

# 4-Methoxydalbergione inhibits esophageal carcinoma cell proliferation and migration by inactivating NF- $\kappa$ B

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**Abstract.** 4-Methoxydalbergione (4-MD) can inhibit the progression of certain types of cancer; however, its effects on esophageal cancer (EC) remain unclear. The present study aimed to investigate the inhibitory effect of 4-MD on EC and its molecular mechanism. ECA-109 and KYSE-105 cells were treated with or without lipopolysaccharide (LPS) and 4-MD. Cell Counting Kit-8 and colony formation assays were used to analyze cell proliferation. Wound healing assay was performed to evaluate cell migration. ELISA and western blotting were performed to measure the expression levels of NF- $\kappa$ B and inflammatory cytokines. In cells treated with 4-MD, proliferation and migration were significantly inhibited, the levels of inflammatory cytokines were downregulated and the NF- $\kappa$ B signaling pathway was inactivated. Notably, proliferation, migration, inflammation and NF- $\kappa$ B were promoted by LPS,

whereas 4-MD reversed the increases induced by LPS in EC cells. In conclusion, 4-MD may attenuate the proliferation and migration of EC cells by inactivating the NF- $\kappa$ B signaling pathway.

## Introduction

It has previously been reported that the incidence of esophageal cancer (EC) is ~604,100 per year, thus accounting for 3.1% of new cancer cases diagnosed and ranking 7th in cancer incidence worldwide. In addition, it has been reported that there are 544,000 cases of EC-associated mortality per year, accounting for 5.5% of cancer-associated deaths and ranking 6th in cancer deaths worldwide (1). EC is divided into squamous cell carcinoma and adenocarcinoma according to histological subtype (2). The highest incidence of esophageal squamous cell carcinoma has been reported to occur in Eastern Asia, especially in China (1). EC is difficult to diagnose at an early stage due to the lack of specific biomarkers; therefore, patients are nearly always diagnosed with advanced EC (3). The overall 5-year survival of patients with EC is 15-20% worldwide (4); therefore, a novel therapeutic strategy to improve the 5-year survival rate of patients with EC is required.

During the COVID-19 pandemic, it was revealed that traditional Chinese medicine (TCM), such as Jinhua Qinggan granules and Lianhua Qingwen capsules, had a decisive role in the clinical treatment of COVID-19 by targeting ACE (5). Furthermore, a large number of studies have demonstrated that TCM serves an important role in the treatment of cancer, including colorectal cancer (6), breast cancer (7), hepatocellular carcinoma (8), lung cancer (9) and EC (10-12). Notably, the active ingredients of TCM are thought to inhibit cancer progression by regulating certain genes or signaling pathways.

4-Methoxydalbergione (4-MD) is a flavonoid with methoxy groups, which is isolated and purified from *Dalbergia sissoo* Roxb. 4-MD has been indicated to have anti-inflammatory effects via inhibition of the NF- $\kappa$ B signaling pathway (13) and antitumor effects due to its high cytotoxicity in tumor

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**Abbreviations:** 4-MD, 4-methoxydalbergione; EC, esophageal cancer; LPS, lipopolysaccharide; CCK-8, Cell Counting Kit-8; OD, optical density; TCM, traditional Chinese medicine; WB, western blotting; RT, room temperature

**Key words:** 4-MD, EC, NF- $\kappa$ B, migration, proliferation

cells (14). In addition, it has been demonstrated that 4-MD can inhibit osteosarcoma (15), human astroglioma (16) and bladder cancer (17) by regulating multiple signaling pathways, including JAK2/STAT3 and Akt/ERK, but not NF- $\kappa$ B. However, the inhibitory effect of 4-MD on EC remains to be determined.

It has been reported that the development of EC is a multistep pathogenic process from inflammation to cancer (18,19). The malignant degree of EC is positively associated with inflammatory factors, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (20), and the survival time of patients with EC has been confirmed to be negatively correlated with inflammation (21,22). Furthermore, EC is accelerated though EC angiogenesis via activation of the NF- $\kappa$ B signaling pathway (23). Therefore, inflammation may accelerate the progression of EC. Whether 4-MD regulates the NF- $\kappa$ B signaling pathway to inhibit EC remains unclear. Previous studies have shown that NF- $\kappa$ B can promote the release of TNF- $\alpha$  (24,25), the synthesis of prostaglandin E2 (PGE2) (26-28), and the expression of cyclin-dependent kinase 1 (CDK1) (29,30), cyclin D1 (31,32) and proliferating cell nuclear antigen (PCNA) (33,34). However, whether 4-MD inhibits EC by suppressing the release of PGE2 and TNF- $\alpha$ , and the expression of proliferation-associated proteins through the NF- $\kappa$ B signaling pathway requires further investigation. Therefore, the present study aimed to explore the effects of 4-MD on cell proliferation and invasion, and to determine the underlying mechanism by which 4-MD regulates the malignant characteristics of EC cells.

