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Amentoflavone Inhibits Metastatic Potential Through Suppression of ERK/NF-κB Activation in Osteosarcoma U2OS Cells

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Abstract. *Aim: The study goal was to investigate effect of amentoflavone on nuclear factor-κB (NF-κB)-modulated metastatic mechanism in osteosarcoma U2OS cells. U2OS cells were treated with amentoflavone, NF-κB inhibitor, protein kinase B (PKB or AKT) inhibitor or mitogen-activated protein kinase (MAPK) inhibitor. Change of cell viability, NF-κB activation, expression of metastasis-associated proteins, signal transduction, and cell migration and invasion were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, NF-κB reporter gene assay, western blotting, and cell migration and invasion assays. The results demonstrated that inhibition of activation of extracellular signal-regulated kinases (ERK) was a key point for suppression of NF-κB-modulated metastatic mechanism. Amentoflavone significantly inhibited NF-κB activation, ERK phosphorylation, expression of metastasis-associated proteins, and cell migration and invasion. Our findings indicate that amentoflavone reduces metastatic potential through suppression of ERK and NF-κB activation in osteosarcoma U2OS cells.*

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Osteosarcoma, the most common type of malignant bone tumor, occurs primarily in children and adolescents, with a second peak of incidence among adults over the age of 50 years (1). The overall survival of patients with osteosarcoma has improved with current treatment methods such as chemotherapy, surgery, and radiotherapy. The 5-year survival rate for patients with localized osteosarcoma is about 65-75% after treatment. Patients with metastatic osteosarcoma often have unsatisfactory response to chemotherapy or radiotherapy and the resultant 5-year survival rate is only 10-20% (2). The majority of patient mortality is due to relentless progression of metastasis (3). Therefore, a treatment strategy focusing on combating metastasis may be beneficial in prolonging disease control.

Cancer metastasis is a multistep process by which cancer cells spread from the primary site distantly to form a secondary tumor (4). Metastasis is modulated by metastasis-associated proteins such as vascular endothelial growth factor (VEGF), urinary plasminogen activator (uPA), matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9). Angiogenesis, or the formation of new blood vessels, is regulated by expression of VEGF and prerequisite for cancer metastasis (5). When uPA, a serine protease, binds to its receptor (uPAR), it can promote conversion of plasminogen to plasmin, which degrades the extracellular matrix (ECM) and activates MMP2 (72 kDa gelatinase A) and MMP9 (92 kDa gelatinase B). ECM degradation has been implicated in cancer invasion and metastasis (3, 6, 7).

Nuclear factor-κB (NF-κB) is an important modulator in tumor progression. Activation of NF-κB contributes to cancer metastasis through inducing expression of metastasis-associated proteins (8). Tang *et al*. found patients whose osteosarcoma had active NF-κB had short median overall survival time as compared to patients whose osteosarcoma had inactive NF-κB (9). Many chemotherapeutic agents, and

radiation, can induce cell-cycle arrest and apoptosis while activating NF-κB signaling, which may hinder treatment effects (10, 11). Inhibitors of NF-κB signaling suppress expression of NF-κB-modulated metastasis-associated proteins and lead to blockage of the metastatic mechanism in osteosarcoma cells, both *in vitro* and *in vivo* (12,13). Therefore, development of an inhibitor of NF-κB signaling as an adjuvant agent may be of potential to help patients with osteosarcoma.

Amentoflavone, a flavonoid extracted from *Selaginella tamariscina*, has been shown to inhibit cell invasion and metastasis *via* suppression of NF-κB activation in breast cancer and melanoma cells both *in vitro* and *in vivo* (14,15). However, whether amentoflavone induces anti-metastatic effect through suppression of NF-κB activation in osteosarcoma cells is unknown. The goal of the present study was to investigate effect of amentoflavone on NF-κB-modulated metastatic mechanism in osteosarcoma cells. Osteosarcoma U2OS cells were used for this study. The effect of amentoflavone on cell viability, NF-κB activation, expression of metastasis-associated proteins, and cell invasion were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, NF-κB reporter gene, western blotting, and matrigel invasion assay. We also verified the role of NF-κB, protein kinase B (PKB or AKT), and mitogen-activated protein kinase (MAPK) signaling inhibition in anti-metastatic mechanism.

