

Antitumor effects of the novel quinazolinone MJ-33: Inhibition of metastasis through the MAPK, AKT, NF- κ B and AP-1 signaling pathways in DU145 human prostate cancer cells

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Abstract. Quinazolinone compounds have been shown to have antitumor activity in many human cancer cell lines. In the present study, we investigated the anti-metastatic activity of MJ-33 (2-(3-ethoxyphenyl)-6-pyrrolidinylquinazolinone), a novel quinazolinone derivate, and the signaling pathway of MJ-33 in human prostate cells. MJ-33 exhibited a growth inhibitory effect on DU145, LNCaP and PC-3 cells by MTT assay. DU145 cells showed greater sensitivity to the growth inhibition of MJ-33 than that of LNCaP and PC-3 cells. MJ-33 also had an inhibitory effect on the invasion, migration and adhesion of DU145 cells using Boyden chamber transwell assays, wound-healing and adhesion assay. In addition, MJ-33 inhibited cell metastasis through the reduction of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and urokinase-type plasminogen activator (u-PA) enzyme activities and protein levels by gelatin zymography assay and western blot analysis, respectively. MJ-33 reduced the protein levels of p-JNK, p-p38, p-ERK, p-AKT and nuclear NF- κ B (p65), c-fos and c-Jun protein levels by western blotting. Using electrophoretic mobility-shift assay (EMSA), we demonstrated that MJ-33 blocked the activation of transcription factor AP-1 (activator protein-1) and NF- κ B, which led to the inhibition of MMP-2 and MMP-9 expression. Collectively, our data showed

that MJ-33 decreased protein levels of MAPKs (mitogen-activated protein kinases), AKT, AP-1 and NF- κ B, resulting in the inhibition of matrix metalloproteinases. Downregulation of MMP-2 and MMP-9 reduces the invasion, migration and adhesion activities of DU145 cells. MJ-33 may be a promising agent against prostate cancer metastasis.

Introduction

Prostate cancer is one of the leading causes of mortality in men (1) and has a highly variable natural history. Prostate cancer may be present as an indolent and silent entity throughout a man's life and then grow rapidly following metastasis to the lymph nodes and bones with a median life expectancy of 24-36 months (2). Therefore, prostate cancer is largely asymptomatic until metastases are present and it is largely a disease of the elderly. It is reasonable to propose that agents which inhibit metastasis could have great therapeutic efficacy.

Quinazoline derivatives are known for multiple effects, such as anti-malarial, anti-inflammatory (3), anti-bacterial, and antitumor activities (4). In recent years, we have designed and synthesized a series of quinazoline derivatives as new anti-mitotic agents (5,6). Our previous study showed that synthesized 6-pyrrolidinyl-4-quinazolinone derivative MJ-29 inhibited tubulin polymerization through binding to β -tubulin at the colchicine-binding site and acted as an anti-mitotic agent (5). Furthermore, we demonstrated that 6-fluoro-(3-fluorophenyl)-4-(3-methoxyanilino)quinazoline (LJJ-10) exhibits anti-metastatic effects in human osteosarcoma U-2 OS cells through targeting the insulin-like growth factor-I receptor (IGF-IR) (6). In the present study, we investigated the effects of MJ-33 on invasion, migration and adhesion in DU145 human prostate cancer cells. MJ-33 inhibited migration and invasion through downregulation of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA) through the AP-1 and NF- κ B signaling pathways.

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Key words: quinazolinone MJ-33, invasion, migration and adhesion, MAPK, AKT, NF- κ B and AP-1 signaling pathways, human prostate cancer DU145 cells

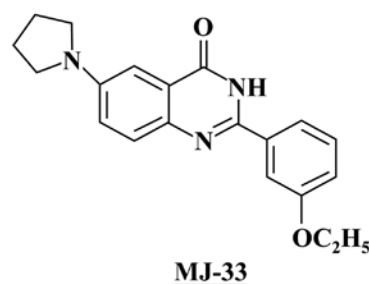
Materials and methods

Chemicals and reagents. MJ-33 was designed and synthesized by Mann-Jen Hour and Sheng-Chu Kuo (China Medical University, Taichung, Taiwan) (Fig. 1). Dimethylsulfoxide (DMSO), potassium phosphates, propidium iodide (PI) and triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco-BRL (Invitrogen, Grand Island, NY, USA). Antibodies against phospho-AKT, phospho-JNK, phospho-ERK and phospho-p38 were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Antibodies against AKT, JNK, ERK, p38, β -actin, MMP-2, MMP-9, u-PA, NF- κ B (p65), c-fos, c-Jun and all peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody against β -actin was purchased from Sigma-Aldrich.

