

Celastrol inhibits migration and invasion through blocking the NF- κ B pathway in ovarian cancer cells

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Abstract. Metastatic ovarian cancer is a major clinical challenge with poor prognosis and high mortality. Celastrol is a natural compound that has exhibits antiproliferative activity; however, its effects on metastasis-related phenotypes in ovarian cancer models are unclear. In the current study, the anti-invasive activities and associated signaling pathways of celastrol were determined in ovarian cancer cells. Cell proliferation was tested by MTT assay. Cell migration was detected by wound healing and Transwell assays, while cell invasion was detected by a Matrigel-coated Transwell method. In addition, nuclear factor (NF)- κ B and matrix metalloproteinase (MMP) expression was examined by western blotting, and MMP-2/-9 activities were determined by gelatin zymography. At sub-toxic concentrations ($<0.5 \mu\text{M}$), celastrol inhibited migration and invasion in a concentration-dependent manner in SKOV-3 and OVCAR-3 cells. At the molecular level, celastrol blocked the canonical NF- κ B pathway by inhibiting I κ B α phosphorylation, and preventing I κ B α degradation and p65 accumulation. Furthermore, the expression and activity of the NF- κ B target protein MMP-9, but not MMP-2, were inhibited by celastrol. Furthermore, celastrol showed no synergistic effect with MG132, an NF- κ B inhibitor. In conclusion, celastrol exhibited significant anti-invasive activities in ovarian cancer cells. Such functions may be mediated via NF- κ B pathway blockade. The results of this *in vitro* study strengthen the value of applying celastrol as a potential clinical intervention modality for delaying ovarian cancer metastasis. This, celastrol warrants further preclinical investigation.

Introduction

Ovarian cancer is one of the most lethal gynecological malignancies with a high case-to-mortality ratio (1). In 2014, the American Cancer Society reported 21,550 cases of epithelial ovarian carcinoma and 14,600 disease-related mortalities, indicating that 69% percent of all patients with ovarian carcinoma succumbed to their disease. The high mortality rate of this cancer is largely due to the fact that many patients present with advanced or metastatic disease (2). In addition, conventional treatment strategies such as chemotherapy and radiation are unlikely to reduce the metastatic frequency in ovarian cancer (3).

Nuclear factor (NF)- κ B is a major transcription factor that is essential for the development of inflammation and cancer (4). It is reportedly involved in many physiological functions of cells, including apoptosis, proliferation, invasion and angiogenesis (5). Under normal conditions, NF- κ B is mainly located in the cytoplasm with a heterotrimer complex comprising p50, p65 and I κ B α . When activated by stimulators and stress, I κ B α is rapidly phosphorylated, ubiquitinated and degraded by the proteasome. NF- κ B then translocates to the nucleus followed by p65 phosphorylation and binding to specific response elements in the DNA, which subsequently activates target gene expression (5).

NF- κ B controls the expression of an array of genes involved in multiple features of cancer, including proliferation, survival angiogenesis and invasion. In invasion and metastasis, NF- κ B regulates the gene transcription of proteolytic enzymes, cytokines and signaling molecules associated with epithelial-mesenchymal transition (EMT) (6). For example, matrix metalloproteinases (MMPs), such as MMP-2 and -9, have been shown to be typical NF- κ B target proteins that are responsible for extracellular matrix breakdown and cell invasion (7). In addition, NF- κ B serves as a key regulator for EMT-associated proteins such as Snail and Slug (8-10).

Traditional herbal medicines have demonstrated great potential in cancer treatment. Several natural materials used in Chinese medicines, including green tea polyphenols, curcumin and triptolide, have been intensively studied and exhibit antitumor efficacy in preclinical models of various cancer types (11). Some leads are currently in early stage clinical trials for a variety of cancers in combination with other interventions. For example, curcumin shows considerable prevention effects when used before chemotherapy in Phase I

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trials in colon cancer patients (12). One reason for the extensive application of traditional medicine in modern cancer therapy is that, in comparison with chemotherapy, natural agents are less toxic to normal tissues and, therefore, patients maintain tolerance to them (13). Notably, many natural compounds have been found to effectively suppress NF- κ B signaling, providing an experimentally validated molecular explanation for their functional outcomes in tumor cells (14).