Materials and Methods

Chemicals. Amentoflavone and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). McCoy's 5A medium was obtained from Hyclone, GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin (PS) were purchased from Gibco/Life Technologies (Carlsbad, CA, USA). NF-κB inhibitor 4-*N*-[2-(4-phenoxyphenyl) ethyl] quinazoline-4,6-diamine (QNZ), AKT inhibitor LY294002, c-Jun *N*-terminal kinase (JNK) inhibitor SP600125, P38 inhibitor SB203580, and extracellular signal-regulated kinase (ERK) inhibitor PD98059 were bought from Selleckchem (Houston, TX, USA). Primary antibodies of VEGF, MMP-9, and pERK were bought from Merck Millipore (Billerica, MA, USA). Primary antibodies to MMP2 and uPA were purchased from OriGene Technologies (Rockville, MD, USA) and Abbiotec (San Diego, CA, USA), respectively. Primary antibody to β-actin was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). D-Luciferin and hygromycin B were bought from Caliper (Hopkinton, MA, USA) and Santa Cruz Biotechnology, respectively. Matrigel was purchased from Corning (Tewksbury, MA, USA). jetPEI™ transfection agent was obtained from Polyplus Transfection (Illkirch, Bas-Rhin, France).

Cell culture. The human osteosarcoma U2OS cells were generously provided by Professor Jing-Gung Chung at Department of Biological Science and Technology, China Medical University, Taichung, Taiwan. Cells were maintained in McCoy's 5A medium supplemented with 10% FBS, 2 mM L-glutamine, and PS (100 Units/ml and 100 μg/ml) and grown at 37˚C in a humidified incubator with 5% CO_2 atmosphere (13).

MTT assay. U2OS cells were seeded into 96-well plates with 2×10^4 cells/well and cultured overnight. Cells were then treated with amentoflavone (50, 75, and 100 μM), QNZ (EVP4593 or NF-κB inhibitor; 0.3, 0.5, 1, 2, and 3 μM), LY294002 (AKT inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), PD98059 (ERK inhibitor, 10 μM), and SB203580 (P38 inhibitor, 10 μM), respectively, for 24 and 48 h. After treatments, cell viability was evaluated with MTT assay as described by Chiang *et al*. (16).

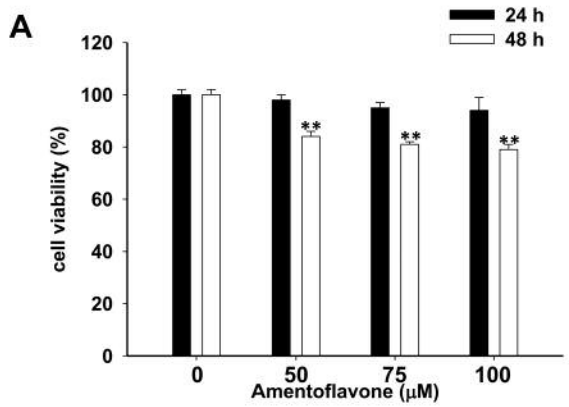
Plasmid transfection. NF-κB-luciferrase2 vector (pNF-κB/*luc2*) was purchased from Promega (Madison, WI, USA). U2OS cells were transfected with pNF-κB/*luc2* using jetPEI™ . Cells (1×10^6) were seeded into 10 cm dish and cultured overnight. Transfection procedure was as described by Tsai *et al*. (17).

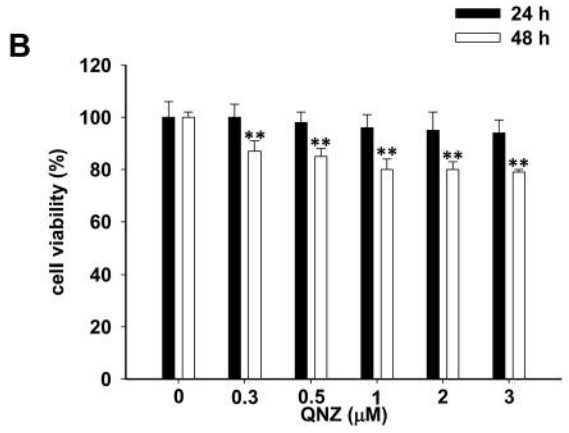
NF-κB reporter gene assay. U2OS cells transfected with pNF-κB/*luc2* were seeded into 96-well plates with 2×10^4 cells/well and cultured overnight. Cells were then treated with amentoflavone (50, 75, and 100 μM), QNZ (EVP4593 or NF-κB inhibitor; 0.3, 0.5, 1, 2, and 3 μM), LY294002 (AKT inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), PD98059 (ERK inhibitor, 10 μM), and SB203580 (P38 inhibitor, 10 μM), respectively, for 24 and 48 h. D-luciferin solution [500 μM D-luciferin in 100 μl phosphate-buffered saline (PBS)] was added to each well for 1 min before imaging. The photon signal from cells was acquired for 1 min using IVIS200 Imaging System (Xenogen, Alameda, CA, USA). After normalizing data with cell viability obtained by MTT assay, relative NF-κB activity was determined as described previously (18).