Cell culture. The DU145, LNCaP and PC-3 human prostate cancer cell lines were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). All cells were individually plated onto 75 cm² tissue culture flasks with 90% RPMI-1640 medium. Cell medium with 2 mM L-glutamine was adjusted to contain 1.5 μ g/ml sodium bicarbonate, supplemented with 10% FBS, 100 Units/ml penicillin and 100 μ g/ml streptomycin. The cells were grown at 37°C under a humidified 5% CO₂ atmosphere.

Cell viability. Approximately 5x10⁴ cells/well of DU145, LNCaP and PC-3 cells were individually grown in 96-well plates for 24 h before different concentrations of MJ-33 were added (0, 50, 100, 250 and 500 nM). Cells were incubated at 37°C, 5% CO₂ and 95% air for 24 and 48 h. Following treatment, the supernatant was discarded before a 100 μ l solution of MTT (500 μ g/ml) was added to each well for 4 h at 37°C. After incubation, the violet formazan crystal produced from MTT was solubilized by the addition of 100 μ l of DMSO. The absorbance of the dissolved formazan grained within the cells was measured at 570 nm by a microplate reader as previously described (6).

Cell invasion assay. Twenty-four-well Transwell inserts with 8 μ m porosity polycarbonate filters (Millipore, Billerica, MA, USA) were pre-coated with 30 μ g Engelbreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel Basement Membrane Matrix) at room temperature for 1 h and then formed a genuine reconstituted basement membrane. DU145 cells (1x10⁴ cells/0.5 ml RPMI-1640) were placed onto the upper compartment and incubated with MJ-33 (0, 50, 100 and 200 nM). The plates were then incubated at 37°C for 24 h in a humidified atmosphere with 95% air and 5% CO₂. The cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and cells that penetrated through the matrigel to the lower surface of the filter were counted under a light microscope at x200. Each treatment was assayed in duplicate, and three independent experiments were carried out as previously described (7,8).



MJ-33
2-(3-Ethoxyphenyl)-6-pyrrolidinylquinazolinone

Figure 1. The chemical structure of MJ-33 (2-(3-ethoxyphenyl)-6-pyrrolidinylquinazolinone).

Cell migration assay. Approximately 5x10⁴ DU145 cells/ml were plated in 6-well plates for 24 h and then the cells in individual wells were wounded by scratching with a pipette tip and incubated with or without FBS free RPMI-1640 medium and treated with or without MJ-33 (0, 50, 100 and 200 nM) for 24 h. The cells were photographed under phase-contrast microscopy (x100) and calculated. Each treatment was assayed in duplicate, and three independent experiments were carried out as previously described (7,8).

Cell adhesion assay. The cell-matrix adhesion assay was used to determine cell adhesion. DU145 cells were plated on a 24-well plate after treatment with or without MJ-33 (0, 50, 100 and 200 nM) for 24 h. Then the individual cells were removed to a plate coated with 150 μ l of type I collagen (10 μ g/ml) and cultured for 30 min. Non-adherent cells were removed by PBS washing, and adherent cells were fixed in ethanol. After staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton-100 and measured at 550 nm by a microplate reader. Each treatment was assayed in duplicate, and three independent experiments were done as previously described (7,8).

Zymography assay. To determine the activity of MMP-2, MMP-9 and u-PA, quantitative gelatin zymography was performed with standard methods. Briefly, cells (1x10⁷ cells/ml) were treated with MJ-33 (0, 50, 100 and 200 nM) for 24 h. Cells were harvested and separated by dilution in zymography sample buffer. Samples were electrophoresed in an 8% SDS-polyacrylamide gel containing 1% gelatin, and incubated in renaturing buffer (2.5% Triton X-100). Electrophoresis was performed at 110 V for 3 h. The gel was incubated with development buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂, 0.02% Brij-35) at 37°C for 18 h, and stained with 0.5% Coomassie blue G-250 for 3 h. The gels were digitized using a scanning digitizing system and analyzed using NIH image software. The u-PA activity was performed by casein-plasminogen zymography. Briefly, 2% casein and 20 μ g/ml plasminogen were added to an 8% SDS-PAGE gel. Samples with a total protein of approximately 30 μ g were then loaded onto the gels. The u-PA activity of cells treated with or without MJ-33 was measured as described for the gelatin zymography assay (7,8).

