

# Evodiamine inactivates NF- $\kappa$ B and potentiates the antitumor effects of gemcitabine on tongue cancer both in vitro and in vivo

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**Objective:** The aim of this study was to investigate whether evodiamine (EVO) could potentiate the antitumor activity of gemcitabine (GEM) in tongue cancer cells and determine its potential underlying mechanisms.

**Materials and methods:** Human Tca8113 and CAL-27 tongue squamous carcinoma cell lines were treated with EVO and GEM in different sequences and doses, after which cell proliferation was measured. Drug interactions were analyzed using the Chou–Talalay method with CompuSyn software. Clonality, apoptosis, and migration were measured using the plate clone formation assay, annexin V/propidium iodide (PI) staining, Hoechst 33342 staining, and the wound-healing test. The activity of the nuclear factor kappa light-chain enhancer of activated B cell (NF- $\kappa$ B) p65 subunit and its downstream proteins was quantified by Western blotting. The effects of the drug combination in vivo were assessed using a CAL-27 heterotopic xenograft model.

**Results:** EVO and GEM had synergistic effects on CAL-27 and Tca8113 cell lines in time- and concentration-dependent manners. Combination of drugs inhibited cell proliferation and migration and reduced the expression of NF- $\kappa$ B p65, B cell lymphoma 2 (Bcl-2), and B cell lymphoma extra large (Bcl-xl) compared with the control and either drug alone. In vivo, combination treatment of the xenograft model with EVO and GEM led to a significant reduction in tumor volume growth and inhibited the activation of NF- $\kappa$ B p65 with no obvious adverse reactions.

**Conclusion:** The results of this study showed that EVO may inhibit cancer cells by suppressing NF- $\kappa$ B activity, and in combination with GEM, it may increase the chemosensitivity of tongue squamous cancer cells, thereby improving the treatment response.

**Keywords:** tongue cancer, evodiamine, gemcitabine, combination index, apoptosis, NF- $\kappa$ B

## Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide,<sup>1</sup> and tongue cancer is estimated to account for more than 50% of oral cancer cases diagnosed each year globally.<sup>2</sup> Research has shown that tongue squamous cell carcinoma is one of the most aggressive cancers of the oral cavity, leading to delays in early diagnosis and a positive prognosis of only 55%–65%.<sup>3</sup> Chemoresistance and systemic adverse reactions from chemotherapeutic agents are the most intractable problems in tumor treatment, so better drugs are needed to target antitumor signaling molecules.

Evodiamine (EVO), a Chinese herb of the *evodia rutaecarpa* family, may inhibit cell proliferation and induce apoptosis in cancer cell lines by downregulating B cell lymphoma 2 (Bcl-2), B cell lymphoma extra large (Bcl-xl), caspase-3, caspase-9, and vascular endothelial growth factor (VEGF), while upregulating Bcl-2-associated X protein (Bax).<sup>4–9</sup> Our recent studies have indicated that EVO inhibits cell growth and

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promotes apoptosis in tongue cancer cells both in vitro and in vivo.<sup>10,11</sup> Takada et al's<sup>12</sup> study showed that EVO abolished the constitutive and inducible activation of nuclear factor kappa light-chain enhancer of activated B cells (NF- $\kappa$ B) by inhibiting I $\kappa$ B $\alpha$  kinase activation, thereby suppressing NF- $\kappa$ B-mediated regulation of antiapoptotic and metastatic gene expression, upregulating apoptosis, and inhibiting invasion. Thus, EVO may be a natural NF- $\kappa$ B inhibitor. Data have shown that many chemotherapeutics and radiation can activate NF- $\kappa$ B, so it may be involved in radiation and chemotherapy resistance.<sup>13</sup> Studies have suggested that NF- $\kappa$ B inhibitors and natural compounds with NF- $\kappa$ B inhibitory activity can induce tumor cell apoptosis.<sup>14,15</sup> When EVO is combined with other chemotherapy drugs, it inhibits the expression of multidrug resistance (*MDR*) gene and P-glycoprotein (P-gp), reversing *MDR* and enhancing inhibition of chemotherapy drugs on tumor cells.<sup>16,17</sup> Inhibitor of apoptosis (IAP) proteins and NF- $\kappa$ B contribute to the prevention of apoptosis, and there is a close interaction between their signaling pathways. It has been reported that EVO can simultaneously target both pathways<sup>18,19</sup> and may augment chemotherapy by directly or indirectly inhibiting the PI3K/Akt pathway and targeting NF- $\kappa$ B, thereby sensitizing pancreatic cancer cells to treatment.<sup>20</sup> However, the use of EVO for the treatment of tongue cancer has not been explored. Thus, we determined whether EVO could increase the anti-tumor effects of chemotherapeutics to treat tongue cancer via inhibition of NF- $\kappa$ B. To this end, we used gemcitabine (GEM), which is the first-line treatment of head and neck cancer,<sup>21</sup> and EVO. GEM can be combined with cisplatin,<sup>22</sup> paclitaxel,<sup>23,24</sup> and oxaliplatin,<sup>25,26</sup> but no standard combination has been recommended. Long-term and heavy use of GEM can induce chemoresistance and ultimately weaken treatment effects. GEM kills cancer cells while activating other protective mechanisms, which may correlate with the upregulation of NF- $\kappa$ B signaling.<sup>27,28</sup> Zhang et al's<sup>29</sup> research showed that pancreatic cancer stem cells developed chemoresistance toward GEM through the NADPH oxidase/ROS (Nox/ROS)/NF- $\kappa$ B/STAT 3 signaling pathway (STAT3).

To study the activity of the EVO/GEM drug combination, we used the Chou–Talalay method, which is based on the median-effect principle. We combined EVO with GEM and treated tongue cancer cell lines to study their synergism and then studied their effects on cell proliferation, apoptosis, and migration. We also studied the potential mechanisms underlying the effects of NF- $\kappa$ B signaling on the apoptosis of tongue cancer cells.

