emodin and cisplatin on A549 and H460 cells behavior was evaluated. The results demonstrated that emodin significantly enhanced the antiproliferative, antidrug efflux, pro-apoptotic and DNA-damaging effects in combination with cisplatin *in vitro*. These data

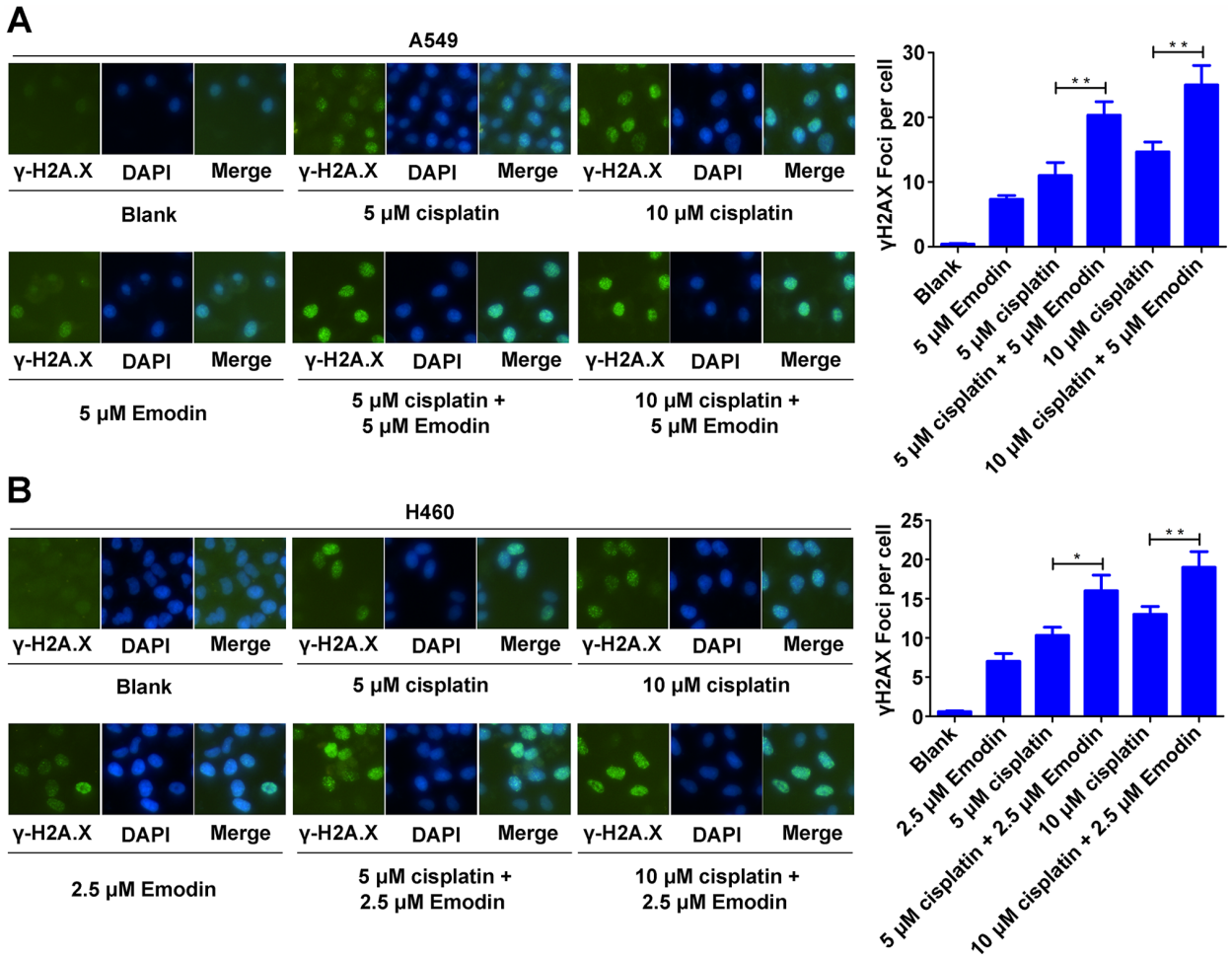

Figure 4. Effect of emodin on DNA damage in A549 and H460 cells. Following A549 and H460 cell treatment with different concentrations of emodin and/or cisplatin for 24 h, the blank control group was treated with equal amounts of vehicle (DMSO), immunocytochemical analysis was used to analyze γ-H2A.X foci formation in (A) A549 and (B) H460 cells. *P<0.05 and **P<0.01.