## Materials and methods

**Cell culture.** ECA-109 esophageal squamous carcinoma cells were purchased from the American Type Culture Collection and KYSE-150 esophageal squamous carcinoma cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. ECA-109 cells were cultured in Dulbecco's modified Eagle's medium (cat. no. SH30021; Hyclone; Cytiva) and KYSE-150 cells were cultured in RPMI 1640 (cat. no. 11875093; Gibco; Thermo Fisher Scientific, Inc.). The media were supplemented with 10% fetal bovine serum (cat. no. SH30396; Hyclone; Cytiva) and 1% penicillin-streptomycin (cat. no. V900929; MilliporeSigma). Cells were incubated in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

**Drug preparation.** 4-MD (cat. no. CB31393122; Chemical Book) was dissolved in DMSO to form a 10 mmol/l solution, which was stored at -20°C. The storage solution was not diluted to the corresponding concentration of the working solution until it was used.

**Cell Counting Kit-8 (CCK-8) assay.** The proliferation of cells was analyzed using the CCK-8 assay. ECA-109 cells were seeded into 96-well plates at 1,000 cells/well, and were treated with different concentrations of 4-MD (0, 3.125, 6.25, 12.5, 25, 50 and 100  $\mu$ mol/l) for 24 h. The optical density (OD) of the cells was evaluated using a spectrophotometer at a wavelength of 450 nm. Using the OD of cells, the half maximal inhibitory concentration (IC<sub>50</sub>) and IC<sub>50</sub> 95% confidence interval

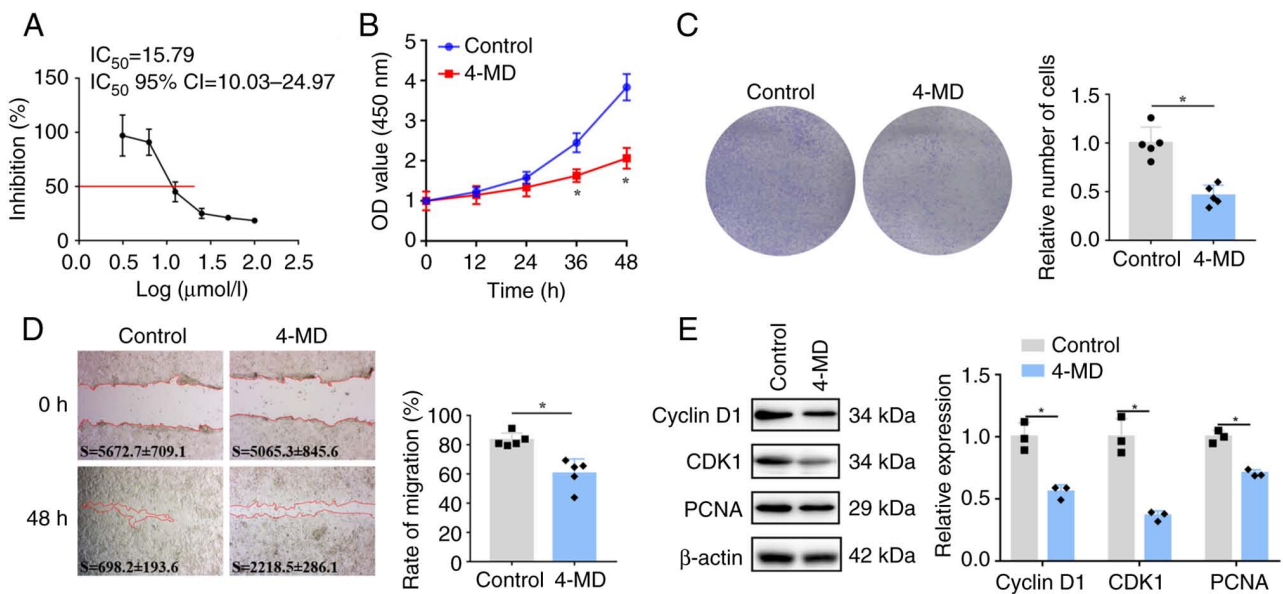
(CI) were calculated using GraphPad Prism 8.3 (GraphPad Software, Inc.). In addition, ECA-109 and KYSE-150 cells were seeded into 96-well plates at 1,000 cells/well, and were pre-incubated with or without 1  $\mu$ g/ml lipopolysaccharide (LPS; cat. no. ST1470; Beyotime Institute of Biotechnology) for 30 min at 37°C in an incubator with 5% CO<sub>2</sub> to activate NF- $\kappa$ B (35), followed by treatment with or without 20  $\mu$ mol/l 4-MD for 12, 24, 36 or 48 h at 37°C in an incubator with 5% CO<sub>2</sub>. Subsequently, 10  $\mu$ l CCK-8 (cat. no. C0038; Beyotime Institute of Biotechnology) solution was added to each well. After culture for 1 h in an incubator, the OD value of the cells was evaluated.