## Materials and methods

### Reagents, cell lines, and animals

EVO (Natural Institutes for Food and Drug Control, Beijing, China) was dissolved in complete medium containing 0.2% dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St Louis, MO, USA) as a 100-mM stock solution and stored at  $-20^{\circ}\text{C}$ . GEM (Eli Lilly and Company, Indianapolis, IN, USA) was reconstituted as a 200- $\mu\text{g}/\text{mL}$  stock solution in sterile normal saline (NS) and stored at  $-20^{\circ}\text{C}$ . Antibodies against NF- $\kappa$ B p65 were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). Anti-Bcl-2, anti-Bcl-xl, and anti- $\beta$ -actin antibodies were obtained from Immune Way (Plano, TX, USA). Human Cal-27 (American Type Culture Collection [ATCC], Manassas, VA, USA) and Tca-8113 (Medical University of the Air Force, Xian, China; approved by the medical ethics committee of Lanzhou University) tongue squamous carcinoma cell lines were cultured in DMEM, supplemented with 10% FBS, and maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Male nude BALB/c mice aged 4–5 weeks old (18–22 g) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). Mice were housed in specific pathogen-free laboratory of Gansu University of Chinese Medicine (Lanzhou, China).

### MTT assay

Cells were plated at a density of  $4 \times 10^3$  cells/well in 96-well culture plates and cultured overnight. After treatment, 20  $\mu\text{L}$  MTT (Sigma-Aldrich Co.) solution was added to each well and incubated for 4 hours. Medium was discarded, MTT formazan was dissolved in 150  $\mu\text{L}$  DMSO, and the absorbance was measured at 570 nm with a microplate reader.

### Drug interaction analysis

Drug interactions between EVO and GEM were assessed using a combination index (CI), where  $\text{CI} < 1$ ,  $\text{CI} = 1$ , and  $\text{CI} > 1$  indicated synergistic, additive, and antagonistic effects, respectively. The CI value was calculated as follows:

$$\text{CI} = \frac{\text{DA}}{\text{IC}_{50}\text{A}} + \frac{\text{DB}}{\text{IC}_{50}\text{B}}$$

where  $\text{IC}_{50}\text{A}$  and  $\text{IC}_{50}\text{B}$  are 50% inhibitory concentrations of cell growth ( $\text{IC}_{50}$ ) of EVO and GEM, respectively, and DA and DB are drug concentrations in combination treatments that inhibit cell growth by 50%.  $\text{IC}_{50}$  and CI were calculated with CompuSyn software.

## Apoptosis assay

After the designated treatment, cells were washed, harvested, and counted. Then,  $1 \times 10^5$  cells were resuspended in 100  $\mu$ L PBS, after which 10  $\mu$ L annexin V and 5  $\mu$ L propidium iodide (PI) were added, and cells were incubated in the dark for 15 minutes at room temperature according to the manufacturer's instructions (Bioseal Biotech, Guangzhou, China). Apoptosis (%) was assayed using flow cytometry. Apoptotic morphology was confirmed by fluorescence microscopy after Hoechst33342 staining (Beyotime, Beijing, China).

## Clonogenic assay

Cells were plated at a density of  $2 \times 10^3$  cells/well in cell culture dishes. After treatment, cells were cultured for another 10 days and then fixed and stained with Giemsa (Abcam, Cambridge, UK). Colonies ( $\geq 50$  cells) were counted to calculate percent growth inhibition.

## Migration assay

Cells were grown to 90%–100% confluence in six-well tissue culture plates. After drug treatment, a wound was made by scraping the middle of the cell monolayer with a 200  $\mu$ L pipette tip. Then, floating cells were removed by washing with PBS, and fresh complete DMEM was added to each well. After 24, 48, and 72 hours of cell migration, wound areas were observed under an inverted microscope. ImageJ software was used to analyze the images.

## Western blot

After treatment, cells were washed with ice-cold PBS and harvested in buffer (Beyotime) containing protease inhibitors (Beyotime). Extracted proteins were resolved with SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Solarbio Science and Technology, Beijing, China). Proteins were probed with specific antibodies as previously described.

## In vivo xenografts

Animal experiments were performed in accordance with the National Regulations on the Management of Experimental Animals (Beijing, China) and approved by the experimental animal ethics committee of School of Stomatology, Lanzhou University (Lanzhou, China). CAL-27 cells were washed with PBS and harvested in serum-free medium. Then,  $\sim 2 \times 10^6$  cells were injected subcutaneously into the right armpit regions of nude mice. When the length of the transplanted tumor reached 5 mm, nude mice were randomized into four groups ( $n=6$ /group) to receive intraperitoneal injections as

follows: control (NS), EVO (10 mg/kg, dissolved in vehicle), GEM (50 mg/kg, dissolved in vehicle), or EVO and GEM twice per week for 5 weeks. Tumor volumes and body weight were measured twice per week. Tumor volume was measured along the longest orthogonal axes and calculated as  $\text{volume} = (\text{length} \times \text{width}^2/2)$ , where width is the shortest measurement. All mice were sacrificed at 35 days after the first day of treatment.

## Transmission electron microscopy (TEM) observations of morphological changes

Morphological changes were observed under TEM. Cells were fixed in 2.5% glutaraldehyde/1% osmium tetroxide for 1.5 hours. After embedding and aggregating, specimens were sliced and stained with uranyl acetate and lead citrate.

## Statistical analyses

All experiments were repeated at least three times, and data are expressed as mean  $\pm$  SD. Differences among groups of cells or mice were analyzed with the chi-squared test or ANOVA, followed by the Student's *t*-test using SPSS v16.0 software (SPSS Inc., Chicago, IL, USA). *P*-values of  $<0.05$  were considered statistically significant.

