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Elemene Induces Apoptosis of Human Gastric Cancer Cell Line BGC-823 via Extracellular Signal-Regulated Kinase (ERK) 1/2 Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AG Pihong Li
B Xiang Zhou
C Weijian Sun
D Weiwei Sheng
F Yangyang Tu
B Yaojun Yu
DE Jianda Dong
BC Bing Ye
BCF Zhiqiang Zheng
E Mingdong Lu

Department of General Surgery, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, P.R. China

Corresponding Authors: Mingdong Lu, e-mail: lu_mingdong126@163.com; Zhiqiang Zheng, e-mail: zhengzhiqiangwz@sina.com
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Background: Elemene is extracted from a traditional herbal medicine and is commonly used in the treatment of cancer in China. However, its effect on gastric cancer cells remains unknown. The goal of this study was to investigate its effect on human gastric cancer cells.

Material/Methods: Human gastric cancer BGC-823 cells and a tumor-bearing mouse model were employed to be divided into 4 groups: control group, elemene group, PD98059 group (an ERK 1/2 signaling pathway inhibitor), and the combined group (elemene plus PD98059). The tumor size, cell proliferation, expression of ERK 1/2 and phosphorylated ERK 1/2 (p-ERK 1/2), Bcl-2 mRNA, and Bax mRNA were measured. Moreover, cell apoptosis was detected and the apoptosis index was calculated.

Results: Elemene and PD98059 each significantly inhibited the proliferation of gastric cancer cells BGC-823, and their combination showed higher synergistic inhibitory effect ($P<0.05$). We also found increased expression levels of p-ERK l/2 protein and Bax mRNA, but reduced level of Bcl-2 mRNA expression ($P<0.05$). Elemene presented higher apoptosis rate in a dose-dependent manner ($P<0.05$). Furthermore, the injection of elemene decreased the weight of transplanted tumors.

Conclusions: Elemene can inhibit the proliferation and induce the apoptosis of gastric cancer cells associated with the ERK 1/2 signaling pathway and expression levels of Bax mRNA and Bcl-2 mRNA.

MeSH Keywords: Apoptosis • Cell Proliferation • Stomach Neoplasms

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Background

Gastric cancer is one of the most common human malignancies and the second leading cause of cancer-related death worldwide [1]. Unfortunately, most gastric cancer patients are diagnosed in the advanced stage, only 30% of which can tolerate surgery; therefore, adjuvant chemotherapy is a major treatment option for advanced gastric cancer [2] However, the treatment outcome of chemotherapy regimens for gastric cancer, such as cisplatin and fluorouracil, has not been satisfactory over the past few decades. Due to the extensive adverse effects and multi-drug resistance in some cases [3], the development of effective and safe anti-cancer drugs is vitally important.

Elemene, a sesquiterpenoid mixture with β-elemene as the main active component, was extracted from Rhizoma Curcumae [4], which is a commonly used traditional Chinese medicine containing essential oils widely prescribed for the treatment of cancer in China. Fu et al. [5] reported the anti-cancer activity of elemene in 1984. Its pharmacological study began primarily in the latter part of the 2000s. Research over the last few decades has shown that elemene can retard cell cycle arrest, induce cell apoptosis, and inhibit metastasis or invasion of tumor cells [6]. In addition, due to its low toxicity to normal cells, elemene has increasingly attracted the interest of researchers [7]. Accumulating evidence shows that β-elemene has potential anti-cancer activity against many types of cancer cells, including ovarian carcinoma, breast cancer, lung cancer, pancreatic carcinoma, hepatocarcinoma, and leukemia [8–14].

A previous study reported that elemene injection combined with chemotherapy has better clinical efficacy and safety than either treatment alone in the treatment of advanced gastric cancer [15]. However, little is known about the exact anti-cancer mechanism of elemene against gastric cancer cells. The present study aimed to explore the therapeutic effect of elemene against human gastric cancer cell line BGC-823 *in vitro* and *in vivo* and to determine its molecular mechanism.

Material and Methods

Cell Lines and mice model

BGC-823 cell line was purchased from the Cell Resource Center of the Shanghai Biological Sciences Institute (Chinese Academy of Sciences, Shanghai, China). A total of 20 male BALB/c athymic nude mice (5 weeks old) were purchased from Shanghai Slac Laboratory Animal Co. LTD (Shanghai, China). All animals received humane care in keeping with the Guide for the Care and Use of Laboratory Animals [Permit Number: SYXK (zhe) 2010-0150] and the protocol was approved for animal experimentation by the Animal Ethics Committee of Wenzhou Medical College.