Celastrol is a natural triterpene derived from the Chinese plant Thunder God Vine (*Tripterygium wilfordii*). It is a pleiotropic compound showing antitumor, anti-inflammatory, antihypertensive and antidiabetic activities (15). With regard to cancer treatment, celastrol has been shown to exert considerable cell-killing effects and to shrink xenografted tumors in tissue and animal models of various malignancies including cancers of the prostate (16-18), breast (19), liver (20,21) and lung (22,23). However, the intervention potential of celastrol on the growth, survival and metastatic features of ovarian cancer cells remains largely unknown.

In the current study, the functional role of celastrol in ovarian cancer cell migration and invasion, two key steps in the metastatic cascade, was evaluated. Furthermore, the possible molecular mechanism underlying the effects of celastrol on the cells was investigated.

Materials and methods

Cell lines. OVCAR-3 and SKOV-3 cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C in a humidified 5% CO₂ incubator. All cells were passaged using 0.05% trypsin/0.02% EDTA (Gibco; Thermo Fisher Scientific, Inc.).

Reagents. Celastrol was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The powder was reconstituted in DMSO to generate a stock concentration of 20 mM and was stored at -20°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and MG132 were also purchased from Sigma-Aldrich (Merck KGaA). Cells were treated with varied concentrations of celastrol (0.125, 0.25, 0.5, 1 and 2 μ M) depending on the experiments. To detect NF- κ B activity, cells were pre-treated with MG132 (0.5 μ M) for 1 h and then treated with celastrol for 24 h. Matrigel was purchased from BD Biosciences (San Diego, CA, USA). Primary antibodies for western blotting were as follows: Antibodies against p-I κ B α (Ser 32) (sc-7977), I κ B α (sc-203), p65 (sc-109), poly ADP ribose polymerase (PARP) (sc-1562), MMP-2 (sc-53630), MMP-7 (sc-8832) and MMP-9 (sc-21733) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-actin antibody (A5441) was obtained from Sigma-Aldrich (Merck KGaA).

Cell proliferation assay. Cell growth was measured by MTT assay (24). Briefly, OVCAR-3 and SKOV-3 cells (5x10³ cells/well) were cultured with serially diluted celastrol

(0.125, 0.25, 0.5, 1 and 2 μ M) in 96-well plates. Control cells were incubated with DMSO in fresh medium. Following incubation for 96 h, the medium was removed and the cells were incubated with 20 μ l MTT solution (5 mg/ml) at 37°C for 4 h. The MTT was then removed, and 100 μ l DMSO was added to each well. After 15 min, the absorbance of each well was measured with a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 570 nm. The viable cell number was proportional to the absorbance. All assays were performed in triplicate.

Wound healing assay. Cell motility was determined using a wound-healing scratch assay. Briefly, OVCAR-3 cells were seeded in a 6-well plate (5x10⁴ cells/well) and grown until confluency. The confluent monolayer was scratched using a sterilized 1-ml pipette tip and incubated with celastrol (0.125, 0.25 and 0.5 μ M) in fresh growth medium for 24 h. The movement of cells to the denuded area was photographed.

Transwell assays. For the migration assay, SKOV-3 cells (5x10³/insert) in DMEM with 10% FBS (Hyclone; GE Healthcare Life Sciences) were seeded into a commercial Transwell insert (membrane pore size, 8 μ m) and incubated with celastrol (0.125, 0.25 and 0.5 μ M). After 24 h, cells that had migrated to the bottom of the filter were stained with crystal violet and counted under a light microscope. For the invasion assay, OVCAR-3 cells were tested using a Transwell insert pre-loaded with Matrigel (BD Biosciences). Inserts were incubated with serum-free DMEM at 37°C for 2 h to allow rehydration of Matrigel. Cells suspended in serum-free DMEM were then loaded onto the top chamber (1x10⁴/insert), and celastrol was added to both upper and lower chambers with equal concentrations (0.125, 0.25 and 0.5 μ M). Complete DMEM with 10% FBS was used in the lower chamber as a chemo-attractant. After 24 h of incubation, the Matrigel was removed and the inserts were stained. Invaded cells on the underside of the filter were counted.