Preparation of whole-cell lysate and nuclear extract. Approximately 1x10⁷ cells were treated with MJ-33 (0, 50,

100 and 200 nM) for 6 or 24 h. The cells were harvested and whole-cell lysed with iced-cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5), and then phenylmethanesulfonyl fluoride (10 mg/ml), leupeptin (17 mg/ml), and sodium orthovanadate (10 mg/ml) were added (9,10) and vortexed for 30 min on ice. The samples were centrifuged at 12,000 g for 10 min. Nuclear extracts were prepared from MJ-33-treated DU145 cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL, USA). Each nuclear pellet was collected and was then re-suspended in nuclear extract buffer (1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 25% glycerol, and 420 mM NaCl). The nuclear suspension was incubated for 20 min on ice then centrifuged at 14,000 g for 5 min. The supernatant (the soluble nuclear fraction) was saved and the remaining pellet was solubilized by sonication in PBS. The protein content in each sample was determined by using Bio-Rad protein assay reagent using bovine serum albumin as the standard. Nuclear extracts were prepared for NF- κ B, c-fos, and c-Jun western determination (11).

Western blotting. Cells were harvested and the total and nuclear proteins were collected as described above. Protein abundance of MMP-2, MMP-9 and u-PA, p-JNK, p-ERK, p-p38, p-AKT, JNK, ERK, p38, AKT, NF- κ B (p65), c-fos and c-Jun were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as previously described (5,12-15).

Electrophoretic Mobility Shift Assay (EMSA). Approximately 1×10^7 cells were treated with MJ-33 (0, 100 and 200 nM) for 6 h. Nuclear extracts were prepared from MJ-33-treated DU145 cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). The protein concentrations were determined and Biotin end-labeled oligonucleotide sequences 5'-Biotin-GATCCAGGGGACTTTCCTAGC-3' corresponded to the consensus site of NF- κ B and Biotin end-labeled oligonucleotide sequences 5'-Biotin-CGCTTGATGACTCAGCCGGAA-3' corresponded to the consensus site of AP-1. Nuclear extract proteins (5 μ g) were used for EMSA with a LightShift Chemiluminescent EMSA Kit according to the manufacturer's protocol. Biotin end-labeled duplex DNA was incubated with a nuclear extract or purified factor and electrophoresed on a 6% polyacrylamide native gel. For competition experiments, a 100-fold excess of unlabeled double stranded oligonucleotide was added to the reaction. The DNA was then rapidly transferred to a positive nylon membrane, UV cross-linked, probed with streptavidin-HRP conjugate and incubated with the substrate of the ECL kit (16,17).

Statistical analysis. Student's t-test was used to analyze differences between treated and control groups. * $p < 0.05$ was considered to indicate a statistically significant difference.

Results

MJ-33 induces growth inhibition effects on human prostate cancer cell lines. We determined the growth inhibition effects of MJ-33 on the human prostate cancer cell lines DU145,