**Colony formation assay.** ECA-109 and KYSE-150 cells were re-suspended in culture medium. Subsequently, 1,000 cells/well were seeded into 24-well plates, and were treated with or without 20  $\mu$ mol/l 4-MD and 1  $\mu$ g/ml LPS for 10 days in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. ECA-109 and KYSE-150 cells were then fixed with 4% paraformaldehyde (cat. no. P0099; Beyotime Institute of Biotechnology) for 30 min at room temperature (RT) and were stained with crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) for 5 min at RT. Finally, the cell colonies containing  $\geq 50$  cells were observed, images were captured under a light microscope and colonies were analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

**Wound-healing assay.** ECA-109 and KYSE-150 cells were used for wound-healing assay. A total of  $1 \times 10^5$  cells/well were seeded into 12-well plates. Once cells reached 100% confluence, a scratch was evenly drawn using a 10- $\mu$ l pipette tip. Subsequently, the cells were incubated in FBS-free medium in a 5% CO<sub>2</sub> incubator at 37°C, and treated with or without 20  $\mu$ mol/l 4-MD and 1  $\mu$ g/ml LPS for a further 36 or 48 h. Finally, the images of wound healing were captured under a light microscope. The wound area was analyzed using ImageJ software. The rate of migration was calculated using the following formula: Rate of migration (%) = (scratch area at 0 h - scratch area at 48 h) / scratch area at 0 h  $\times 100$ .

**ELISA.** The levels of TNF- $\alpha$  and PGE2 in the culture supernatant of ECA-109 cells treated with or without LPS and 4-MD were analyzed using TNF- $\alpha$  (cat. no. PT518; Beyotime Institute of Biotechnology) and PGE2 (cat. no. HB833-Hu; Shanghai Hengyuan Biotechnology Co., Ltd.) ELISA kits according to the manufacturers' protocols. OD value was measure at 450 nm using a spectrophotometer.

**Antibodies.** All antibodies were purchased from Beyotime Institute of Biotechnology. The antibodies used for western blotting (WB) were as follows: Cyclin D1 rabbit monoclonal antibody (1:1,000; cat. no. AF1183), CDK1 rabbit polyclonal antibody (1:500; cat. no. AF0111), PCNA rabbit monoclonal antibody (1:1,000; cat. no. AF1363), phosphorylated (p)-IKB $\alpha$  (Ser32) rabbit monoclonal antibody (1:1,000; cat. no. AF1870), IKB $\alpha$  rabbit monoclonal antibody (1:1,000; cat. no. AF1282), P65 rabbit monoclonal antibody (1:1,000; cat. no. AF1234), p-NF- $\kappa$ B P65 (Ser536) rabbit polyclonal antibody (1:500; cat. no. AF5881),  $\beta$ -actin rabbit monoclonal antibody (1:2,000;



**Figure 1.** 4-MD inhibits the proliferation and migration of ECA-109 cells. (A) ECA-109 cells were treated with different concentrations of 4-MD for 24 h. The IC<sub>50</sub> of 4-MD was 15.79 μmol/l, as determined by the CCK-8 assay. Cells were then treated with 20 μmol/l 4-MD. (B) Proliferation and (C) colony formation (x40 magnification) were analyzed by CCK-8 and colony formation assays, respectively. (D) Migration was measured by a wound-healing assay (x40 magnification); 4-MD inhibited the migration of ECA-109 cells. S represents the scratch area in the images; there was no significant difference in the scratch area at 0 h between the groups. (E) Expression levels of cyclin D1, CDK1 and PCNA were evaluated by WB in ECA-109 cells. Data were obtained from three (for WB) or five (for CCK-8, colony formation and wound-healing assays) independent experiments, and are presented as the mean ± SD. \*P<0.05 vs. control (unpaired Student's t-test). 4-MD, 4-methoxydalbergione; CCK-8, Cell Counting Kit-8; CDK1, cyclin-dependent kinase 1; IC<sub>50</sub>, half maximal inhibitory concentration; OD, optical density; PCNA, proliferating cell nuclear antigen; WB, western blotting.

cat. no. AF5003) and HRP-labeled goat anti-rabbit IgG (1:1,000; cat. no. A0208).

**WB.** ECA-109 cells were harvested and lysed in pre-chilled RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) on ice for 30 min. The supernatants were collected after centrifugation of lysates at 13,300 × g for 30 min at 4°C. The concentrations of proteins were quantified using a NanoDrop ND-2000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). Subsequently, ~20 μg proteins were separated by SDS-PAGE on 10% gels. The proteins were then transferred onto PVDF membranes (cat. no. IPVH00010; MilliporeSigma) and blocked with 5% skim milk (cat. no. P0216; Beyotime Institute of Biotechnology) for 2 h at room temperature (RT). The membranes were incubated with primary antibodies overnight at 4°C, then incubated with the secondary antibody for 2 h at RT. Finally, the bands were observed by chemiluminescence using SuperSignal™ West Pico PLUS (cat. no. 34577; Thermo Fisher Scientific, Inc.), and images were captured using a gel imaging system (Chemidoc MP; Bio-Rad Laboratories, Inc.) and blots were analyzed using ImageJ software (version 1.8.0; National Institutes of Health).