Western blotting assay. U2OS cells (3×10^6) were seeded into 10 cm diameter dishes, cultured overnight, and then treated with different concentrations (75, 100 μM) of amentoflavone, 3 μM QNZ, or 10 μM PD98059, respectively, for 24 h. Lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM phenylmethanesulfonyl fluoride) was used to extracted total cell protein from each treatment group. Protein levels of uPA, VEGF, MMP2, and MMP9 were evaluated with western blotting assay as described by Ting *et al*. (19). Protein bands were quantified by using ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD, USA).

Migration assay. Transwell inserts (8 μm pore size) were obtained from Corning (Tewksbury, MA, USA). A total of 3×10^6 U2OS cells were resuspended with 1 ml serum-free McCoy's 5A medium and treated with 100 μM amentoflavone, 3 μM QNZ, or 10 μM PD98059, respectively. Cell suspension (100 μl) was placed in the apical chamber of the transwell insert and cells were incubated for 24 h. McCoy's 5A medium with 10% serum was added to the basolateral chamber. After treatment, the migrated cells in the permeable membrane of transwells were fixed with mixture of methanol and acetic acid in a ratio (3:1) for 15 min and then stained with 0.5% crystal violet staining solution. Fixed cells were photographed under a light microscope at ×100 and then counted using ImageJ software version 1.50 (National Institutes of Health) (20).

Invasion assay. Transwells (8 μm pore size) were coated with 50 μl matrigel solution (25 μl matrigel in 25 μl serum-free McCoy's 5A





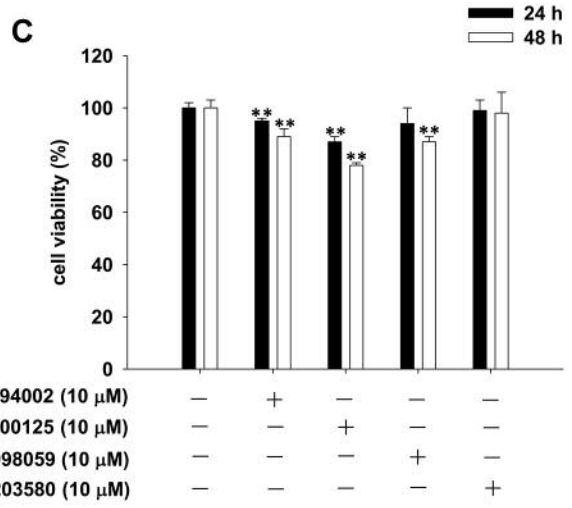
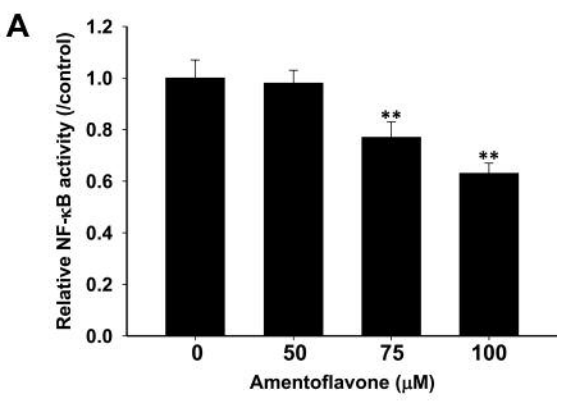
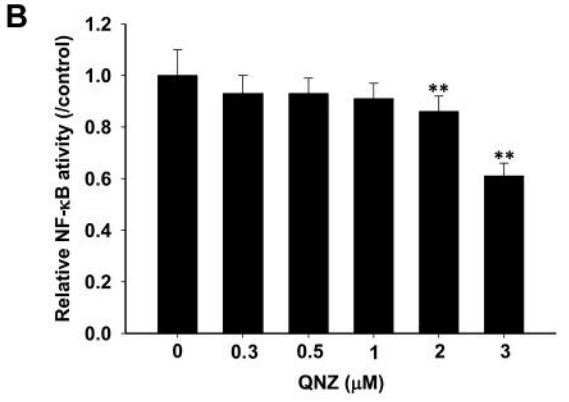