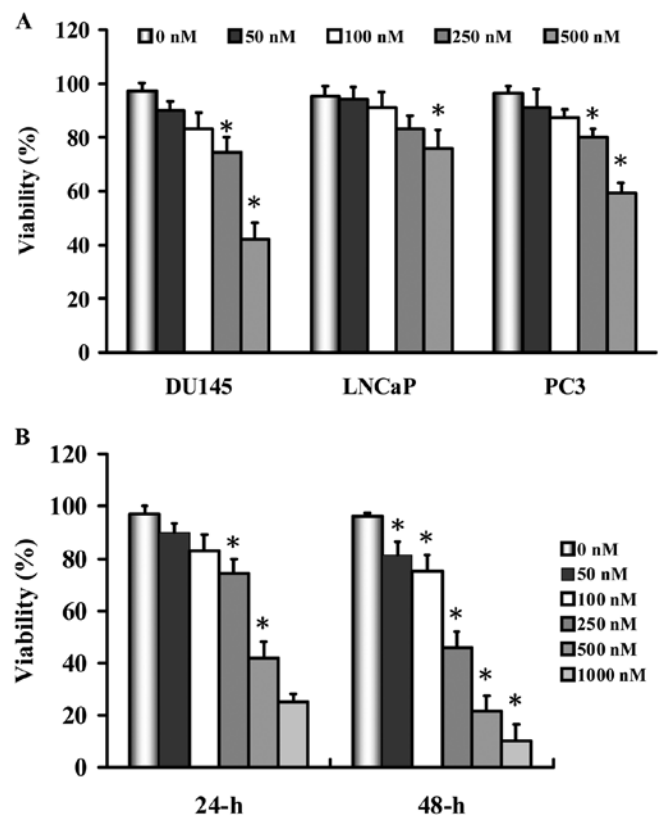


Figure 2. MJ-33 induces cytotoxic effects in human prostate cancer cell lines. (A) Cells (DU145, LNCaP and PC-3) were treated with MJ-33 (0, 50, 100, 250 and 500 nM) for 24 h then viable cells were determined by MTT assay. (B) DU145 cells were treated with MJ-33 (0, 50, 100, 250, 500 and 1000 nM) for 24 and 48 h and the viable cells were determined by MTT assay, as described in Materials and methods. Each point is the mean \pm SD of three experiments. * $p < 0.05$ indicates a significant difference compared to the control.

LNCaP and PC-3. As shown in Fig. 2, MJ-33 inhibited the cell growth of the three cell lines in a concentration-dependent manner. DU145 cells were more sensitive by MJ-33 than that of the other two cell lines. We therefore investigated whether or not MJ-33 could induce a concentration- and time-dependent growth inhibition effect on DU145 cells. As seen in Fig. 2B, MJ-33 decreased the percentage of viable DU145 cells in a concentration- and time-dependent manner, but we selected less than 200 nM of MJ-33 for further works in this study.

MJ-33 inhibits invasion, migration and adhesion of DU145 cells. The effects of MJ-33 on cell invasion were examined using Matrigel-coated Transwell assay in DU145 cells. As shown in Fig. 3A, MJ-33 (50-200 nM) significantly inhibited cell invasion in a concentration-dependent manner; the percentage of inhibition ratio was 15-70%. The inhibition of DU145 cell migration by MJ-33 was examined using the wound-healing assay. As shown in Fig. 3B, MJ-33 (50-200 nM) significantly inhibited cell migration in a concentration-dependent manner; the percentage of inhibition ratio was 30-75%. The inhibition of DU145 cell adhesion by MJ-33 was examined by using cell adhesion assay. As shown in Fig. 3C, MJ-33 (50-200 nM) significantly inhibited cell adhesion in a concentration-dependent manner; the percentage of inhibition ratio was 10-45%. MJ-33 did not affect cell viability at