Reagents

Elemene was purchased from Dalian Holley Golden Port Industry Co. LTD (Dalian, China). RMPI l640 was purchased from Gibco (Carlsbad, CA, USA). ERK 1/2 signaling pathway inhibitor PD98059 and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA) and 0.9% saline was obtained from the Manufacturing Laboratory, the Second Affiliated Hospital of Wenzhou Medical University. The PrimeScript RT reagent kit was purchased from Takara (Japan). SYBR Green was purchased from Roche (USA). Rabbit anti-human REK 1/2, p-REK 1/2, and HRP-labeled goat anti-Rabbit antibody were purchased from Cell Signaling Technology (CST, USA).

Cell culture

BGC-823 cell line cells were cultured in RMPI-1640 medium (Beyotime), into which 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 μg/ml streptomycin (Beyotime), and 100 U/ml penicillin (Beyotime) were supplemented. A humidified atmosphere containing 5% CO_2 at 37°C was applied for the incubation of the cultures.

MTT cell viability assay

Cells were plated in 96-well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) with 4000 cells per well. After being cultured in a humidified incubator for 24 h, cells were randomized into 4 groups: 0.9% saline (group A, control), elemene (group B, 200mg/kg), ERK 1/2 inhibitor PD98059 (group C, 1 mg/kg), and elemene plus PD98059 (group D), respectively, administered peritoneally.

Moreover, cells were separately exposed to different concentrations of elemene (0.02, 0.04, 0.08, and 0.16 mg/ml) in group B, PD98059 (25, 50, 75, and 100 μmol/L) in group C, and 0.08 mg/ml elemene combined with 50 μmol/L PD98059 in group D, at 6, 12, 24, and 48 h. Each well was incubated for 4 h with addition of 20 μl of MTT solution (5 mg/ml). After incubation, the supernatant was discarded and 150 μl DMSO was added and mixed. The absorbance was determined at a wavelength of 492 nm and expressed as A value, and the proliferation rate of cell was calculated. All assays were performed with 5 replicates.

Cell apoptosis assay

The apoptosis was detected using the TUNEL method. The cells (1×10^4/ml) in control and experimental groups were plated in 6-well plates, cover-slipped, and incubated for 24 h. They were

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subsequently exposed to various testing agents as described above. The whole procedure was based on the instructions of the TUNEL detection kit. The main steps were: firstly, coverslips with cells were taken out, fixed with 4% paraformaldehyde for 1 h at room temperature, and washed with PBS 3 times ×2 min. Then, 0.3% H_2O_2 was added for 30 min and washed with PBS 3 times ×5 min. We then added 1% Triton and the mix was incubated for 2 min at 4 °C. PBS was used for washing 3 times ×5 min, followed by addition of 50 µl TUNEL. After incubating in a wet box at 37°C for 1 h, cells were washed with PBS, then we added peroxide enzyme conversion agent and incubated the mix at 37°C for 30 min. Thereafter, cells were colored using DAB developer, and passed a series of orderly process including counterstaining, dehydration, transparency, and mounting. Electron microscopy was used to observe the morphological changes of the apoptotic cells. Cells that stained brown were consonant with changes of apoptotic cells in morphology. The apoptosis index was calculated by the ratio of positive cells divided by the total cells ×100%.

Western blot analysis

After treating with different testing agents for 24 h, cells were placed in 100 ml of ice-cold RIPA lysis buffer for incubation at 4°C for 1 h. Further clarification was performed at 12 000 rpm for 20 min at 4°C. Subsequently, according to the manufacturer's instructions, a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was used to quantify the protein content. The separation of 30 µg protein was performed on a 10% SDS-PAGE gel followed by transfer to polyvinylidene difluoride (PVDF) membranes. Then, 0.05 g/ml nonfat dry milk was used to block the blots, and ERK 1/2 (1: 1 000), p-ERK l/2 (1: 1000) was incubated overnight at 4°C, and then incubated with HRP-conjugated secondary antibody IgG (1: 6000) for 2 h at room temperature. Expression of ERK 1/2 and p-ERK l/2 proteins was assessed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Real-time PCR

Cells in control and experimental groups were plated in 6-well plates with 1×10^4 cells per well. After growing with adherence, the cells were treated with the aforementioned testing agents for 24 h. Thereafter, the Trizol method was performed to extract RNA. Then, reverse transcription was carried out strictly following the instructions of Takara [16] and cDNA was obtained. The real-time PCR conditions were: 95°C for 15 min initial denaturation, followed by 45 cycles of amplification at 95°C, denaturation for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, followed by 95°C for 1 min, 55°C for 1 min, and 95°C for 1 min. The expression levels of Bcl-2 and Bax mRNA were determined.