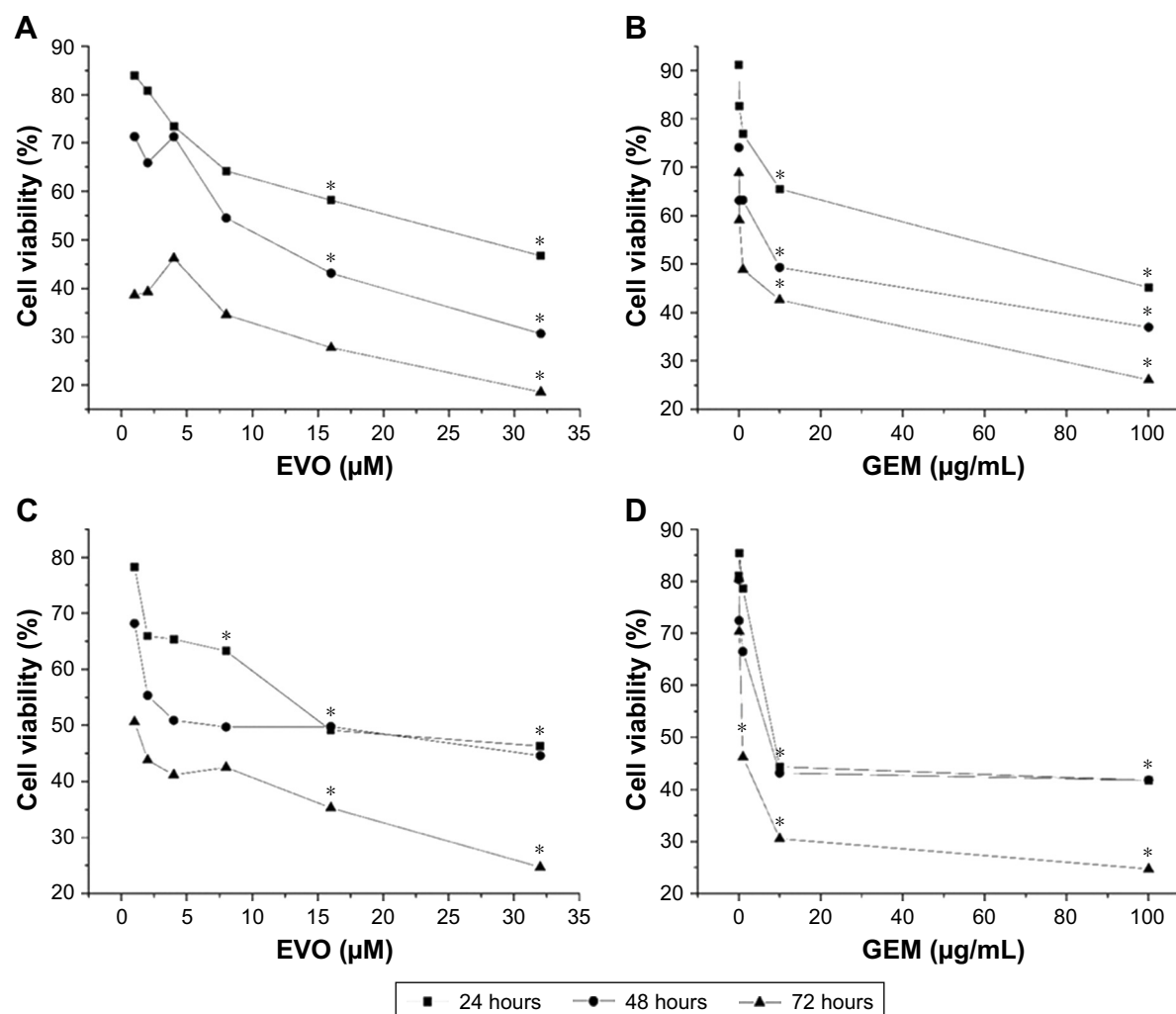
## Results

### Synergistic inhibition of proliferation with EVO and GEM in vitro

CAL-27 and Tca8113 cells were treated with chosen dosages of GEM (0, 0.01, 0.1, 1, 10, and 100  $\mu$ g/mL), EVO (0, 1, 2, 4, 8, 16, and 32  $\mu$ M), or their combinations. Figure 1 shows that EVO and GEM inhibited the proliferation of tongue squamous cells in dose- and time-dependent manners; the  $IC_{50}$  data are given. CI data showed that different treatment sequences and dose ratios induced different drug interactions. Table 1 summarizes that sequential exposure to EVO for 24 hours followed by GEM for 24 hours at a 2:1 ratio of EVO:GEM produced synergistic effects in both cell lines, although the effects were more pronounced in Tca8113 cells.

### Effect of EVO and GEM on apoptosis in vitro

CAL-27 and Tca8113 cells were treated with GEM (1.8 and 2.8  $\mu$ g/mL, respectively), 5  $\mu$ M EVO, or their combination for 48 hours. EVO and GEM increased apoptosis in CAL-27 cells ( $P<0.01$ ). It was shown that pretreatment with EVO followed by GEM treatment induced apoptosis in a greater



**Figure 1** Inhibition of cell proliferation was assessed by MTT assay.

**Notes:** EVO and GEM inhibit CAL-27 (A, B) and Tca8113 (C, D) cell proliferation in a dose- and time-dependent manner. Mean  $\pm$  SD.  $n=3$ ,  $*P<0.05$ .

**Abbreviations:** EVO, evodiamine; GEM, gemcitabine.

proportion when compared with treatment with either drug alone (Figure 2A,  $P<0.05$ ). Figure 2B shows that the nuclei of the controls were regular and oval. Part of the nucleus dissolved, was irregularly shaped, and stained bright blue after treatment with a single drug. The number of cells significantly decreased after combination treatment, and nuclei had significant shrinkage, fragmentation, dissolution, and irregularities.

## Effect of EVO and GEM on cloning and migration in vitro

CAL-27 and Tca8113 cells were treated with GEM (0.35 and 0.55  $\mu\text{g/mL}$ , respectively), 1  $\mu\text{M}$  EVO, or their combination for 48 hours. Figure 3 shows the clones of CAL-27 cells in each treatment group and that combination treatment inhibited clone formation more than single drug treatment. This effect was more pronounced in Tca8113 cells ( $P<0.01$ ).