Figure 1. *Effects of amentoflavone, nuclear factor-κB (NF-κB) inhibitor, protein kinase B (PKB or AKT) inhibitor, and mitogen-activated protein kinase (MAPK) inhibitors on cell viability in U2OS cells. Cell viability 24 and 48 h after treatment with amentoflavone (A), QNZ (EVP4593 or NF-κB inhibitor) (B), and LY294002 (AKT inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor), PD98059 (extracellular signal-regulated kinases inhibitor), and SB203580 (P38 inhibitor) (C) as evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Significantly different at p<0.01 compared to the control (0 μM).*





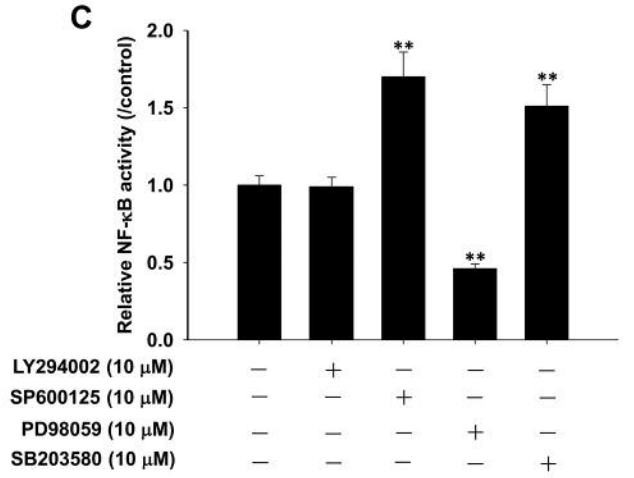


Figure 2. *Effects of amentoflavone, nuclear factor-κB (NF-κB) inhibitor, protein kinase B (PKB or AKT) inhibitor, and mitogen-activated protein kinase (MAPK) inhibitors on NF-κB activation in U2OS cells. Twenty-four and 48 h after treatment with amentoflavone (A), QNZ (EVP4593 or NF-κB inhibitor) (B), and LY294002 (AKT inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor), PD98059 (extracellular signal-regulated kinases inhibitor), and SB203580 (P38 inhibitor) (C) as evaluated with NF-κB reporter gene assay. **Significantly different at p<0.01 compared to the control (0 μM).*