Western blotting. The protocol for western blotting was as previously reported (25) with minor modifications. Following treatment with celastrol, OVCAR-3 cells were harvested and lysed in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.25% sodium deoxycholate and 1 mM EDTA with protease inhibitor cocktail, 1 mM NaF and 1 mM Na₃VO₄] at 4°C for 20 min. Whole cell lysates were subjected to protein quantification by Bradford assay. Equal amounts (10 μ g/lane) of lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and then electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). The membrane was probed with primary antibodies against p-I κ B α (Ser 32) (1:200), I κ B α (1:200), p65 (1:200), PARP (1:200), MMP-2 (1:100), MMP-7 (1:100), MMP-9 (1:100) or Actin (1:5,000) at 4°C overnight, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG1-HRP, ab193651; anti-rabbit IgG1-HRP, ab191866; Abcam, Shanghai, China) with 1:5,000 dilution at room temperature for 1 h, and detected with an enhanced chemiluminescence substrate (Amersham; GE Healthcare Life Sciences).

Cytosol-nuclei fractionation. To detect the subcellular distribution of NF- κ B proteins, cytoplasmic and nuclear compartments of the cells were prepared as described previously (26). Briefly, following treatment with celastrol, OVCAR-3 cells (3×10^6) were suspended in hypotonic lysis buffer (10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and protease inhibitors) and incubated at 4°C for 15 min. Nonidet P-40 (10%) was then added to a final concentration of 0.5%. Immediately after NP-40 addition, samples were vortexed and centrifuged at 13,000 \times g for 1 min, and supernatant (cytosolic extract) was collected. For the precipitation, Laemmli lysis buffer (Santa Cruz Biotechnology, Inc.) was used to reconstitute the pellet followed by ultrasonic homogenization on ice to generate the nuclear extract. Subcellular proteins were quantified by Bradford assay and employed for western blot analysis.

Gelatin zymography. OVCAR-3 cells were treated with celastrol (0.25 or 0.5 μ M) for 24 h. Supernatants were collected and loaded on a 10% SDS-PAGE gel with gelatin (1 mg/ml). Following electrophoresis, the gels were washed for 15 min in 2.5% Triton X-100 and then incubated overnight in the same buffer at room temperature. After washing with deionized water, the gels were incubated for an additional 24 h at 37°C in a calcium-zinc renaturation buffer (1610765; Bio-Rad Laboratories, Inc.). The gel was stained with Coomassie blue R-250. Clear bands appearing at the expected locations for matrix metalloproteinase (MMP)-9 and MMP-2 on the basis of molecular weight markers were visualized using a transilluminating densitometer. The number of pixels per band was used to determine the enzyme activity in each group. The zymography experiment was repeated three times with independent samples.

Statistical analysis. All data are expressed as the mean \pm standard deviation (SD). Inter-group analyses were performed using analysis of variance with Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Anti-proliferative effect of celastrol on ovarian cancer cells. The impact of celastrol on cell proliferation was investigated. SKOV3 and OVCAR-3 ovarian cancer cells were treated with serial concentrations of celastrol and cell proliferation was tested by MTT assay. Celastrol markedly inhibited cell proliferation when concentrations $\geq 1 \mu$ M were used in the two cell lines, while at $< 1 \mu$ M, the drug yielded only modest effects (Fig. 1). In order to exclude the antiproliferative effect of celastrol from its effects on cell migration and invasion, drug concentrations $< 1 \mu$ M were used for subsequent experiments.

Celastrol inhibits ovarian cancer cell migration. The effect of celastrol on cell movement was evaluated by wound-healing scratch assay and Transwell assays. When used at subtoxic concentrations (0.125-0.5 μ M), celastrol clearly decreased the number of OVCAR-3 cells that moved into the denuded area (Fig. 2A). Consistent with this, in the Transwell migration

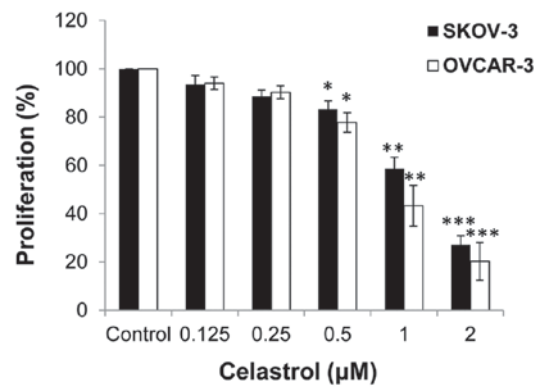


Figure 1. Effect of celastrol on cell proliferation. SKOV-3 and OVCAR-3 cells were seeded into a 96-well plate and treated with various concentrations of celastrol. Cell viability was determined by MTT assay after 96 h incubation. Results are presented as the mean \pm standard deviation ($n=3$). $^*P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ vs. untreated control.