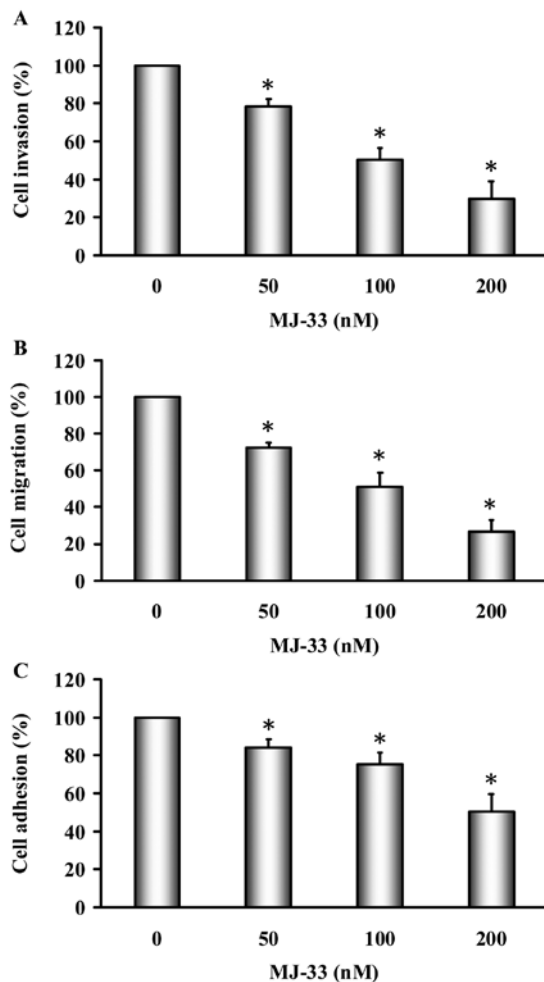


Figure 3. MJ-33 inhibited cell invasion, cell migration, and adhesion activities of DU145 cells *in vitro*. (A) Cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and counted under a light microscope at $\times 200$. (B) Cells were plated in 6-well plates, were then wounded by scratching with a pipette tip and incubated with FBS free RPMI-1640 medium with or without MJ-33 for 24 and 48 h. The cells were calculated as the percentage of inhibition of migration. (C) Cells were seeded on a 24-well plate and coated, and were then cultured for 30 min. Non-adherent cells were removed and adherent cells were fixed in ethanol to examine the percentage of adhesion cells as described in Materials and methods. * $p < 0.05$ indicates a significant difference compared to the control.

50-200 nM of 24-h treatment. On the other hand, the EC_{50} is 458.32 ± 6.96 nM for 24 h in MJ-33-treated DU145 cells. Our results demonstrated that MJ-33 inhibited the effects of cell invasion, migration and adhesion in DU145 cells. Also, the inhibitory effects of MJ-33 on invasion, migration and adhesion are independent of cellular cytotoxicity.

MJ-33 inhibits MMP-2, MMP-9 and u-PA enzyme activities of DU145 cells. We investigated the mechanisms of cell invasive phenotype by determining the involvement of MMP-2, MMP-9 and u-PA. DU145 cells were treated with MJ-33 (0, 50, 100 and 200 nM) for 24 h. The MMP-2, MMP-9 and u-PA activities were determined by gelatin or casein zymography. As shown in Fig. 4, we found that MJ-33 inhibited individual activity of MMP-2, MMP-9 (Fig. 4A) and u-PA (Fig. 4B). Reductions in activity are consistent with decreases in protein abundance of MMP-2, MMP-9 and u-PA, as shown in Fig. 5A.

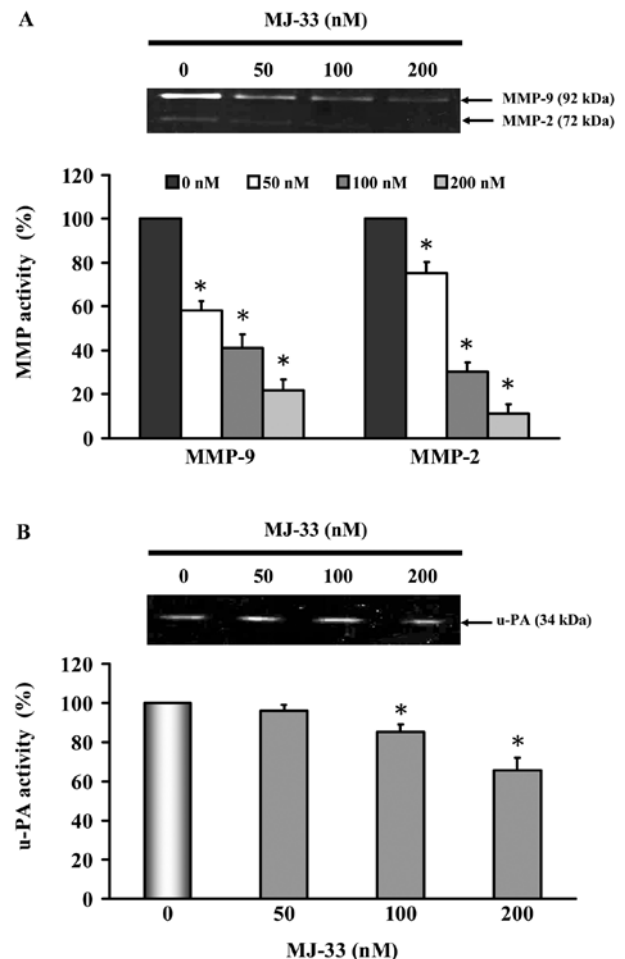


Figure 4. MJ-33 inhibited the activities of MMP-2, MMP-9 and u-PA of DU145 cells. Cells were treated with various concentrations (0, 50, 100 and 200 nM) of MJ-33 for 24 h. The conditioned media were collected, and MMP-2, MMP-9 (Panel A) and u-PA activities (Panel B) were determined by gelatin or casein zymography. MMP-2 and u-PA activities were quantified by densitometric analysis. The densitometric data are expressed as the mean \pm SD of three independent experiments. * $p < 0.05$ indicates a significant difference compared to the control.