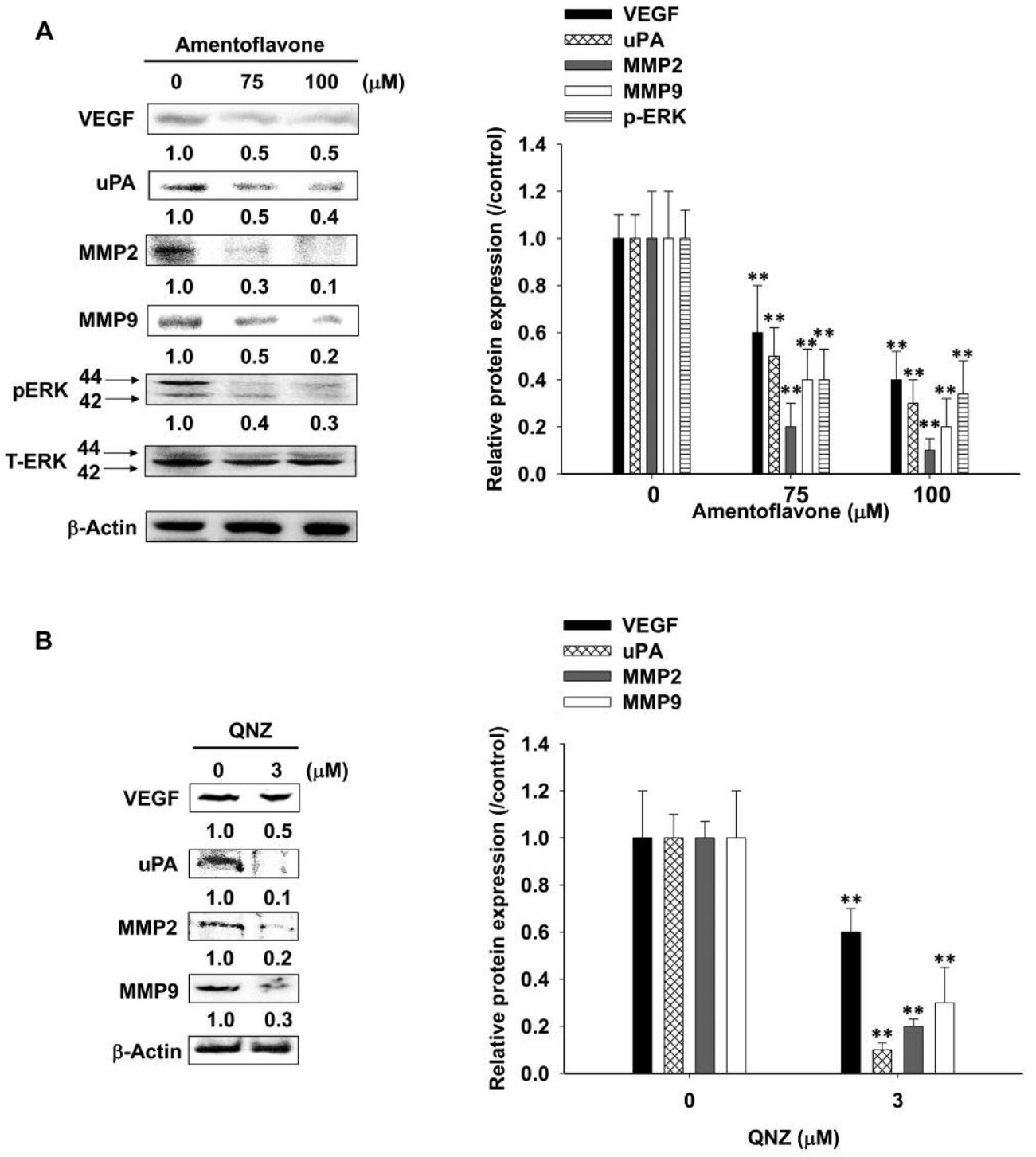


Figure 3. *Continued*

medium) and incubated overnight at 37˚C in a humidified incubator. U2OS cells (3×10^6) were resuspended with 1 ml serum-free McCoy's 5A medium and then treated with 100 μM amentoflavone, 3 μM QNZ, or 10 μM PD98059, respectively. Cell suspension (100 μl) was placed in the apical chamber of the transwell insert and cells were incubated for 24 h. McCoy's 5A medium with 10% serum was added to the basolateral chamber. After treatment, a sterile cotton swab was used to remove matrigel on the permeable membrane of the transwell. The invasive cells in the permeable membrane were fixed with a mixture of methanol and acetic acid (3:1) for 15 min and then stained with