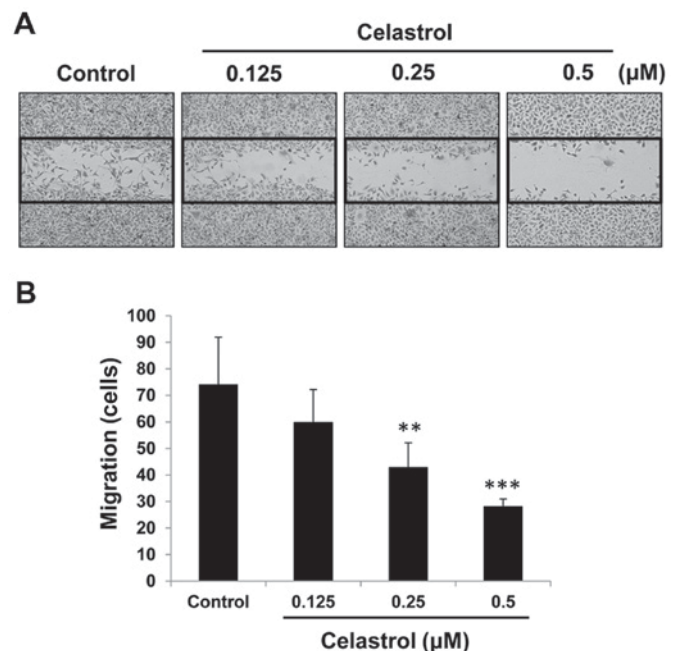


Figure 2. Effect of celastrol on the cell migration of ovarian cancer cells. (A) Confluent OVCAR-3 cell monolayers were scratched and treated with celastrol. After 24 h, the migration of cells into the denuded area was visualized. Typical images of cell migration are shown (original magnification, $\times 50$). Data are from three independent experiments. (B) SKOV-3 cells were seeded into Transwell inserts (5×10^3 /insert) with or without celastrol. The underside of the filter was stained 24 h after cell loading. Migrating cells were scored from 10 random fields (original magnification, $\times 100$). Results are presented as the mean \pm standard deviation ($n=10$). $^{**}P < 0.005$ and $^{***}P < 0.001$ vs. untreated control.

assay, the number of SKOV-3 cells that passed through the Transwell membrane was significantly reduced by celastrol in a concentration-dependent manner (Fig. 2B).

Celastrol suppresses ovarian cancer cell invasion. The effect of celastrol on the invasive capacity of OVCAR-3 cells was investigated. In a Matrigel-coated Transwell assay, celastrol at concentrations of 0.25 and 0.5 μ M significantly inhibited cell invasion (Fig. 3). The inhibition rates were 39.6 ± 5.2 and $51.7 \pm 3.5\%$ for 0.25 and 0.5 μ M celastrol, respectively.