Tumor-bearing mouse model

In this study, 20 male nude mice were randomly divided into 4 groups: control group (group A, n=5), elemene group (group B, n=5), PD98059 group (group C, n=5), and PD98059 combined with elemene group (group D, n=5). All mice were inoculated with human gastric cancer BGC-823 cells to establish the tumor-bearing mouse model.

Therapeutic efficacy in established tumors *in vivo*

Two weeks later, agent treatment was carried out when the injected cell mass reached a mean volume of 200 mm^3 and the tumors could be palpated. The tumor-bearing mice was injected peritoneally with 0.9% saline only in the untreated control group, elemene (200 mg/kg/day, every 3 days) in group B, PD98059 (1 mg/kg/day, every 3 days) in group C, and elemene and PD98059 (200 mg/kg/day and 1 mg/kg/day, every 3 days) in group D. Subsequently, the weight of the mice and the size of the transplanted tumors were measured every 3 days, and the volume of tumors was calculated. After 15 days of treatment, the were killed and tumors were removed. The final tumor dimensions were measured and the tumor inhibitory rate (IR) was calculated using the following formula: IR (%)=[1–tumor weight in experimental group (g)/tumor weight in control group (g)]×100%.

Statistical analysis

All statistical analyses were performed using the SPSS 20.0 package and all quantitative assays were performed in triplicate. Data are expressed as means and standard deviations (mean ±SD). Comparisons among groups were tested using analysis of variance (ANOVA), least significant differences (LSD) post hoc tests, or Dunnett's T3 test. P values less than 0.05 were considered statistically significant.

Results

Elemene inhibited proliferation of gastric cancer BGC-823 cells *in vitro*

The curves in Figure 1A demonstrate the inhibited effects of elemene on the proliferation of BGC-823 cells, as time went by and the dose increased, indicating that elemene has concentration- and time-dependent inhibitory activities against gastric cancer. As shown in Figure 1B, the inhibition effects did not change when the concentration of PD98059 was more than 50 µmol/L, suggesting that PD98059 inhibited growth of BGC-823 cells in a time-dependent manner, but not in a dose-dependent manner. As we expected, Figure 1C showed that the inhibition effects of elemene combined with PD98059 group

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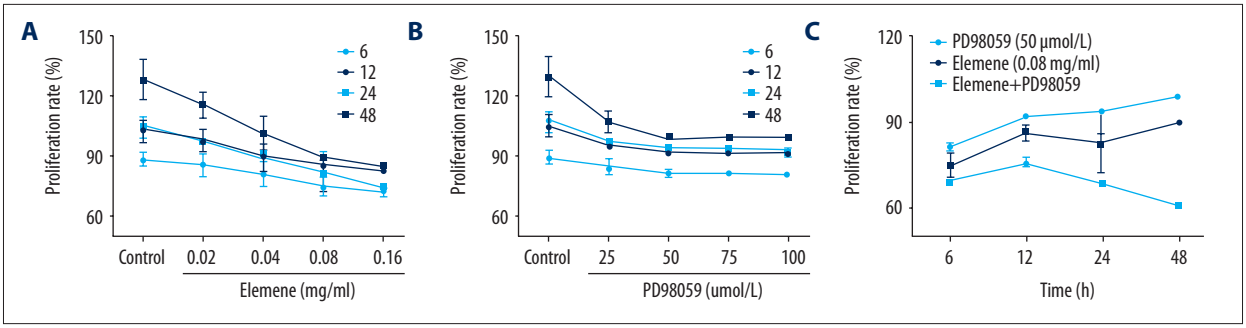

Figure 1. MTT cell viability assay of gastric cancer BGC-823 cells treated with elemene, PD98059, or the combination of the 2 drugs.