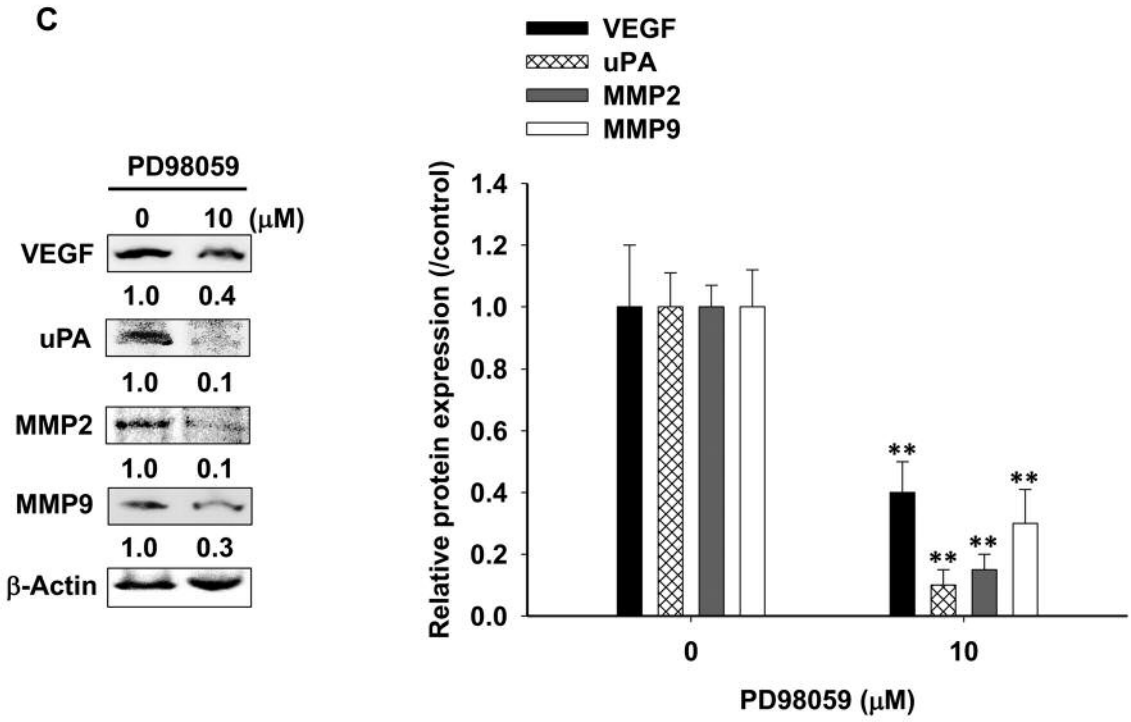


Figure 3. *Effects of nuclear factor-κB (NF-κB) inhibitor, extracellular signal-regulated kinases (ERK) inhibitor, and amentoflavone on expression of metastasis-associated proteins in U2OS cells. Cells were treated with different concentrations of amentoflavone (A), QNZ (EVP4593 or NF-κB inhibitor) (B), and PD98059 (ERK inhibitor) (C) for 24 h. Proteins expression of vascular endothelial growth factor (VEGF), urinary plasminogen activator (uPA), matrix metalloproteinase-2 (MMP2), MMP9, and phosphorylated ERK 24 h after treatment were analyzed with western blotting assay. **Significantly different p<0.01 as compared to the control (0 μM).*

0.5% crystal violet staining solution. Fixed cells were photographed under a light microscope at ×100 and then counted using ImageJ software version 1.50 (National Institutes of Health) (21).

Statistical analysis. Student's *t*-test was used to test the significance of differences in mean values between different groups. *p*-Values of 0.05 or less were considered statistically significant. Each experiment was carried out three times.

Results

Effect of amentoflavone, NF-κB inhibitor, AKT inhibitor, and MAPK inhibitor on viability of U2OS cells. Figure 1A indicates that amentoflavone induced cytotoxicity in a time-dependent manner. Cell viability remained relatively unchanged when cells were treated with different concentrations (50, 75, and 100 μM) of amentoflavone for 24 h. After treatment with amentoflavone at the same concentration for 48 h, cell viability was significantly reduced as compared to the control. Figure 1B indicates that QNZ inhibited cell growth in a time-dependent manner. Cell viability remained unchanged when cells were treated with different concentrations (0.3, 0.5, 1, 2, and 3 μM) of QNZ for 24 h. After treatment with QNZ for 48 h, cell viability was significantly decreased as compared to the control. Figure 1C showed that cell viability significantly decreased after treatment with LY294002 or SP600125 for 24 h as compared to the control. In addition, after treatment with 10 μM LY294002, SP600125, PD98059, or SB203580 for 48 h, only SB203580 did not significantly influence cell viability.

Amentoflavone, NF-κB inhibitor, and ERK inhibitor reduce NF-κB activation in U2OS cells. Figure 2A shows that amentoflavone treatment at 75 and 100 μM significantly inhibited NF-κB activation by 23-37% as compared to the control. Figure 2B shows QNZ treatment at 2 and 3 μM significantly reduced NF-κB activation by 14-39% as compared to the control. Figure 2C indicates that after treatment with 10 μM AKT, JNK, ERK, or P38 inhibitor for 24 h, only ERK inhibitor (PD98059) significantly inhibited NF-κB activation, by 50% as compared to the control, and can be referred to as a NF-κB signal inhibitor.