**Statistical analysis.** All data from three or five independent experiments are presented as the mean ± SD, and were analyzed by SPSS 23.0 (IBM Corp.) or GraphPad Prism 8.3 software (GraphPad Software, Inc.). Unpaired Student's t-test was performed to analyze the differences between two groups. One-way ANOVA followed by Sidak's multiple comparisons test was used to analyze the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**4-MD alleviates the proliferation and migration of ECA-109 cells.** To explore the inhibitory effect of 4-MD on EC cells, ECA-109 cells were treated with different concentrations of 4-MD for 24 h. As shown in Fig. 1A, the IC<sub>50</sub> of 4-MD was 15.79 μmol/l and the 95% CI of IC<sub>50</sub> was 10.03–24.97 μmol/l. Subsequently, 20 μmol/l 4-MD was used for further studies. ECA-109 cells were treated with 4-MD, and the proliferation of cells was analyzed by CCK-8 and colony formation assays. Compared with those in the untreated control group, the OD values of cells treated with 4-MD were significantly lower at 36 and 48 h (Fig. 1B). In addition, the colonies of cells treated with 4-MD were fewer and smaller than those in the control group (Fig. 1C). A wound-healing assay was performed to evaluate the migration of ECA-109 cells treated with or without 4-MD for 48 h. The migration rate of control cells was ~80%, whereas the migration rate of cells treated with 4-MD was ~60%, thus suggesting that the migration of ECA-109 cells was suppressed by 4-MD (Fig. 1D). The expression levels of proliferation-associated proteins were determined by WB. The results revealed that the protein expression levels of cyclin D1, CDK1 and PCNA were significantly downregulated in cells treated with 4-MD compared with those in the control group (Fig. 1E). These results indicated that 4-MD inhibited proliferation and migration, and downregulated the expression levels of cyclin D1, CDK1 and PCNA in ECA-109 cells.

**4-MD reduces the production of inflammatory cytokines in ECA-109 cells.** To investigate the inhibitory effects of 4-MD on inflammation, the levels of inflammatory cytokines, including TNF-α and PGE2, were measured in ECA-109 cells by ELISA.