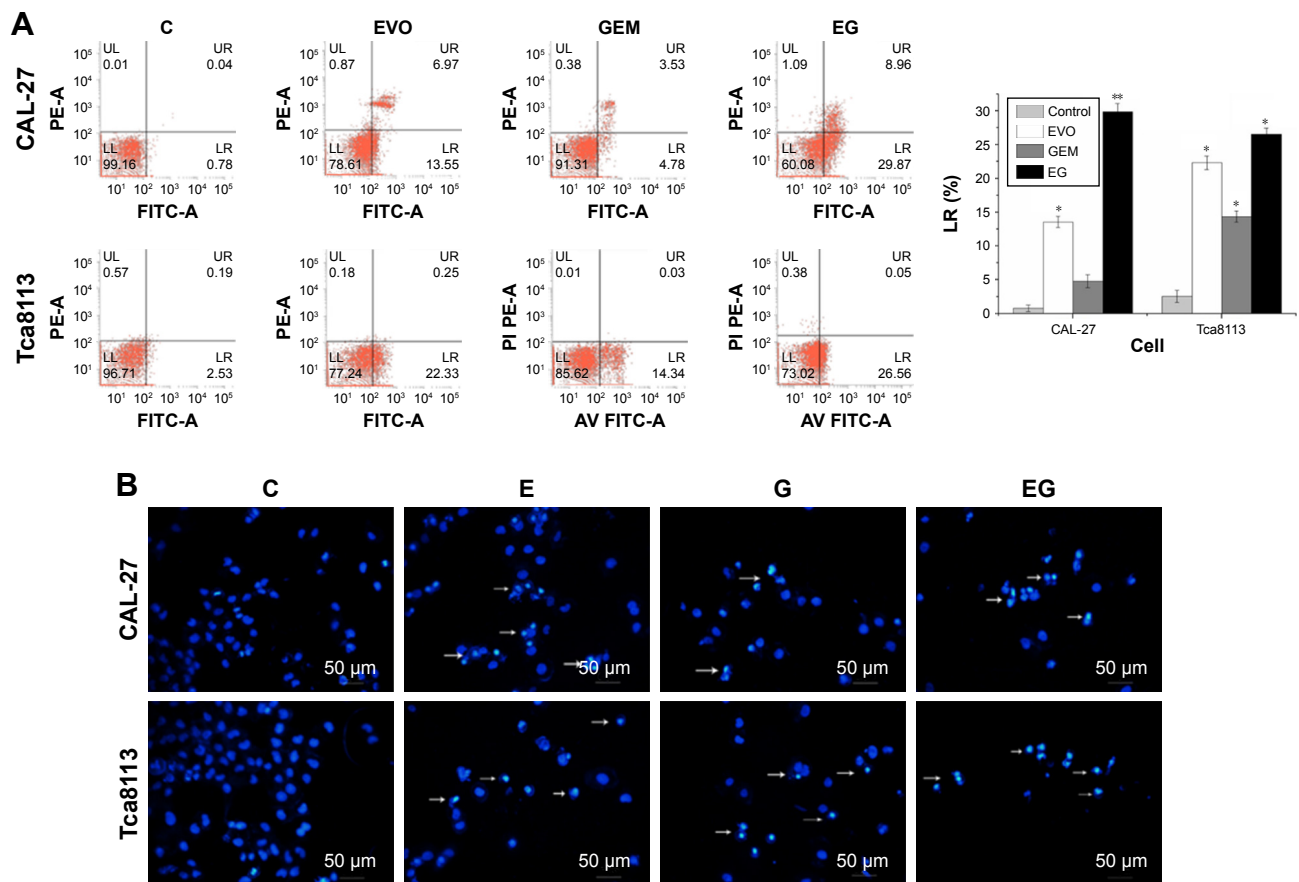
**Table 1** CI of drug combination

CI	CAL-27			Tca8113		
	G:E=1:2	G:E=1:1	G:E=2:1	G:E=1:2	G:E=1:1	G:E=2:1
G E	0.73451	3.44608	3.2348	5.18286	5.75601	7.09234
E G	0.66848	1.62307	2.6346	0.12937	0.9953	0.4497
G+E	1.20678	2.00714	2.54105	1.31941	2.26136	2.71772

**Notes:** CI of CAL-27 and Tca8113 cells. GE sequential exposure to GEM for 24 hours followed by EVO for 24 hours. EG sequential exposure to EVO for 24 hours followed by GEM for 24 hours. G+E simultaneous exposure to EVO and GEM for 24 hours followed by no drug culture for 24 hours. "G" represents "GEM" and "E" represents "EVO".

**Abbreviations:** CI, combination index; EVO, evodiamine; GEM, gemcitabine.





**Figure 2** The apoptosis rate (%) and the apoptotic morphology of cells were determined by FCM (A) and Hoechst33342 staining (B).

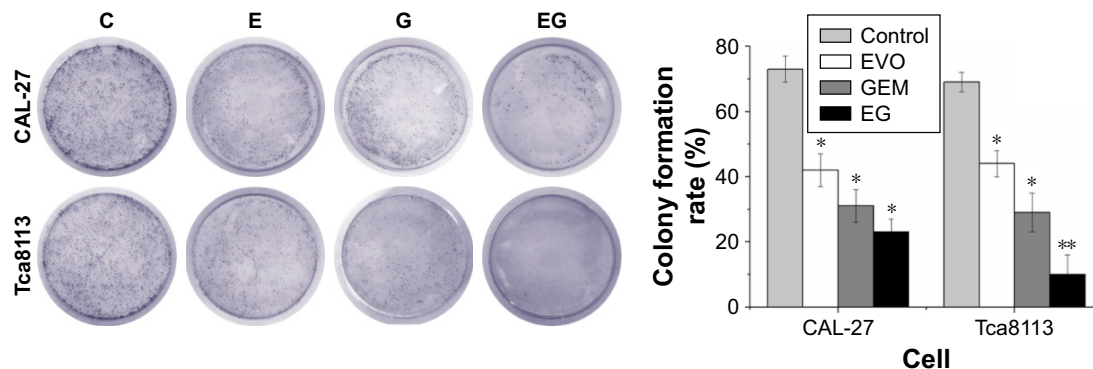
**Notes:** "C", "E", and "G" represent "control", "EVO" and "GEM", respectively. "EG" represents that the cells were sequential exposed to EVO for 24 hours followed by GEM for 24 hours. The "LR" represents "lower right quadrant", indicating the ratio of early apoptotic cells. \* $P < 0.05$  and \*\* $P < 0.01$ .