MJ-33 inhibits the MAPKs and AKT signaling pathways in DU145 cells. We investigated the effects of MJ-33 on metastatic protein levels in DU145 cells by western blotting. As shown in Fig. 5A, we determined DU145 cells after exposure to MJ-33 (0, 50, 100 and 200 nM) for 24 h. MJ-33 reduced the protein levels of MMP-2, MMP-9 and u-PA. To clarify the possible upstream signaling pathways in MJ-33-treated DU145 cells, we evaluated the related protein levels in the MAPK (JNK, p38 and ERK) and AKT signaling pathways by western blotting. We determined DU145 cells after exposure to MJ-33 (0, 50, 100 and 200 nM) for 6 h. We found that incubation of cells with MJ-33 reduced the protein levels of p-JNK, p-ERK, p-p38 and p-AKT.

MJ-33 inhibits the AP-1 and NF- κ B signaling pathways in DU145 cells. Numerous studies have reported that MMP-9, MMP-2 and u-PA promoters have several transcription binding motifs such as NF- κ B and AP-1. In order to clarify the involvement of NF- κ B and AP-1 proteins in the mechanisms of MJ-33's action, we evaluated the related protein levels in NF- κ B, c-fos

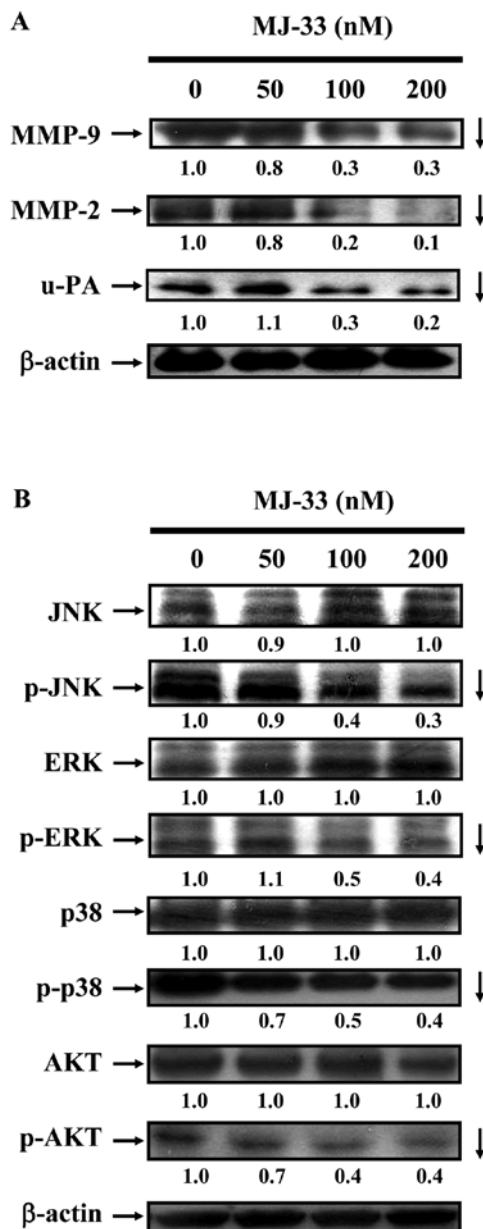


Figure 5. MJ-33 affects the levels of associated proteins in metastasis of DU145 cells *in vitro*. Cells were placed onto the plates with or without various concentrations of MJ-33 (0, 50, 100 and 200 nM) treatment for 24 h and were then isolated from each sample. The total and nuclear proteins were then prepared and were detected by western blotting as described in Materials and methods. The levels of (A) MMP-2, MMP-9, u-PA, and (B) p-JNK, p-ERK, p-p38, p-AKT expressions were examined by western blotting.