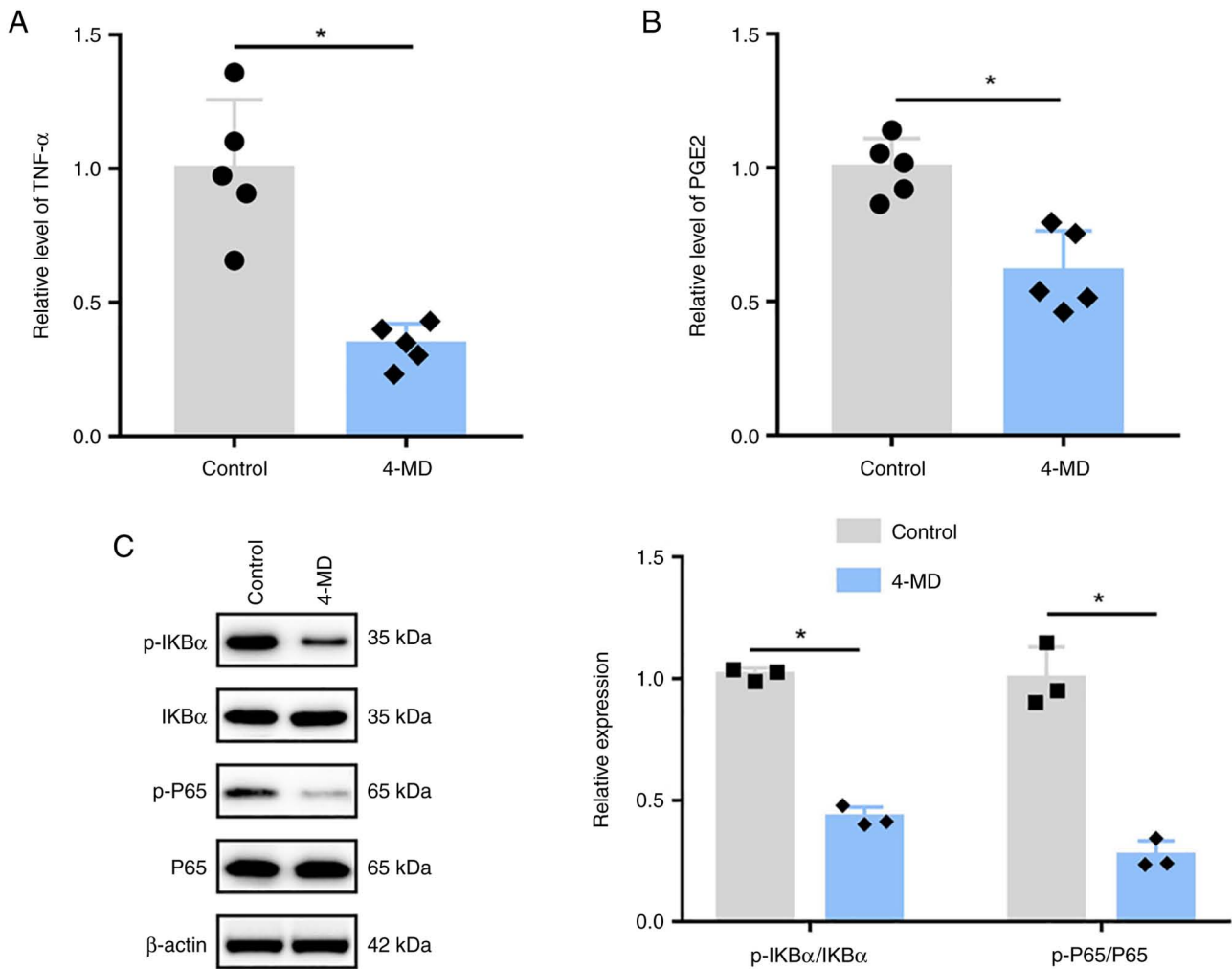


Figure 2. 4-MD downregulates the levels of inflammatory cytokines and inactivates NF- $\kappa$ B in ECA-109 cells. ECA-109 cells were treated with 4-MD for 48 h. Subsequently, the production of (A) TNF- $\alpha$  and (B) PGE2 were analyzed by ELISA, which indicated that 4-MD downregulated the levels of inflammatory cytokines. (C) Expression levels of NF- $\kappa$ B-related proteins were analyzed by WB, which indicated that 4-MD inactivated the NF- $\kappa$ B signaling pathway in the ECA-109 cells. Data were obtained from three (for WB) or five (for ELISA) independent experiments, and are presented as the mean  $\pm$  SD. \* $P$ <0.05 (unpaired Student's t-test). 4-MD, 4-methoxydalbergione; p-, phosphorylated; PGE2, prostaglandin E2; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; WB, western blotting.

As shown in Fig. 2A and B, the production of TNF- $\alpha$  and PGE2 was significantly decreased in ECA-109 cells treated with 4-MD. In addition, WB was performed to measure the expression levels of IKB $\alpha$  and P65. Compared with those in the control groups, the expression levels of p-IKB $\alpha$  and p-P65 were decreased in cells treated with 4-MD, thus suggesting that 4-MD could inactivate NF- $\kappa$ B in ECA-109 cells (Fig. 2C).

**4-MD reverses the LPS-induced proliferation and migration of EC cells.** To further assess whether 4-MD inhibits the proliferation and migration of EC cells by inactivating NF- $\kappa$ B, ECA-109 cells were treated with LPS to activate NF- $\kappa$ B, and subsequently treated with 4-MD. Results of the CCK-8 assay revealed that the proliferation of cells pre-incubated with LPS was significantly increased compared with that in the control group, whereas cell proliferation was inhibited following treatment with LPS and 4-MD, thus indicating that 4-MD partly inhibited the LPS-induced increase in cell proliferation (Fig. 3A). Furthermore, the size and number of cell colonies was increased in the LPS group compared with that in the control group, whereas the colonies in the group treated with LPS and

4-MD were fewer and smaller compared with in the group treated with LPS alone, indicating that 4-MD suppressed the acceleration of cell proliferation induced by LPS in ECA-109 cells (Fig. 3B). Cell migration was analyzed by wound healing. The migration rate of the control cells was  $\sim$ 80% 48 h after scratching; however, the migration rate was  $\sim$ 100% in the group of cells treated with LPS, and was  $\sim$ 90% in the group of cells treated with LPS and 4-MD, which suggested that 4-MD could partly abolish the LPS-induced increase in cell migration (Fig. 3C). To fully demonstrate the inhibitory effect of 4-MD on another EC cell line, KYSE-105 cells were treated with LPS and 4-MD, and proliferation and migration were measured. Notably, the results of CCK-8, colony formation and wound-healing assays in KYSE-105 cells were consistent with the results in ECA-109 cells, which indicated that 4-MD could inhibit the LPS-induced increase in the proliferation and migration of KYSE-105 cells (Fig. 3D-F).

**4-MD inhibits the production of TNF- $\alpha$  and PGE2, and the expression levels of proliferation-related proteins by inactivating NF- $\kappa$ B in ECA-109 cells.** To elucidate the