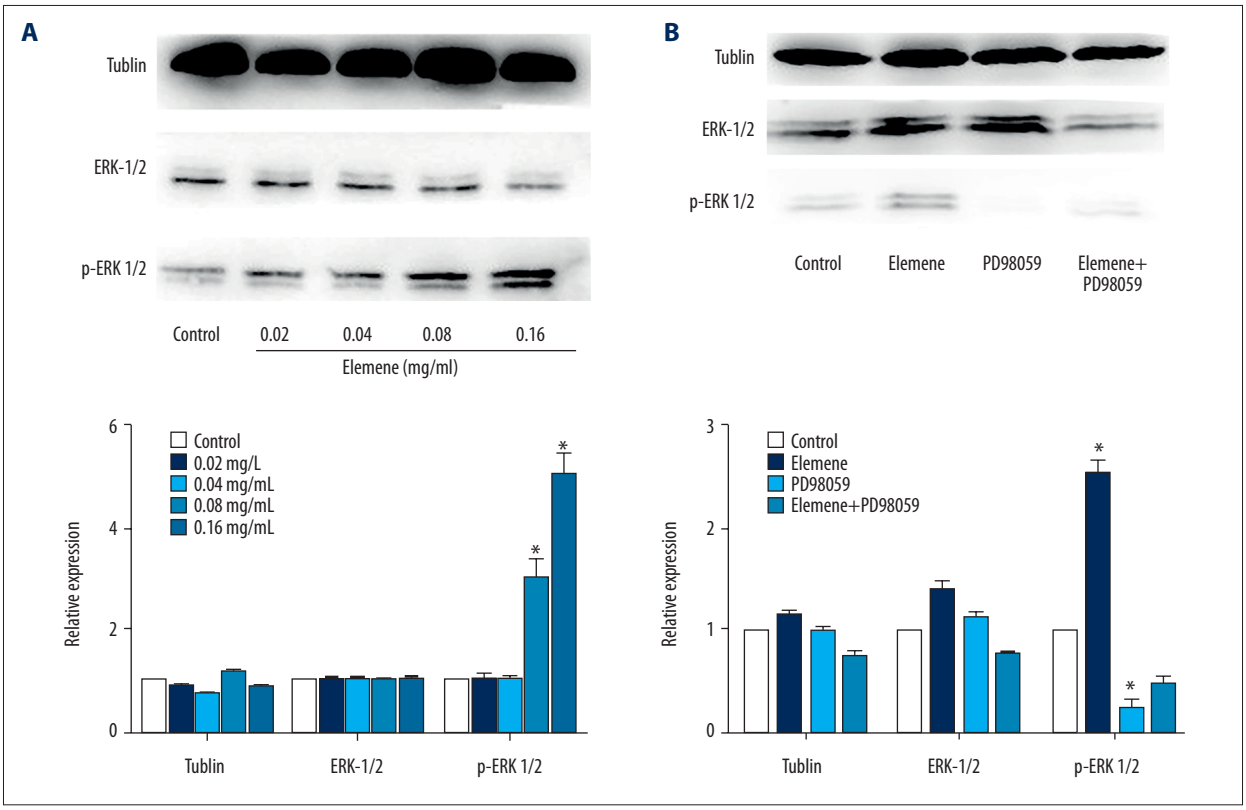

Figure 2. Western blot analysis of Tubulin, ERK 1/2, and p-ERK 1/2 protein levels in gastric cancer BGC-823 cells treated with elemene, PD98059, or the combination of the 2 drugs.

were obviously superior to that of the single action, suggesting elemene and PD98059 had the synergistic antitumor effect against gastric cancer *in vitro*.

Expression of ERK 1/2 and p-ERK 1/2 in different groups

Figure 2A shows that elemene upregulated the expression of p-ERK 1/2 in a dose-dependent manner. In addition, p-ERK 1/2 protein was quickly activated when the concentration of elemene was more than 0.08 mg/ml. We compared the expression of ERK 1/2 and p-ERK 1/2 among different experimental groups and the control group. The results demonstrate that p-ERK 1/2 was significantly increased in the elemene group with the concentration of 0.08 mg/ml, and was significantly decreased in the PD98059 group with the concentration of 50 μmol/L, when compared with the control group, but no significant difference was observed in the elemene plus PD98059 group (Figure 2B).