and c-Jun by western blotting. In addition, the effects of MJ-33 on DNA binding of NF- κ B and AP-1 were determined using EMSA. We determined DU145 cells after exposure to MJ-33 (0, 50, 100 and 200 nM) for 6 h. We found that incubation of cells with MJ-33 reduced protein levels of NF- κ B, c-fos and c-Jun. The results shown in Fig. 6B demonstrate that MJ-33 inhibited NF- κ B and AP-1 DNA binding in a dose-dependent manner. Binding of NF- κ B and AP-1 were particularly inhibited by treatment with 100 and 200 nM of MJ-33. Therefore, our study proposes that MJ-33 might block the invasion, migration and adhesion in DU145 cells by inhibiting AKT and MAPKs as well as suppressing the NF- κ B signaling.

Discussion

Previous reports have found that quinazoline derivatives exert antitumor activity against seven types of cancer cells both *in vitro* and *in vivo* (18), and also induce apoptosis and inhibit metastasis in the U-2 OS human osteogenic sarcoma cell line (19). There is, however, no information on the effects of MJ-33 on invasion, migration and adhesion in human prostate cancer cells. Initially, three human prostate cancer cell lines (DU145, LNCaP and PC-3) were examined and it was observed that MJ-33 reduced DU145 cell viability more compared with the other two cell lines. Based on these findings, DU145 cells were used to examine the effects of MJ-33 on invasion, migration and adhesion. We found that MJ-33 can induce growth inhibition effects and inhibit invasion, migration and adhesion of DU145 cells (Fig. 3). Furthermore, these effects were associated with inactivation of the MAPKs (ERK, JNK, p38) and AKT (Fig. 5), inhibitory effects on NF- κ B, c-fos, and c-Jun transcriptional factors (Fig. 6). This effect on AP-1 and NF- κ B transcription factors was consistent with less DNA binding of NF- κ B and AP-1 DNA (Fig. 6B). MAPKs are intricately involved in the expression of the components involved in MMPs or u-PA promoter induction through NF- κ B, AP-1 and its association with c-fos and c-Jun (20). Numerous studies from different cell types have suggested the MAPKs play a central role in regulating the activities of MMPs or u-PA (21-23). Inhibition of the MAPKs pathway might have the potential of preventing angiogenesis, proliferation, invasion, and migration occurring with a wide range of tumors.

Our findings reinforce the potential of MJ-33 as a new strategy for antitumor therapy, especially in the inhibition of cancer metastasis which is a major cause of mortality in cancer patients. MMP-2 and u-PA promoters have several transcription factor binding motifs, including NF- κ B and AP-1 (24). Thus, multiple pathways leading to activation of NF- κ B and AP-1 binding factors in tumor cells may contribute to MMP-2, MMP-9 and u-PA transcription and metastatic enhancement. We found that MJ-33 inhibited cell invasion, migration and adhesion through the downregulation of MMP-2 and MMP-9 protein abundance in DU145 cells. This is in agreement with our previous study that LJJ-10 (a novel quinazoline derivative) inhibited the invasion of human osteosarcoma U-2 OS cells through inhibition of MMP-2 and MMP-9. There is evidence that growth factors and cytokines affect MMP-9 expression through acting on the transcription factors NF- κ B and AP-1 through the Ras/MAPK and PI3K/AKT signaling pathways (25). NF- κ B and AP-1 binding to the MMP-2 and MMP-9 promoter are centrally involved in the induction of *MMP-2* and *MMP-9* gene expression associated with tumor cell invasion (26-28). To further explore how MJ-33 inhibits invasion, migration and adhesion, we used gelatin or casein-plasminogen zymographic assays to detect activities of MMP-2, MMP-9, and u-PA. In this study, MJ-33 significantly decreased the levels of MMP-2, MMP-9 and u-PA activity (Fig. 4). These results indicate that the anti-metastatic effect of MJ-33 is associated with the inhibition of enzymatically degradative processes of tumor metastasis. Furthermore, we used a wound-healing and a Boyden chamber assay to quantify the migratory potential of DU145 cells.