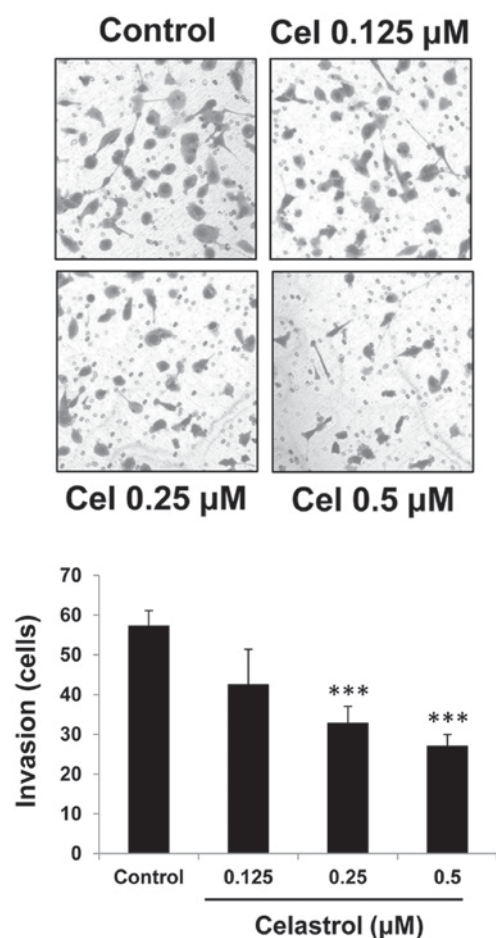


Figure 3. Effect of celastrol on ovarian cancer cell invasion. OVCAR-3 cells were seeded into Matrigel-coated Transwell inserts (1×10^4 /insert) with or without celastrol in the serum-free medium. Complete medium was loaded in the bottom chamber as a chemo-attractant. The underside of the filter was stained 24 h after cell loading. Invading cells were scored from 8 random fields (original magnification, $\times 100$). Representative images are shown. Quantified data are from three independent experiments. Results are presented as the mean \pm standard deviation ($n=6$). *** $P<0.001$ vs. untreated control. Cel, Celastrol.

Celastrol strongly blocks the NF- κ B pathway. To elucidate the molecular mechanisms by which celastrol affects ovarian cancer cells, proteins in the NF- κ B pathway were analyzed, since this pathway is indicated to be one of the predominant mechanisms underlying the antitumor efficacy of celastrol (27). As shown in Fig. 4, celastrol clearly inhibited p-I κ B α (S32) and induced I κ B α accumulation in the cytosol, and consistent with this, reduced nuclear p65 recruitment in OVCAR-3 cells, suggesting blockade of the canonical NF- κ B pathway. The levels of cytosolic p65 and nuclear I κ B α were undetectable, with or without drug treatment, indicating that celastrol did not affect the translocation of these proteins. Notably, nuclear-specific protein PARP was undetectable in the cytosolic compartment, indicating the precision of the cytosol-nuclei fractionation.

MMP-9 expression and activity are reduced by celastrol. NF- κ B controls the gene expression of many proteolytic enzymes involved in cell invasion and metastasis, such as urokinase-type plasminogen activator and MMP-9 (5). To test whether celastrol impacts the expression of MMPs, three typical MMPs (MMP-2, MMP-7 and MMP-9) were detected in OVCAR-3 cells following

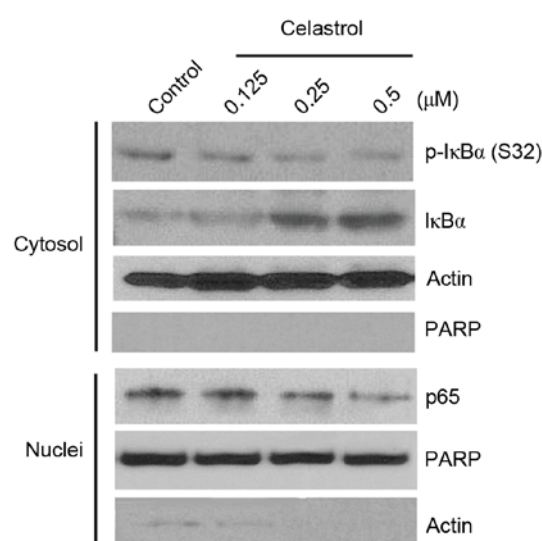


Figure 4. Effect of celastrol on the nuclear factor- κ B pathway. OVCAR-3 cells were treated with celastrol for 6 h. Cytosolic and nuclear extracts were subjected to western blotting with the indicated antibodies. Cytosolic extract was probed with the antibody against I κ B α , with actin as a loading control, while the nuclear compartment was detected by an anti-p65 antibody, with PARP as a loading control. PARP, poly ADP ribose polymerase; p, phosphorylated.