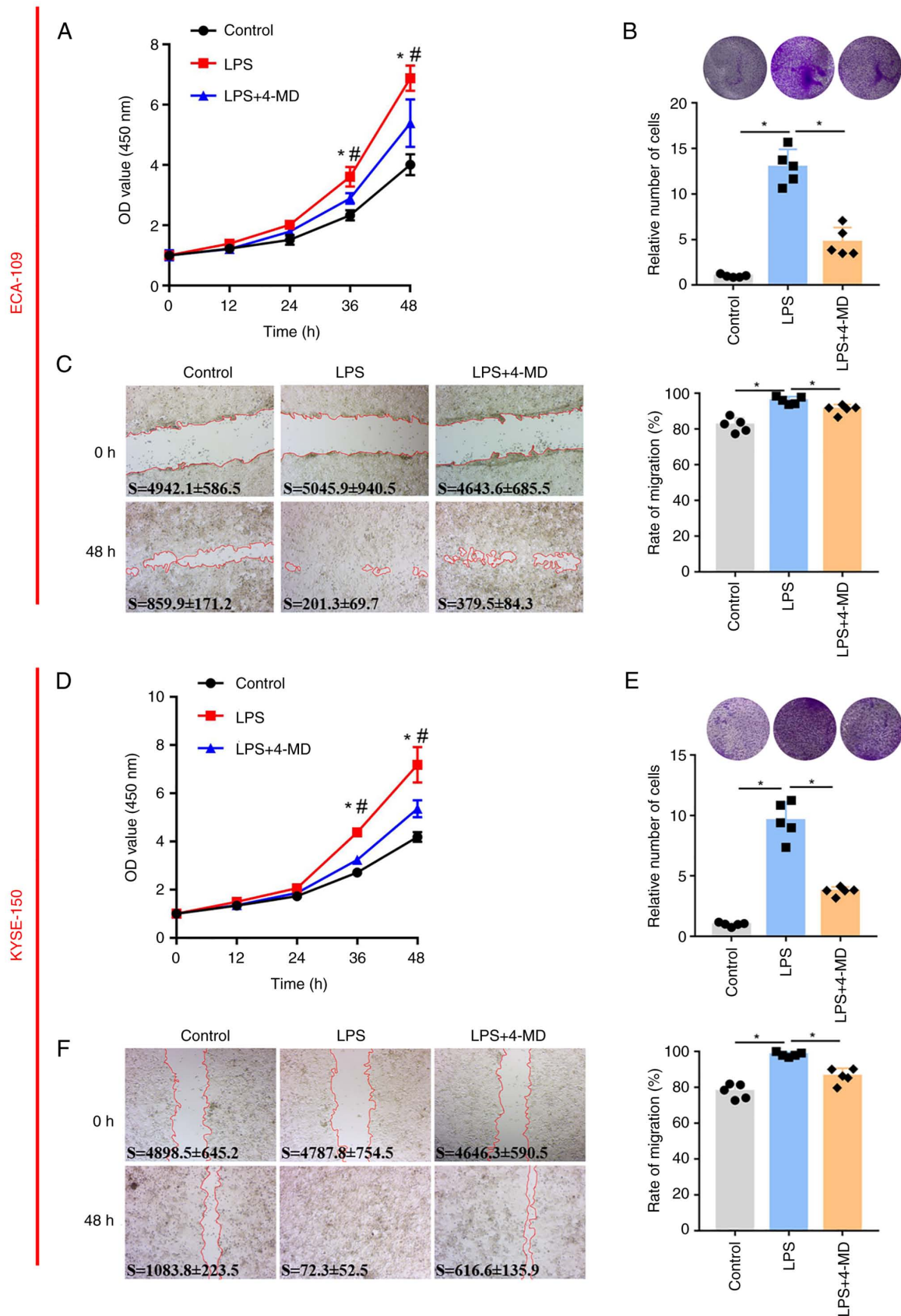


Figure 3. 4-MD partially abrogates the LPS-induced proliferation and migration of esophageal cancer cells. (A-C) ECA-109 and (D-F) KYSE-105 cells were pre-incubated with LPS, and were then treated with or without 4-MD. The proliferation of (A) ECA-109 and (D) KYSE-105 cells was analyzed using the Cell Counting Kit-8 assay, which indicated that 4-MD could reverse LPS-induced proliferation. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. LPS + 4-MD (one-way ANOVA). The proliferation of (B) ECA-109 and (E) KYSE-105 cells was also measured by colony formation assay (x40 magnification), which revealed that 4-MD inhibited the increase in proliferation induced by LPS. The migration of (C) ECA-109 and (F) KYSE-105 cells was assessed by wound-healing assay (x40 magnification), which demonstrated that 4-MD reversed LPS-induced migration. S represents the scratch area in the images; there was no significant difference in the scratch area at 0 h among the groups. Data were obtained from five independent experiments and are presented as the mean  $\pm$  SD. \* $P < 0.05$  (one-way ANOVA). 4-MD, 4-methoxydalbergione; LPS, lipopolysaccharide; OD, optical density.