Assessment of apoptosis in BGC-823 cells

At the end of the 24-h incubation period, we observed the morphological changes of the apoptotic cells by electron microscopy and assessed the apoptotic rate. Figure 3 shows that all agent groups were effective in significantly inducing the apoptosis of BGC-823 cells. Compared with the control group, the apoptotic

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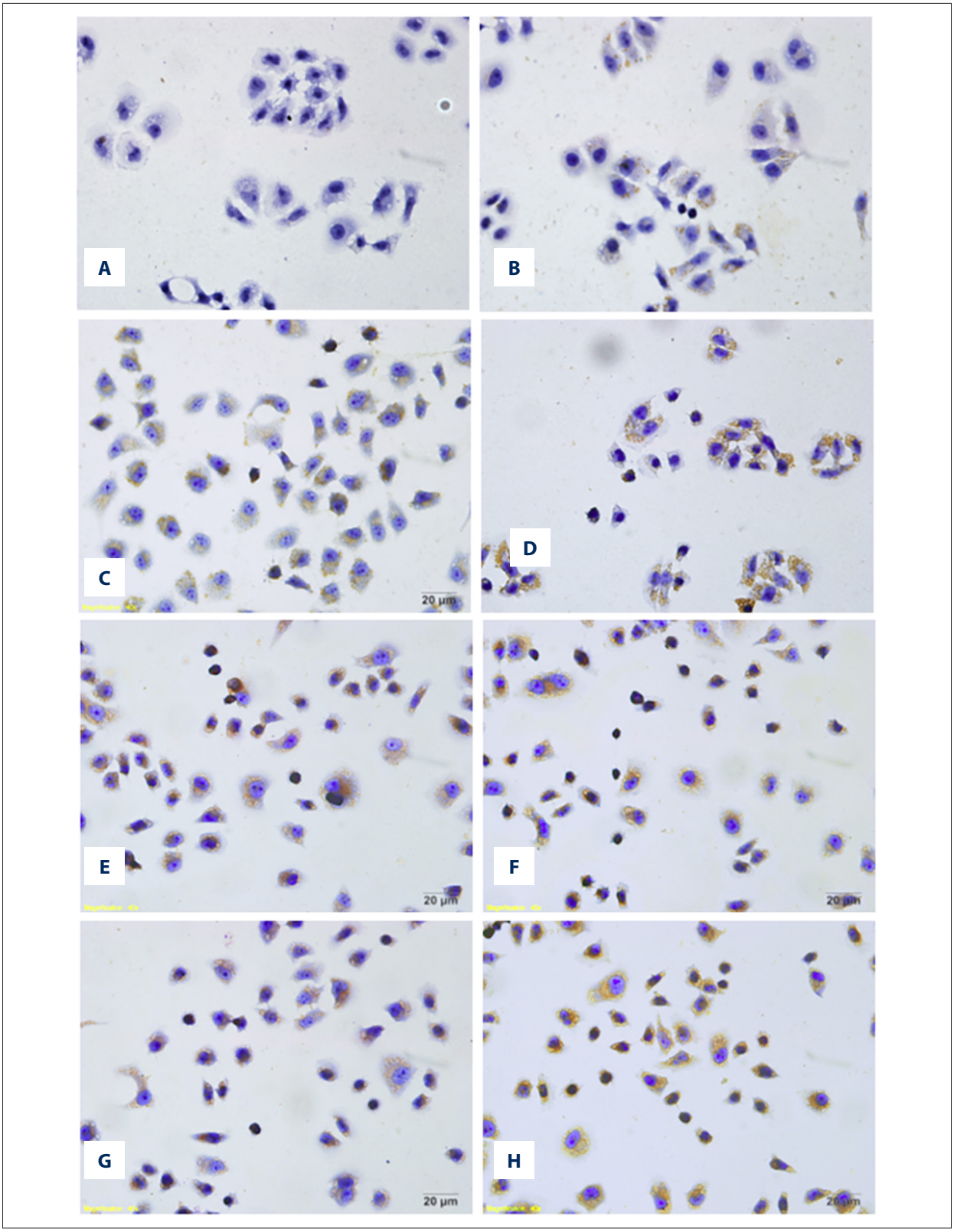

Figure 3. TUNEL staining of tumor tissues in different groups. (**A**) Negative controls; (**B**) Controls; (**C**) Elemene 0.02 mg/mL; (**D**) Elemene 0.04 mg/mL; (**E**) Elemene 0.08 mg/mL; (**F**) Elemene 0.16 mg/mL; (**G**) PD98059 50 μmol/L; (**H**) Elemene 0.08 mg/mL plus PD98059 50 μmol/L.