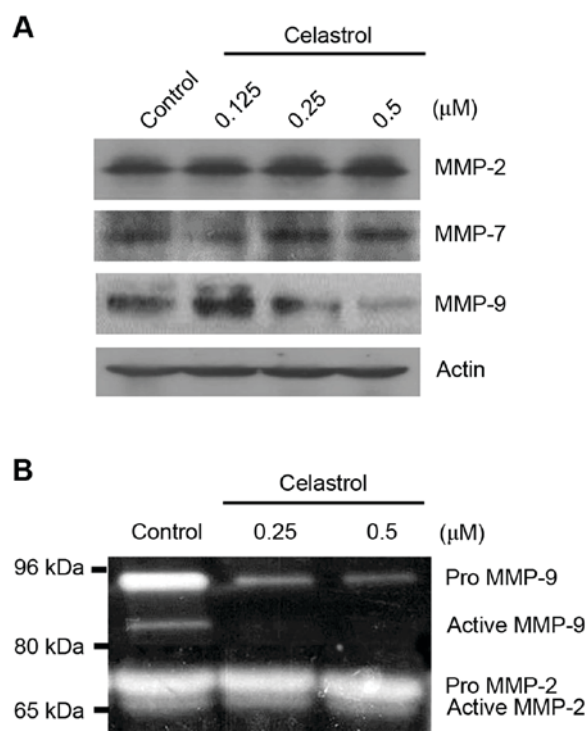


Figure 5. Effect of celastrol on MMP expression and activity. (A) OVCAR-3 cells were treated with celastrol for 24 h. Whole cell lysates were processed for western blotting with primary antibodies against MMP-2, -7 and -9. Actin was used as a loading control. (B) Representative zymographic image showed reduced intensity of gelatin bands associated with active MMP-9 (92 kDa) but not MMP-2 (72 kDa). MMP, matrix metalloproteinase.

celastrol treatment. MMP-9 protein was clearly inhibited by celastrol, particularly at higher concentrations (Fig. 5A). By contrast, no inhibition of MMP-2 and MMP-7 was observed (Fig. 5A). Furthermore, in the zymography assay, the expression

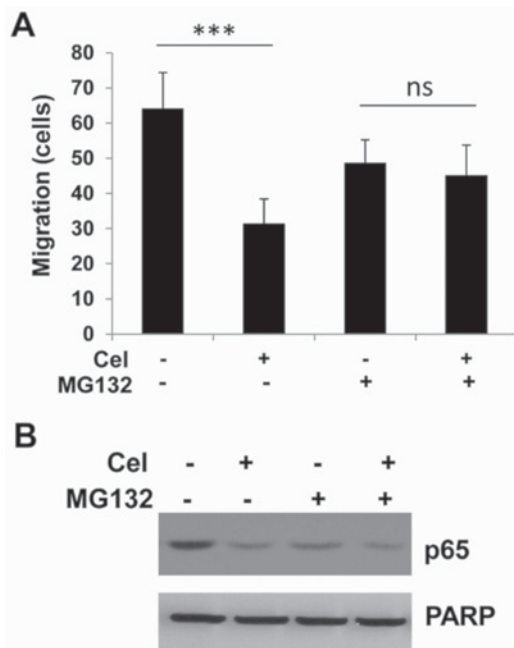


Figure 6. Impact of the NF- κ B inhibitor MG132 on celastrol-induced effects. OVCAR-3 cells were pre-treated with MG132 (0.5 μ M) for 1 h and then treated with celastrol (0.5 μ M) for 24 h. (A) Cells were seeded into Transwell inserts (5x10³/insert) in triplicate and the filter membrane was stained 24 h after cell seeding. Migrating cells were scored from 10 random fields (original magnification, x100). Results are presented as the mean \pm standard deviation (n=10). ***P<0.001 vs. untreated control. ns, not significant. (B) Expression of p65 in the nuclear compartment after subcellular fractionation was detected using western blotting, with PARP as a loading control. PARP, poly ADP ribose polymerase; cel, celastrol; ns, not significant.

of gelatin (the substrate of MMP-2 and MMP-9), was decreased at 92 kDa (for Pro MMP-9) and 83 kDa (for active MMP-9), but not 72 kDa (for Pro MMP-2) and 66 kDa (for active MMP-2) by celastrol treatment, suggesting that celastrol inhibits the proteolytic activity of MMP-9, but not that of MMP-2 (Fig. 5B).