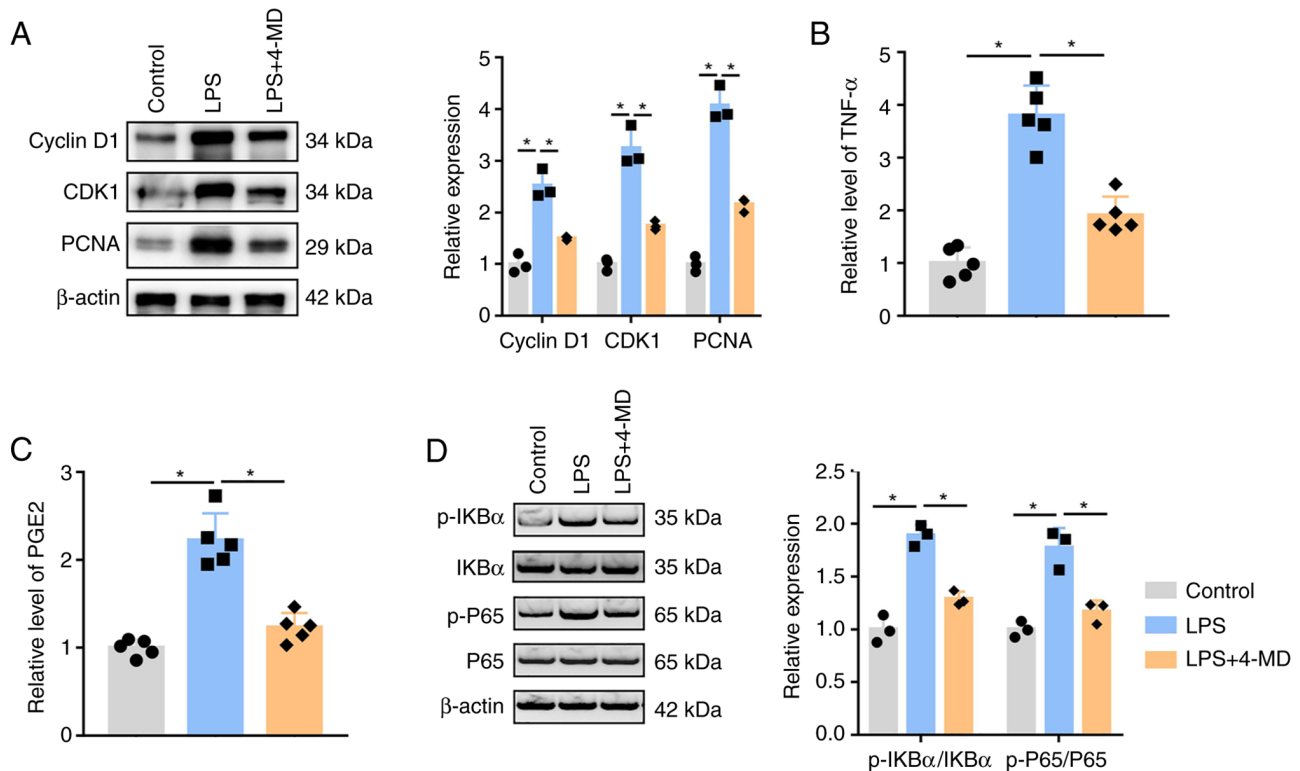


Figure 4. 4-MD downregulates the levels of TNF- $\alpha$ , PGE2 and proliferation-related proteins by inhibiting NF- $\kappa$ B in ECA-109 cells. (A) Expression levels of cyclin D1, CDK1 and PCNA were measured by WB, which indicated that 4-MD inhibited the LPS-induced upregulation of proliferation-related proteins in ECA-109 cells. (B) TNF- $\alpha$  and (C) PGE2 levels were evaluated by ELISA in the culture medium of ECA-109 cells treated with or without LPS and 4-MD; 4-MD inhibited the LPS-induced increase in the levels of TNF- $\alpha$  and PGE2. (D) Expression levels of NF- $\kappa$ B-related proteins were analyzed by WB, which indicated that 4-MD abolished the LPS-induced activation of the NF- $\kappa$ B signaling pathway in ECA-109 cells. Data were obtained from three (for WB) or five (for ELISA) independent experiments, and are presented as the mean  $\pm$  SD. \* $P < 0.05$  (one-way ANOVA). 4-MD, 4-methoxydalbergione; CDK1, cyclin-dependent kinase 1; LPS, lipopolysaccharide; p-, phosphorylated; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; WB, western blotting.

potential mechanism underlying the suppressive effects of 4-MD on the proliferation and migration of EC cells, ECA-109 cells were incubated with LPS and 4-MD. As shown in Fig. 4A, the expression levels of cyclin D1, CDK1 and PCNA were increased in cells treated with LPS compared with those in the control group; meanwhile, the expression levels of these proteins were downregulated in cells treated with LPS and 4-MD compared with those in cells treated with LPS alone.

The production of TNF- $\alpha$  and PGE2 was analyzed by ELISA. The levels of TNF- $\alpha$  (Fig. 4B) and PGE2 (Fig. 4C) were increased in cells treated with LPS compared with those in the control group. By contrast, compared with in cells treated with LPS alone, the levels of TNF- $\alpha$  and PGE2 were decreased in cells treated with LPS and 4-MD. Compared with in the control cells, the protein expression levels of p-IKB $\alpha$  and p-P65 were increased in cells treated with LPS, whereas p-IKB $\alpha$  and p-P65 expression levels were significantly decreased in cells treated with LPS and 4-MD compared with those in cells treated with LPS alone (Fig. 4D), which indicated that 4-MD could inhibit LPS-induced activation of NF- $\kappa$ B.

Taken together, these results suggested that 4-MD inhibited the proliferation and migration of EC cells by decreasing TNF- $\alpha$  and PGE2 production, and downregulated cyclin D1, CDK1 and PCNA expression through inactivating the NF- $\kappa$ B pathway.

## Discussion

Approximately 25% of cancer is caused by inflammation, including EC (36). EC is an aggressive malignant tumor of the digestive system, which is seriously life-threatening and has a complex pathogenesis. Studies have shown that microbial infections serve a catalytic role in EC development (37-39). Furthermore, it has been suggested that inflammation triggered by infections is a notable cause of cancer (40,41). Increasing evidence has shown that inflammation is the main cause of EC induction (19). In the present study, the inflammatory inducer LPS (42,43) was used to promote inflammation in EC cells, which simulated the inflammatory response of EC cells.

Esophagectomy is one of the main treatments for advanced EC but is likely to impair quality of life (44). In a previous study, the single-cell transcriptomic analysis of EC indicated that genes related to macrophages and neutrophils were activated in early esophageal tissue, which created a chronic inflammatory environment that accelerated the progression of EC (18). During the follow-up of patients with EC, it has been revealed that the levels of inflammatory cytokines in recurrent EC are significantly higher than those in patients without recurrence, and inflammation is positively associated with the recurrence of EC, thus suggesting that inflammation could accelerate EC recurrence (45). Under inflammatory stimulation, the expression levels of microRNA-302b have been reported to be

decreased in EC cells, resulting in increased expression levels of ERBB4, IRF2 and CXCR4, which may promote tumor cell growth (46). These findings suggested that inflammation could promote the development and recurrence of EC. Therefore, inhibiting inflammation may be a therapeutic strategy for the treatment of EC. Patients with EC undergoing continuous oral administration of vitamin C exhibited reduced drug resistance via the inactivation of NF- $\kappa$ B (47). Furthermore, curcumin has been shown to enhance the sensitivity of EC to chemotherapy by reducing the expression levels of cyclooxygenase-2 and lipoxygenase through inhibiting the inflammatory response (48). Natural astaxanthin (49) and lycopene (50) may also significantly inhibit the occurrence of EC by suppressing NF- $\kappa$ B activity. These results indicated that NF- $\kappa$ B may be an effective molecular target for the treatment of EC.