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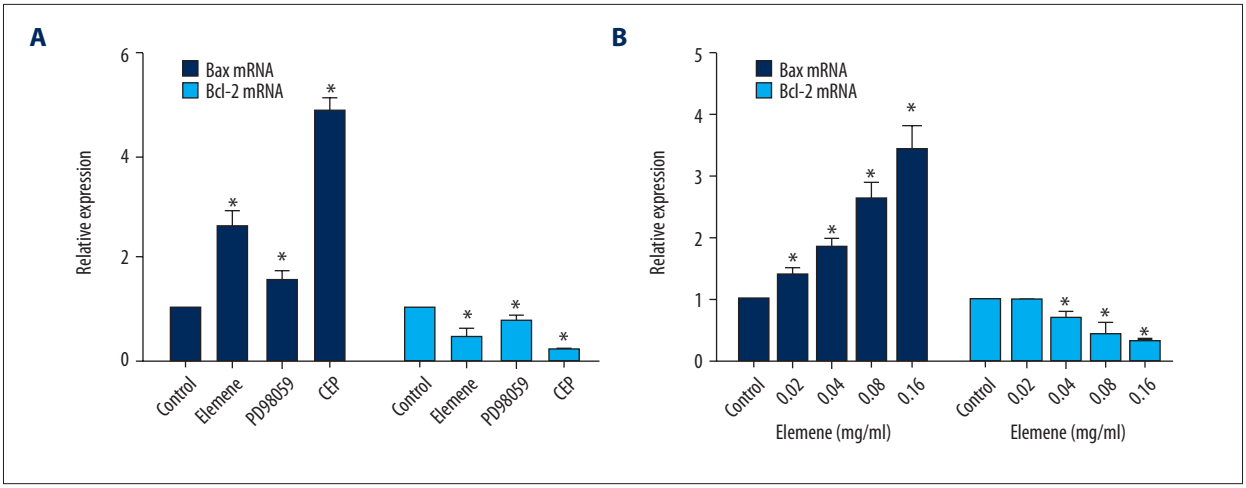

Figure 4. Real-time PCR analysis of Bax and Bcl-2 mRNA levels in gastric cancer BGC-823 cells treated with elemene, PD98059, or the combination of the 2 drugs. CEP – Elemene plus PD98059.

index significantly increased across the increasing concentrations of elemene (Figure 3C–3F). We also observed a markedly increased apoptosis rate of BGC-823 cells in the PD98059 group and in the combination of elemene and PD98059 group, compared with the control group (Figure 3G, 3H).

Effect of elemene on mRNA expression of Bcl-2 and Bax in BGC-823 cells

Real-time PCR analysis was used to assess the expression levels of Bcl-2 and Bax mRNA in BGC-823 cells treated with different drugs. As shown in Figure 4, with the increase of elemene, the Bax mRNA expression significantly increased and the Bcl-2 mRNA expression significantly decreased. Furthermore, elemene together with PD98059 caused the synergistic effect with the highest expression of Bax mRNA and the lowest expression of Bcl-2 mRNA.

Inhibitory effects of elemene on gastric cancer BGC-823 cells *in vivo*

We further explored the inhibitory effects of elemene on tumor formation *in vivo*. Body weight and tumor volume and weight were measured. As shown in Figure 5A, the body weights of the elemene-treated and control mice were significantly increased 2 weeks after treatment, but no significant increase was observed in mice treated with PD98059 and the combination of elemene and PD98059, indicating that elemene efficiently retards tumor growth in nude mice without apparent adverse effects on the hosts, while PD98059 showed toxicity to the growth of hosts. Table 1 lists the tumor weights of each treatment in nude mice, showing the strongest inhibition effect with the combination. As shown in Figure 5B, compared with the negative control group, tumor volume had a decreasing trend over time in the elemene group. Interestingly, the combination of elemene and PD98059 exhibited more potent growth inhibition of tumors compared to the other 3 groups. Although PD98059 slowed tumor growth, tumor volume in this group was not different from the result obtained in the control group.

Discussion

Gastric cancer is the second leading cause of cancer death worldwide, and the incidence and mortality of gastric cancer is second only to that of lung cancer in China [1,17]. Most patients are managed with chemotherapy because they are initially diagnosed in the advanced stage and cannot tolerate surgery [18]. Despite the efficacy of chemotherapy, these treatments cause significant adverse effects and enormous economic and social costs, as well as reducing patient quality of life and decreasing the overall efficacy of chemotherapy for gastric cancer.