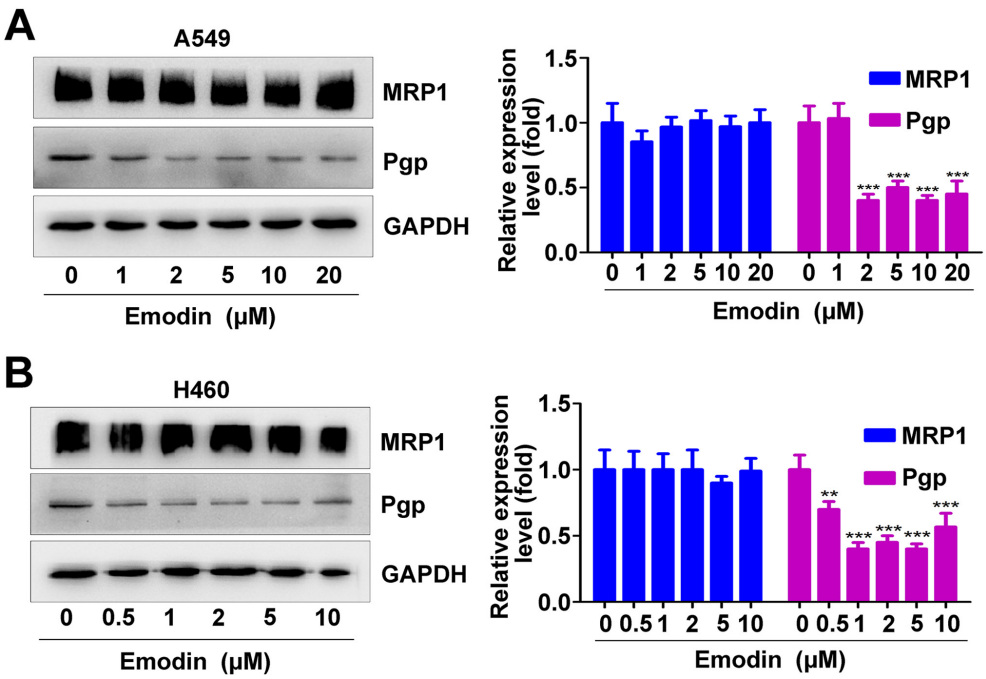

Figure 5. Effect of emodin on the expression of Pgp and MRP1 in A549 and H460 cells. Following A549 and H460 cell treatment with different concentrations of emodin for 24 h, western blotting was used to detect Pgp and MRP1 expression in (A) A549 and (B) H460 cells. **P<0.01 and ***P<0.001. Pgp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1.

suggested that emodin may enhance cisplatin-induced anti-tumor activity in A549 and H460 cells in a dose-dependent manner. Previous studies have reported that 5 μM emodin slightly promotes the proliferation of bladder cancer cells, although there was no significant difference (15,32). In addition, emodin significantly decreases the antitumor effect of tamoxifen in $HER2^+$ breast cancer cells (36). Emodin may likely show different levels of antitumor activity depending on the type of tumor and the antitumor activity of different concentrations of emodin could be different in the same tumor. In the present study, different concentrations of emodin had different effects on the proliferation of A549 cells. Emodin at 1 μM had a slight promoting effect on the proliferation of A549 cells, while emodin at >5 μM significantly inhibited the proliferation of A549 cells. Different concentrations of emodin had a certain inhibitory effect on H460 cell proliferation. Therefore, emodin exerted an anti-tumor effect in a concentration-dependent manner in NSCLC.

MDR is an important defense mechanism of tumor cells against chemical drugs (37). However, multiple factors are associated with MDR, including the efflux pump mechanism of drug-resistant proteins [Pgp, MRP and lipoprotein receptor-related protein-1 (LRP1)], the decrease in DNA topoisomerase activity, and the abnormal DNA repair (38). In particular, the drug protein pump mediated by Pgp, MRP and other drug resistance-related proteins, such as BCRP and LRP1, is the main mechanism by which tumors develop MDR (27). Previous studies have demonstrated that emodin and cisplatin alone or in combination can significantly decrease the expression of Pgp and MRP1 in bladder cancer cells (32,35,39). Furthermore, emodin and doxorubicin significantly decrease the expression of Pgp and MRP1 in colon cancer cells (39). In the present study, the effect of emodin on the expression of Pgp and MRP1 in A549 and H460 cells was investigated. The results demonstrated that emodin enhanced the sensitivity of NSCLC cells toward cisplatin by decreasing the expression of Pgp but not of MRP.

The present study did have some limitations. First, the effect of emodin on the mRNA expression of Pgp and MRP1 in A549 and H460 cells, and the effect of emodin on the expression of Pgp and MRP1 in combination with cisplatin, were not investigated. These topics need to be investigated in future. Secondly, the present study did not investigate the effect of emodin on the chemotherapy sensitivity of NSCLC cells in xenograft animal models. Although emodin/cisplatin administration has been found to have no significant effect on body weight and histological findings in treated mice (tissue structure, cell morphology and vascular distribution) (32), this does not imply that emodin is not toxic. Future work will perform pharmacodynamic, acute toxicity, long-term toxicity and irritability tests in order to verify the safety of emodin. To the best of our knowledge, there is currently no clinical study on the combination of cisplatin and emodin. Clinical trials and long-term follow-up are needed to fully assess the toxicity of combination therapy in the future.

In summary, the present study demonstrated that emodin could increase the sensitivity of A549 and H460 cells to cisplatin by downregulating Pgp expression. The results suggested that emodin may be considered as an effective sensitizer for cisplatin-based chemotherapy in patients with NSCLC.

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Availability of data and materials

Not applicable.

Authors' contributions

XZD and QK designed the present study. SP, JW and CL performed all the experiments, analyzed the data and prepared the figures. XD and ZX were responsible for the initial manuscript and interpretation of data. JJC was involved in drafting the manuscript, analysis and interpretation of data. All authors read and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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