Amentoflavone, NF-κB inhibitor, and ERK inhibitor suppress expression of metastasis-associated proteins in U2OS cells. QNZ and PD98059 were used to verify the effect of NF-κB and ERK inactivation on expression of metastasis-associated

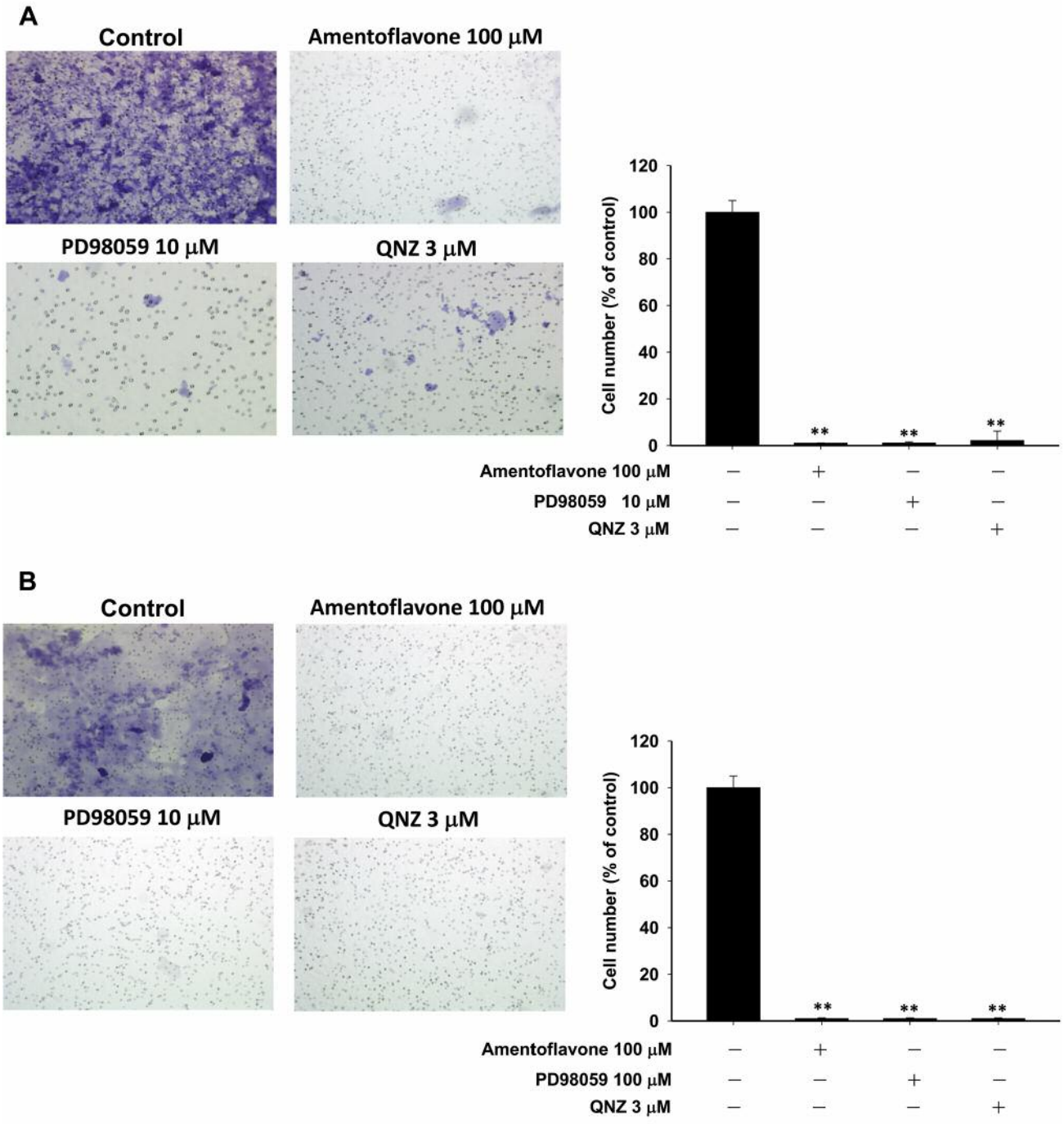


Figure 4. *Effect of treatment for 24 h with amentoflavone, QNZ (EVP4593 or nuclear factor-κB inhibitor), and PD98059 (extracellular signal-regulated kinases inhibitor) on cell migration and invasion by U2OS cells. A: Numbers of migrated cells were evaluated with migration assay. B: Numbers of invading cells were evaluated with matrigel invasion assay. **Significantly different at p<0.01 as compared to the control (0 μM).*

proteins in U2OS cells. We found amentoflavone treatment (75 and 100 μM) significantly reduced expression of metastasis-associated proteins VEGF, uPA, MMP2, and MMP9 and ERK phosphorylation by 60-90% as compared to the control (Figure 3A). Figure 3B and C show that treatment with 3 μM QNZ or 10 μM PD98059 significantly inhibited expression of VEGF, uPA, MMP2, and MMP9 by 40-90% as compared to the control.