Elemene, as an anti-cancer adjuvant drug, was shown in recent studies to exhibit broad-spectrum anti-cancer activity against cancer cell lines of different tumor types [8–14,19–21]. In addition, increasing evidence shows that the addition of elemene injection in chemotherapy not only improved the efficacy but also reduced the adverse effects of these drugs in the treatment of gastric carcinoma. These previous studies demonstrated that elemene had potential clinical benefits both alone and in combination with other drugs. Our current data further demonstrate that elemene not only alone, but also together with PD98059, can be effective in inhibiting the proliferation and inducing apoptosis of BGC-823 gastric cancer cells.

In the current study, the inhibitory effects of elemene on human gastric cancer BGC-823 cells were assessed both *in vitro*

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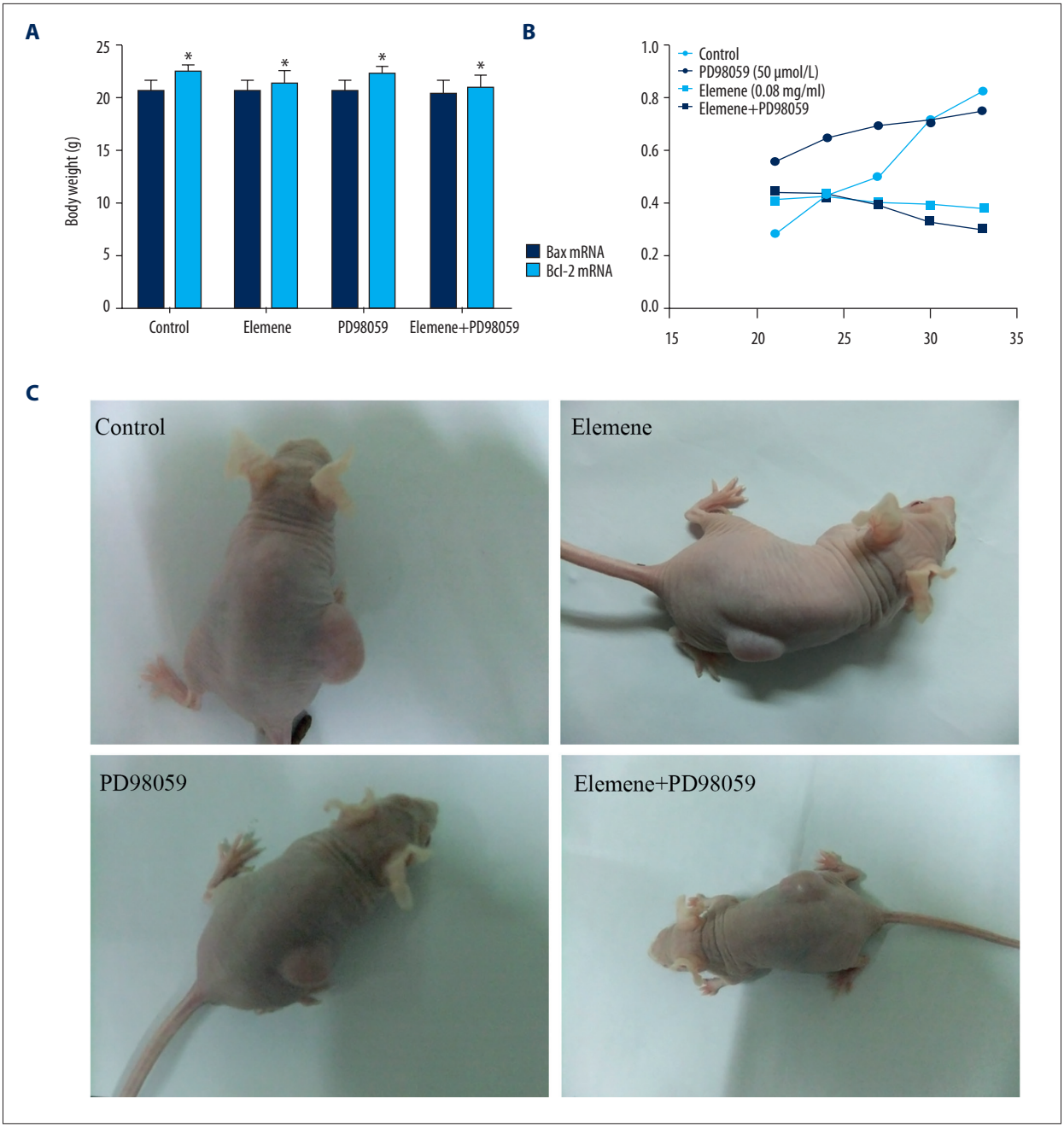