NF- κ B pathway inhibitor MG132 attenuates celastrol-induced cell migration and p65 inhibition. In order to investigate whether the mechanism for the inhibition of migration and invasion by celastrol involves NF- κ B, the proteasome inhibitor MG132 was used as an NF- κ B inhibitor; MG132 is widely used as a tool to study NF- κ B signaling and associated molecular mechanisms (28). As shown in Fig. 6A, both celastrol and MG132 inhibited cell migration when applied alone. However, when these two agents were used together, the celastrol-induced suppression was not further increased by MG132; the celastrol-induced movement of tumor cells was attenuated by MG132. Consistently, at the molecular level, MG132 together with celastrol did not show stronger p65 suppression compared with either celastrol or MG132 alone, suggesting that the celastrol-mediated functions proceed via the same pathway as MG132, that is, through NF- κ B pathway (Fig. 6B).

Discussion

Celastrol has been shown to have anti-invasive and antimetastatic activities in preclinical models of prostate cancer (17), breast cancer (19), colon cancer and pancreatic cancer (29). The current study provides new evidence for the inhibitory

effect of celastrol on certain functions of ovarian cancer cells. The *in vitro* data indicate that celastrol may be a suitable candidate for preventing tumor cell migration and invasion in ovarian cancer. Furthermore, such functional consequences are associated with blockade of the NF- κ B/MMP-9 pathway, which is widely associated with malignancy and aggressiveness (30,31).

Natural medicines have been studied in cancer management for many years, usually in combination with traditional interventions. Products from the plant *Tripterygium wilfordii*, including celastrol and triptolide, have exhibited impressive anticancer activities in a variety of cancer models, and therefore are amongst the traditional herb medicines considered to have the most potential in modern cancer therapy (11). For the treatment of ovarian cancer, while very limited studies have focused on celastrol, triptolide, which exhibits similar biological activities to celastrol, has been demonstrated to exert efficacy in preclinical models (32). Ou *et al* reported that triptolide effectively inhibits ovarian cancer cell proliferation by blocking the HER2/PI3K/Akt/NF- κ B pathway (33). In addition, triptolide has been shown to potently inhibit the migration and invasion of ovarian cancer cells, and such suppression is strongly associated with the reduced transcription and translation of several MMPs (34). The results of the present study indicate that celastrol, an analog of triptolide, impairs ovarian cancer cell migration and invasion, and inhibits the NF- κ B/MMP-9 pathway; such molecular findings are consistent with previous studies in other cancers (7,19). Together, these findings provide information useful for future studies on these active ingredients from the plant *Tripterygium wilfordii*, in cancer treatment.

Various molecular mechanisms have been described for the antitumor effects of celastrol. While many signaling pathways are highly associated with survival and apoptosis, NF- κ B is the predominant pathway that plays a pleiotropic role in controlling multiple cell functions, including proliferation, survival, cell death, invasion and angiogenesis (5). The NF- κ B pathway serves as one of the major mechanisms underlying the cancer-killing effects of natural compounds.

In the current study, the results indicated that the NF- κ B pathway may be an important in the functional changes induced by celastrol. In particular, they suggest that celastrol may inhibit the classical NF- κ B pathway by preventing I κ B α degradation, blocking p65 translocation and suppressing MMP-9 expression downstream of NF- κ B. These results are consistent with those of previous studies (18,19,27), although other studies have suggested different mechanisms for the effects of celastrol on invasiveness (22,35-38). It should be noted that alternative mechanisms to the NF- κ B pathway are also likely to contribute to the effects of celastrol on migration and invasion in the current models. For example, Rac/Rho GTPase is a key player involved in cell skeletal rearrangement and cell polarity, while the Src/FAK axis is a major pathway controlling focal adhesion, cell movement and invasion (39). Notably, celastrol is able to suppress the constitutive phosphorylation of Src kinase at least in multiple myeloma cells (40), suggesting that Src may serve as another potential molecular contributor. Future studies will focus on detailed delineation of the signaling pathways interrupted by celastrol.

In summary, the results of the present study illustrate the potential of celastrol for impairing tumor invasiveness in ovarian cancer, therefore broadening the possible applications of this natural agent. The results of these *in vitro* experiments supports the use of celastrol as a potential clinical intervention modality for preventing and delaying ovarian cancer metastasis. Therefore, celastrol warrants further preclinical investigation.

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