**Abbreviations:** EVO, evodiamine; FCM, flow cytometry; GEM, gemcitabine.

The results of the wound healing assay showed that cell coverage in each groups increased over time, with coverage in the control group increasing the fastest which scratches almost completely healing by 72 hours (Figure 4,  $P < 0.05$ ). Cell coverage between controls and treatments was statistically different at 72 hours in both cell lines ( $P < 0.05$ ).

## EVO enhanced GEM-induced apoptosis by regulating NF- $\kappa$ B and Bcl-2 family protein

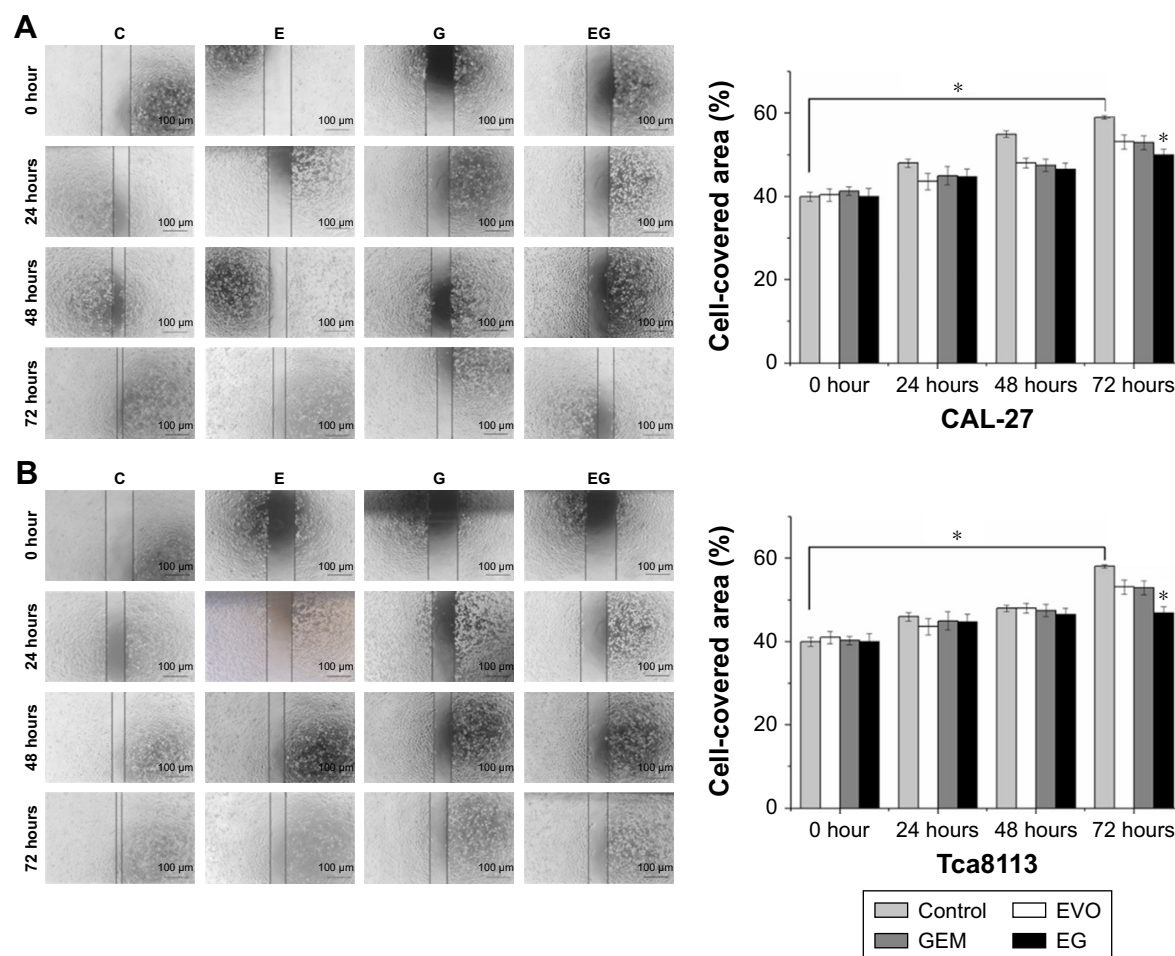
We investigated whether the inhibitory effects of EVO on tongue cancer cell survival were mediated via the activity of NF- $\kappa$ B-related proteins. Protein extracts of untreated, EVO-



**Figure 3** Cell colony formation ability was assessed by plate clone formation assay.

**Notes:** Combination group showed less clone formation compared with single drug in tongue squamous cancer cells. "C" represents control group, "E" represents cells were treated with EVO for 48 hours. "G" represents cells were treated with GEM for 48 hours. "EG" represents cells were sequential exposed to EVO for 24 hours followed by GEM for 24 hours. \* $P < 0.05$  and \*\* $P < 0.01$ .

**Abbreviations:** EVO, evodiamine; GEM, gemcitabine.



**Figure 4** Migration capabilities were assessed by wound-healing test.

**Notes:** The combination of EVO and GEM on CAL-27 (A) and Tca8113 (B) cell. "C" represents control group, "E" represents cells were treated with EVO for 48 hours. "G" represents cells were treated with GEM for 48 hours. "EG" represents cells were sequential exposed to EVO for 24 hours followed by GEM for 24 hours. \* $P < 0.05$ .

**Abbreviations:** EVO, evodiamine; GEM, gemcitabine.

treated (5  $\mu$ M), GEM-treated (2  $\mu$ g/mL), and dual-treated cells were analyzed by Western blotting to assess the expression of p65 and downstream apoptosis-related proteins (Figure 5). CAL-27 and Tca8113 cells had NF- $\kappa$ B signal activation, but GEM treatment did not significantly activate NF- $\kappa$ B signaling. EVO inhibited NF- $\kappa$ B signaling and GEM-induced NF- $\kappa$ B activity in CAL-27 cells ( $P < 0.05$ ). The effects were modest in Tca8113 cells. EVO and GEM downregulated Bcl-2 and Bcl-xl in both cell lines ( $P < 0.05$ ).