Figure 5. Effects of elemene on gastric tumors in mice. (**A**) Body weight of mice treated with different treatments are measured; (**B**) Tumor volume of mice treated with different treatments are measured; (**C**) Representative images from each treatment group are shown.

and *in vivo*. The *in vitro* findings demonstrated that elemene suppresses the proliferation of human gastric cancer cells by promoting cell apoptosis with concentration- and time-dependent inhibitory activities, but only time-dependent inhibitory activity was observed in the PD98059 group. In an *in vivo* study, the antitumor activity of elemene was similar to that of an *in vitro* study; the elemene group showed marked growth-inhibitory action against the growth of gastric cancer BGC-823 cells compared with the control group. Moreover, elemene in combination with PD98059 caused a more potent growth inhibition of BGC-823 cells than each agent alone *in vitro*. Importantly, the body weight of elemene-treated mice in all groups was significantly increased after treatment, without any significant difference; however, mouse growth was inhibited in the PD98059 and PD98059 plus elemene groups, suggesting that elemene effectively inhibits tumor growth in

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Table 1. Effects of different treatments on tumor weights in mice.

	Tumor weight (g)	Tumor inhibitory rate (%)
Control	0.7890±0.3150	
PD98059	0.4880±0.1314	38.15%
Elemene	0.3414±0.2187	56.72%
Elemene+PD98059	0.1898±0.0885	75.59%

nude mice, without apparent adverse effects of PD98059 inhibiting mouse growth. Therefore, elemene has the potential to be a novel and safe therapeutic method against gastric cancer.

MAPK-Activated Protein Kinases (MAPK) pathways play an important role in regulating diverse cellular activities, including motility, survival, apoptosis, and differentiation [22]. To date, the ERK 1/2 signaling pathway, as an MAPK pathway, has been proved to be a key regulator of cell proliferation, and inhibitors of the ERK pathway have been used in clinical trials as potential anti-cancer agents [23]. The ERK 1/2 signaling pathway was reported to be involved in the growth, proliferation, apoptosis, invasion, metastasis, and differentiation of gastric cancer cells [24–26]. Lin et al. [24] found that Euphol from Euphorbia tirucalli could selectively inhibit the growth of human gastric cancer cells through the induction of ERK1/2-mediated apoptosis. Another study found that TGF-beta promotes the invasion and metastasis of gastric cancer cells via ERK and JNK signal pathways [25]. Previous studies have reported that the inhibition effect of β-elemene on cancer cell proliferation is mainly due to apoptotic cell death and cell cycle arrest [7,27,28]. Another study reported that β-elemene rapidly induces the phosphorylation of Akt and ERK involved in β-elemene-induced cell apoptosis [29]. Bcl-2 families play a vital role in the process of apoptosis. Of the Bcl-2 families, Bcl-2 and Bax are the 2 most representative antiapoptotic and proapoptotic proteins, and the ratio of Bcl-2 and Bax is used to determine the apoptosis or survival of the cell after receiving stimulus signals [30]. The mitochondria-mediated apoptotic pathway is reported to be regulated by members of the Bcl-2 family [31], and the overall consequence is dictated by the balance of the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax. Remarkably, Zhang et al. showed that β-elemene combined with etoposide could enhance anti-cancer activity, which may be mediated by the upregulation of Bax protein [11]. In addition, another study found that β-Elemene exposure decreased the levels of Bcl-2 protein [27]. In the present study, we observed an obvious activation of the p-ERK 1/2 signaling pathway, upregulation of Bax mRNA expression, and downregulation of Bcl-2 mRNA. Collectively, our results demonstrate the possible molecular mechanism of elemene in inducing the apoptosis of gastric cancer cells, which is associated with activation of the p-ERK 1/2 signaling pathway through influencing the expression of Bcl-2 and Bax.

Conclusions

The present study suggests that elemene may be an effective and safe therapeutic strategy to inhibit the growth of gastric cancer BGC-823 cells by inducing apoptosis. The mechanism may be associated with the activation of the p-ERK 1/2 signaling pathway through upregulation of Bax mRNA expression and downregulation of Bcl-2 mRNA expression. In addition, elemene may function synergistically with the ERK 1/2 signaling pathway inhibitor PD98059 in the induction of gastric cancer cells apoptosis *in vitro* and *in vivo*. Further studies are warranted to elucidate the detailed mechanisms by which elemene contributes to BGC-823 cell apoptosis.

Competing interests

The authors declare that they have no competing interests.

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