Notably, the active ingredients of TCM have attracted attention due to their inhibitory effects on cancer. A previous study showed that sulforaphene activated the GADD45B/MAP2K3/p38/p53 feedback loop, and down-regulated the expression levels of SCD and CDH3 to alleviate the progression of EC (51). Echinatin has also been shown to inhibit the proliferation and invasion of EC cells by inducing AKT/mTOR signaling pathway-dependent autophagy and apoptosis (52). These findings indicated that the active ingredients of TCM may act on different genes/signaling pathways to inhibit the progression of EC. 4-MD was isolated and purified from *D. sissoo* Roxb. It has been shown that 4-MD can suppress the proliferation and induce apoptosis of human osteosarcoma cells by downregulating the JAK2/STAT3 pathway *in vitro* and *in vivo* (15). 4-MD can also effectively arrest the cell cycle of astrogloma cells in G<sub>2</sub> phase, and regulate multiple genes that enrich the cell cycle and the p53, TNF and MAPK signaling pathways (16). 4-MD has been reported to attenuate the proliferation of bladder cancer cells by inducing autophagy and suppressing the Akt/ERK signaling pathway *in vitro* (17). The present study revealed that the OD value and the number of cell colonies were reduced, the size of colonies was smaller, and wound healing was significantly inhibited in EC cells treated with 4-MD. Furthermore, WB showed that the expression levels of cyclin D1, CDK1 and PCNA were decreased in EC cells treated with 4-MD. These results indicated that 4-MD may be a potential drug for the treatment of EC that functions by downregulating cyclin D1, CDK1 and PCNA.

It has previously been reported that 4-MD can suppress inflammatory responses in addition to being a potent inhibitor of cancer. 4-MD has been demonstrated to exert cytoprotection by inducing anti-inflammatory effects, specifically via inhibiting the LPS-induced production of nitric oxide and PGE2 in microglia (53) and reducing LPS-induced inflammation in RAW264.7 cells (54,55). In the present study, the levels of TNF- $\alpha$  and PGE2 were decreased in EC cells treated with 4-MD, as determined by ELISA. Subsequently, the related proteins in the NF- $\kappa$ B signaling pathway were analyzed by WB, and the results revealed that p-IKBA and p-P65 expression levels were significantly reduced, thus suggesting that 4-MD reduced inflammatory cytokines by inactivating NF- $\kappa$ B in EC cells. To confirm that 4-MD inhibited the proliferation and migration of EC cells by suppressing inflammation, EC cells were treated with the NF- $\kappa$ B agonist LPS. Proliferation, migration, and TNF- $\alpha$  and PGE2 levels were markedly

increased, and the NF- $\kappa$ B signaling pathway was activated in response to LPS. These results were consistent with the finding that LPS can promote the proliferation of EC cells (35,56). Following activation of NF- $\kappa$ B, EC cells were treated with 4-MD. Notably, 4-MD partially reversed the LPS-induced increases in proliferation, migration and inflammatory cytokines (TNF- $\alpha$  and PGE2), and activation of the NF- $\kappa$ B signaling pathway.

In conclusion, the results of the present study demonstrated that 4-MD significantly inhibited proliferation and migration by inactivating the NF- $\kappa$ B signaling pathway in EC cells, thus providing a novel strategy for 4-MD-induced inhibition of NF- $\kappa$ B in the treatment of EC. However, the present study has the following limitations: First, esophageal inflammation is one of the pathogeneses that accelerate the occurrence of EC. 4-MD can inhibit the malignant characteristics of EC cells by reducing the release of inflammatory factors; however, the present study does not show that the antitumor effect of 4-MD is superior to other antitumor drugs. Second, the present study revealed that 4-MD inhibited EC by inactivating the NF- $\kappa$ B signaling pathway; however, whether the inactivation of NF- $\kappa$ B is the dominant role of 4-MD in tumor suppression requires further investigation. Third, 4-MD has only been demonstrated to inhibit the proliferation of EC cells by suppressing NF- $\kappa$ B *in vitro*; therefore, further studies are needed to explore the inhibitory effect of 4-MD on EC *in vivo*.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

QZ and FC designed the study and revised the manuscript. ML wrote the manuscript, performed cell culture and migration assays, and analyzed the data. YB, PY, LW, TZ, ZX and XL performed the experiments, including the proliferation assay and WB. KZ performed the WB experiment shown in Fig. 4A, analyzed these data, described the relevant results and financially sponsored this study. All authors read and approved the final manuscript. ML and FC confirm the authenticity of all the raw data.

**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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