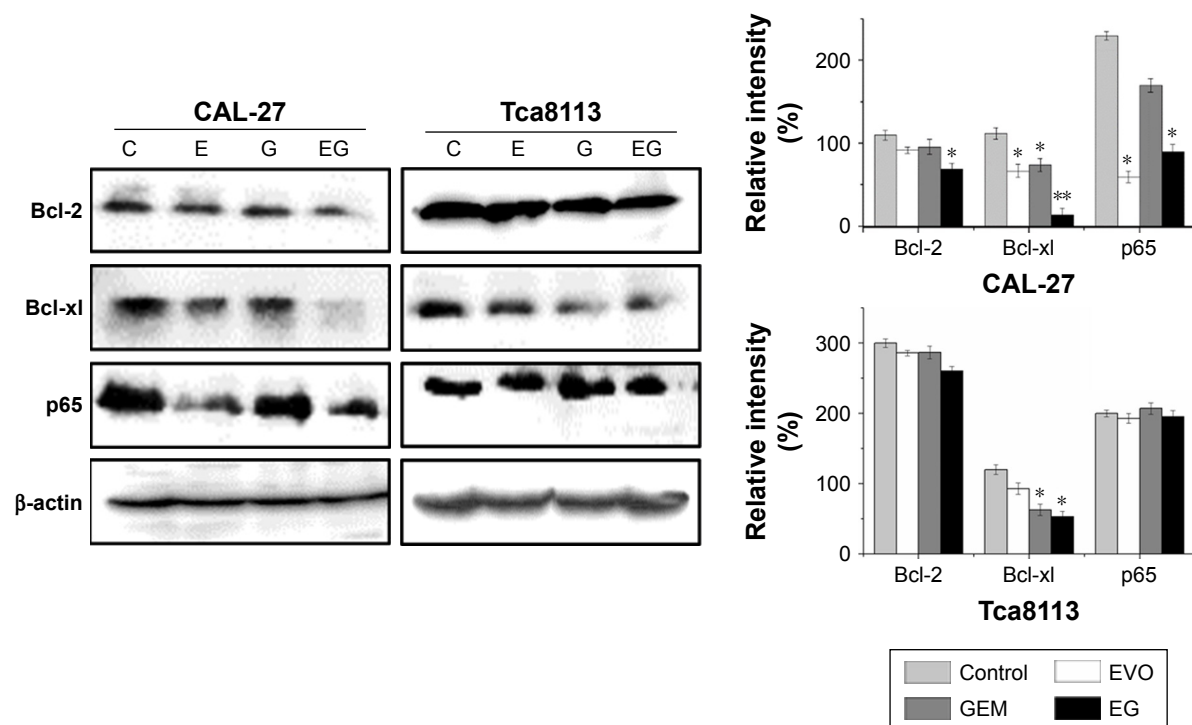
## Antitumor effect of EVO plus GEM on the growth of implanted tongue tumor in vivo

We evaluated the in vivo antitumor activity of EVO in a xenograft tongue cancer model. Tumor growth was significantly inhibited in mice treated with EVO ( $P < 0.05$ ) or GEM ( $P < 0.05$ ) compared with vehicle, and tumor growth after EVO + GEM treatment was slower than with any single agent ( $P < 0.05$ ). Mouse weight

slowly increased in each group, and there was no significant difference among groups. Protein extracts from the specimens of all groups were also analyzed by Western blotting to assess NF- $\kappa$ B p65, Bcl-2, and Bcl-xl expression, and the data were in accordance with the in vitro data. TEM showed that tumor cell nuclei in the controls were intact, clear, and homogeneous. Cell volume was reduced, and chromatin was concentrated, showing early apoptosis in the single drug treatment groups. Nuclear heterochromatin fragmentation and concentration at the nuclear membrane were observed in the combined treatment group, which showed delayed apoptotic characteristics.

## Discussion

EVO significantly decreased the growth of human tongue cancer cells, sensitized tongue cancer cells to chemotherapy, and inhibited NF- $\kappa$ B signaling. In vitro experiments revealed that growth inhibitory effects of EVO in combination with GEM were time dependent. Sequential exposure to EVO



**Figure 5** Combination of two drugs can reduce the expression level of Bcl-2 and Bcl-xl of tongue squamous cancer cells.

**Notes:** EVO can reduce the expression of NF- $\kappa$ B p65 level of CAL-27 cells. The expression of NF- $\kappa$ B p65 has no significant difference in the four groups in Tca8113 cells. "C" represents control group, "E" represents cells were treated with EVO for 48 hours. "G" represents cells were treated with GEM for 48 hours. "EG" represents cells were sequential exposed to EVO for 24 hours followed by GEM for 24 hours. \*P < 0.05.

**Abbreviations:** Bcl-xl, B cell lymphoma extra large; EVO, evodiamine; GEM, gemcitabine.

for 24 hours followed by GEM for 24 hours at a ratio of 2:1 produced synergistic effects in both cell lines. Other regimens resulted in additive or antagonistic effects, suggesting that the drug combination was almost equally or as weakly cytotoxic as each drug alone, in accordance with the literature,<sup>27,28,30,31</sup> suggesting that sequential administration of small molecules that suppress NF- $\kappa$ B activation followed by GEM may be preferable, and simultaneous administration may be inappropriate. The Chou–Talalay method can quantify the synergistic, additive, and antagonistic effects of two or more drugs and help with optimal combination selection as it reveals single and combination drug dose–response curves.

The mechanisms underlying the antagonistic or synergistic interaction with sequential exposure are unclear. The antagonistic effects of EVO and GEM suggest that they cancel out their individual cytotoxic effects. EVO and GEM are cell cycle specific. EVO inhibits cells at the G2/M phase,<sup>27,32</sup> and GEM prevents cells from entering the S phase, arresting the cell cycle at the G1 phase.<sup>28</sup> Thus, drug effects may be associated with cell proliferation kinetics. In addition, EVO and GEM at a dose ratio of 2:1 offer better synergistic effects, perhaps due to the rapid growth of tongue squamous cells, the longer G2/M phase, and better EVO inhibition.