Amentoflavone, NF-κB inhibitor, and ERK inhibitor prevent cell migration and invasion by U2OS cells. Amentoflavone, PD98059, and QNZ all significantly prevented cell migration and invasion of U20S cells by 95-98% (Figure 4A and B) compared to the control.

Discussion

Expression of metastasis-associated proteins, cell invasion and metastasis are linked to NF-κB activation in osteosarcoma. Liao *et al*. used shRNA to knockdown NF-κB in order to abolish cell invasion and metastasis in osteosarcoma both *in vitro* and *in vivo* (12). In this study, we also used QNZ (NF-κB inhibitor) to suppress NF-κB activation leading to inhibition of expression of metastasis-associated proteins, cell migration, and cell invasion in U2OS cells (Figure 2B, 3B and 4). AKT and MAPK signaling have been proposed to regulate NF-κB activation and metastasis in osteosarcoma cells. Some anticancer agents diminish NF-κB-modulated mechanism of metastasis by inhibiting AKT and MAPK signaling in osteosarcoma cells (22-24). Zhu *et al*. found inhibition of AKT/NF-κB activation reduced cell invasion and migration by osteosarcoma cells *in vitro* (22). We used AKT and MAPK inhibitors to investigate the role of AKT and MAPK signaling in NF-κB-modulated mechanism of metastasis in U2OS cells. We found only the ERK inhibitor PD98059 reduced NF-κB activation. We also found PD98059 inhibited expression of metastasis-associated proteins, cell invasion and metastasis (Figures 3C and 4). Based on the above data we suggest blockage of ERK/NF-κB activation leads to inhibition of metastasis-associated protein expression, cell migration, and cell invasion in U2OS cells. Li *et al*. also found down-regulation of ERK/NF-κB pathway resulted in inhibition of invasive properties in U2OS cells (24).

ERK (MAPK) plays a major role in the hallmarks of cancer, which include evasion of apoptosis, tumor growth, angiogenesis, and metastasis (25). Na *et al*. demonstrated overexpression of phosphorylated ERK was associated with poor prognosis in patients with osteosarcoma (26). Inhibition of ERK phosphorylation impedes cell invasion and metastatic potential in osteosarcoma (25). Sorafenib (Nexavar), an oral multikinase inhibitor of platelet-derived growth factor (PDGF), VEGF receptor, and rapidly accelerated fibrosarcoma kinases, is indicated for use in patients with recurrent high-grade osteosarcoma based on a phase II clinical trial with a 14% overall tumor response rate, 49% disease control rate, and expected 4-month progression-free survival (27,28). Pignochino *et al*. showed that sorafenib reduced protein expression of VEGF, MMP2, and pERK in different types of osteosarcoma cells (27). Our results show amentoflavone inhibited NF-κB activation, ERK phosphorylation, expression of metastasis-associated proteins, cell migration, and cell invasion. In our previous study, amentoflavone induced anti-metastatic effect through suppression of NF-κB activation (14). Therefore, we suggest amentoflavone reduces metastatic potential through suppression of ERK/NF-κB signaling pathway (Figure 5) in osteosarcoma U2OS cells.

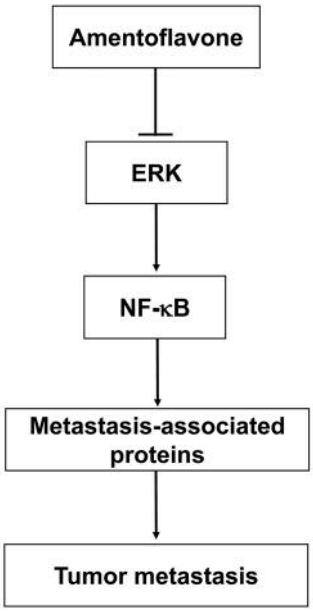


Figure 5. *The suggested mechanism of action for amentoflavone reduction of metastatic potential through suppression of nuclear factor-κB extracellular signal-regulated kinases (ERK)/nuclear factor-κB (NF-κB) activation in U2OS osteosarcoma cells*.

Conclusion

This study indicated amentoflavone inhibited expression of metastasis-associated proteins, cell migration, and cell invasion through suppression of ERK/NF-κB signaling pathway in osteosarcoma U2OS cells. We propose the potential application of amentoflavone for patients with osteosarcoma as adjuvant treatment.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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