MDR may arise from ATP-binding cassette (ABC) transporter irregularities including P-gp, MDR1, and breast cancer resistance protein, as cell membranes of chemotherapy-insensitive tumors tend to overexpress ABC transporters.<sup>33</sup> These transporters can increase drug efflux to reduce the therapeutic concentration. In addition, blockade of pro-survival signaling pathways can increase tumor cell sensitivity to chemotherapy. Enhanced antiapoptotic activity may be a mechanism of MDR. In addition, autophagy-induced chemotherapy resistance, tumor stem cell-regulated MDR, micro-RNA-regulated MDR, hypoxia-induced MDR, DNA repair mechanism-enhanced MDR, and MDR epigenetic regulation may contribute to resistance. Studies have shown that EVO can target tumor stem cell-like cells,<sup>34</sup> and EVO in combination with other drugs may offer synergistic inhibition of cancer cells.<sup>35,36</sup> Therefore, EVO may target tumor stem cells and survival-related pathways, but its effects on ABC transporter are unclear. EVO may synergize with other drugs to overcome MDR without increasing toxicity and could be used as adjuvant chemotherapy for tongue squamous cell carcinoma.

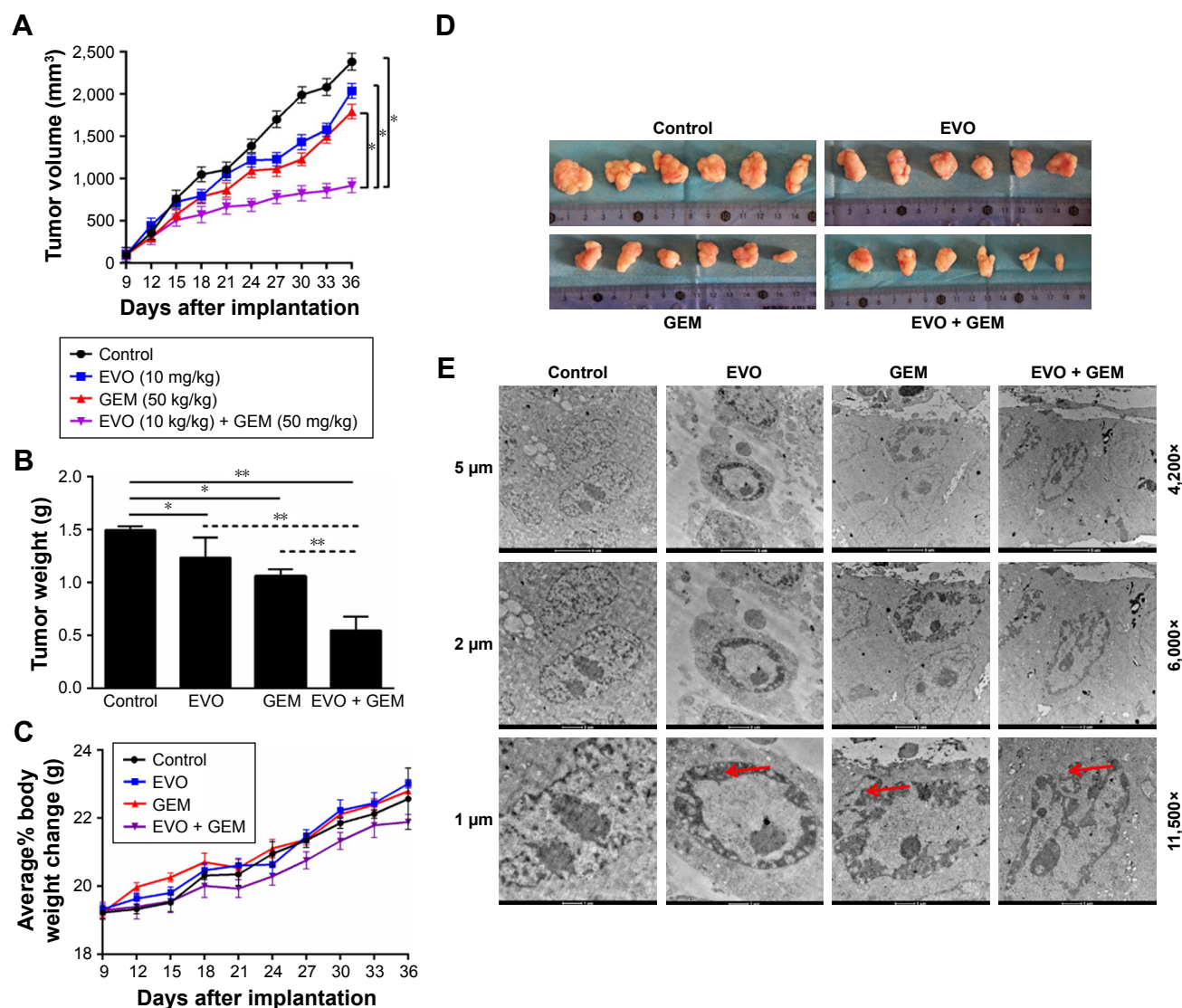
Activation of the NF- $\kappa$ B signaling pathway regulates antiapoptotic and survival activity in tumor cells. Studies have shown that an in vitro colon cancer model of



5-fluorouracil chemotherapy resistance is related to NF- $\kappa$ B activation.<sup>37</sup> In addition, resistance in acute myeloid leukemia is associated with the activation of NF- $\kappa$ B, and it can be used as a target for treatment.<sup>38</sup> Overexpression of NF- $\kappa$ B is positively correlated with chemotherapeutic resistance and poor prognosis in serous ovarian epithelial carcinoma.<sup>39</sup> Thus, its overactivation may cause minimal residual disease (MRD) in tumor cells, and in some cancers such as ovarian cancer, NF- $\kappa$ B has dual regulatory effects.<sup>40</sup> Hong et al<sup>41</sup> showed that high NF- $\kappa$ B expression was correlated with poor differentiation and high metastasis of tongue squamous cell carcinoma. Su et al<sup>42</sup> concluded that the overactivation of NF- $\kappa$ B was correlated with the invasiveness of tongue

cancer according to NF- $\kappa$ B expression in clinical specimens of tongue cancer. Similarly, Wang et al's<sup>43</sup> showed that NF- $\kappa$ B was correlated with epithelial–mesenchymal transition (EMT) in SCC9 and SCC25 cell lines. Thus, tongue squamous cell carcinoma has NF- $\kappa$ B activation itself, promoting the survival of cancer cells and increasing metastasis. Our Western blotting data were consistent with this hypothesis that CAL-27 and Tca8113 cells have the activation of NF- $\kappa$ B p65.

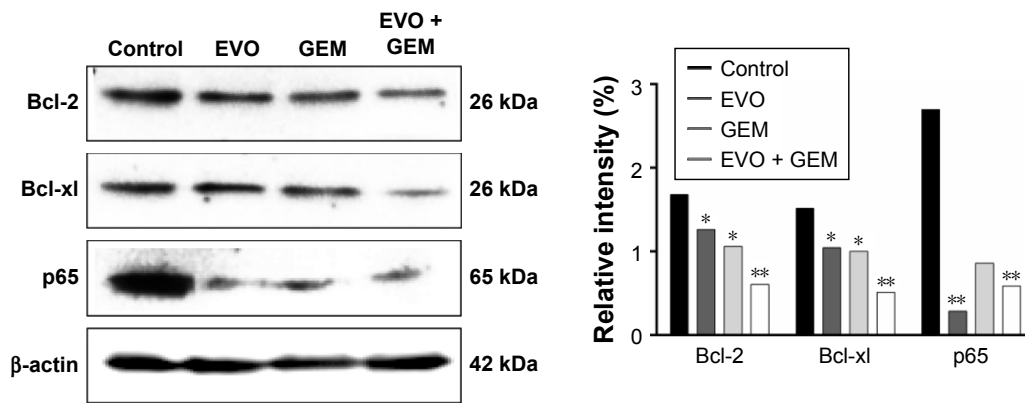
Some studies have suggested that drugs that inhibit NF- $\kappa$ B combined with GEM can improve the therapeutic effects of GEM on pancreatic and gallbladder cancers.<sup>30,44</sup> Small interfering RNA knockout of NF- $\kappa$ B improved GEM's



**Figure 6** Measurements of tongue tumor volume at the indicated time points, depicting the in vivo therapeutic efficacy of EVO and GEM (A) with mean tumor weight (B) and representative picture of a tumor at the end of the experiment (D). Detection of body weight at the indicated time points (C), depicting the in vivo side effects of EVO and GEM. Morphological changes of specimens were observed by TEM (E). \* $P < 0.05$  and \*\* $P < 0.01$ .

**Abbreviations:** GEM, gemcitabine; EVO, evodiamine; TEM, transmission electron microscopy.





**Figure 7** Combination of two drugs can reduce the expression level of Bcl-2 and Bcl-xl in vivo. EVO can reduce the expression of NF- $\kappa$ B p65 level alone or in combination of CAL-27 cells. \* $P < 0.05$  and \*\* $P < 0.01$ .

**Abbreviations:** Bcl-xl, B cell lymphoma extra large; EVO, evodiamine; GEM, gemcitabine.

effects on pancreatic cancer cells;<sup>45</sup> thus, NF- $\kappa$ B may be a promising target for reducing MRD.

The Bcl-2 family includes antiapoptotic and pro-apoptotic proteins, which are major regulators of the mitochondrial membrane potential.<sup>46</sup> The antiapoptotic proteins are Bcl-2 and Bcl-xl, whereas Bax and Bid are distributed in the cytoplasm to promote apoptosis. There is a significant positive correlation between Bcl-2 family and NF- $\kappa$ B.<sup>47,48</sup> Many tumor-associated signaling pathways are associated with Bcl-2 family proteins, and data have shown that EVO plus GEM significantly reduced the expression of Bcl-2 and Bcl-xl, suggesting increased apoptosis. EVO may not only inhibit NF- $\kappa$ B but also may regulate the apoptotic pathways of cancer cells.

Studies have indicated that GEM alone in gallbladder cancer cells can activate NF- $\kappa$ B signaling, causing chemotherapeutic resistance,<sup>49</sup> which may also occur after lengthy treatment of pancreatic cancer with GEM, whereby the NF- $\kappa$ B signaling is activated and promotes the invasion and metastasis of pancreatic cancer cells.<sup>50</sup> Thus, GEM treatment alone may induce NF- $\kappa$ B, leading to diminished therapeutic effects.

In this study, GEM treatment alone in tongue cancer cells did not cause the activation of NF- $\kappa$ B in either cell line, possibly due to the lower concentration of GEM or shorter treatment time. EVO alone significantly reduced NF- $\kappa$ B activity in CAL-27 cells, and NF- $\kappa$ B p65 expression in the combination treatment group was significantly reduced compared with the controls, but these effects were not obvious in Tca8113 cells. Figures 6 and 7 show that mouse weight decrease did not detect, so toxicity did not increase with dual therapy.

## Conclusion

EVO plus GEM may be effective for treating tongue cancer. In vitro data were confirmed in a mouse model in which EVO and GEM significantly blocked tumor growth compared with treatment with either agent alone. In conclusion, the results of this study showed that EVO can potentiate the antitumor activity of GEM in human tongue cancer cells both in vitro and in vivo, and this may be due to EVO-induced suppression of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products. More studies are needed to confirm these data and improve tongue cancer treatment options.

## Ethics approval and consent to participate

This article does not contain any studies with human participants. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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## Author contributions

Professor Jing Wang and Jian Chen contributed to the design of the study. Qi Guo and Yanmei Liu participated in the sequence alignment and drafted the manuscript. Jiayuan Zhao carried out the animal experiments. Jing Wang participated in the cell culture work. Yue Li performed the statistical analysis. Yunqing Pang conceived of the study, participated in its design

and coordination, and helped to draft the manuscript. All the authors read and approved the final manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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