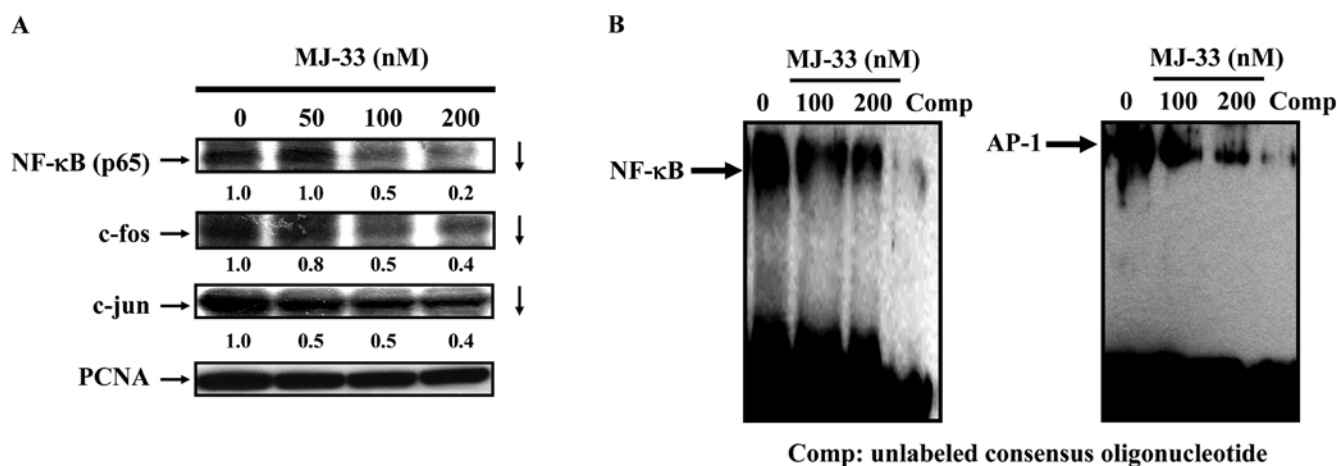


Figure 6. MJ-33 affects the DNA binding activities of NF- κ B and AP-1 in DU145 cells. (A) NF- κ B, c-fos and c-Jun expressions were examined by western blotting. (B) Cells were treated with 0, 100 and 200 nM of MJ-33 for 24 h, and then nuclear extracts were prepared and analyzed for NF- κ B and AP-1 DNA binding activity using biotin labeled consensus NF- κ B and AP-1 specific oligonucleotides, and then EMSA assay was performed as described in Materials and methods. Lane 1, control; lane 2, 100 nM MJ-33; lane 3, 200 nM MJ-33; lane 4, unlabeled consensus oligonucleotides. Excess free probe is indicated at the bottom. Results from three repeated and separate experiments were similar.

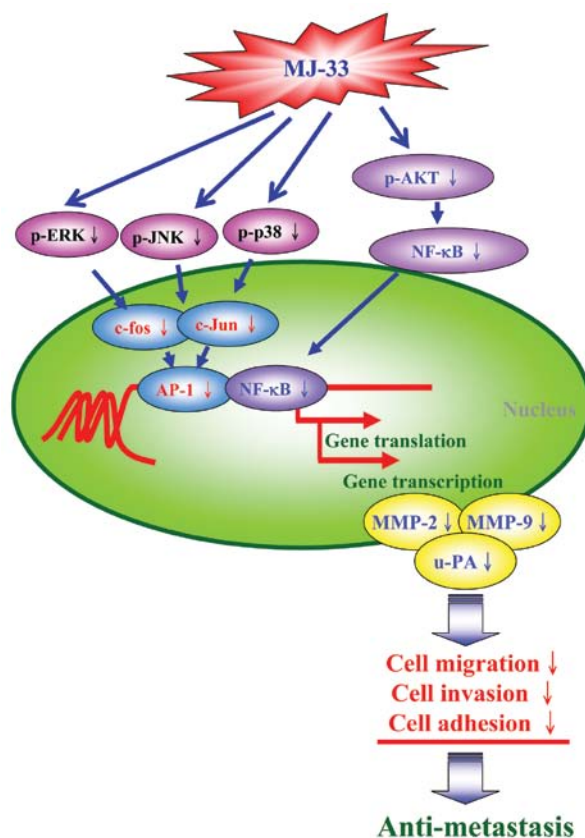


Figure 7. The proposed signaling pathways of MJ-33-inhibited invasion and migration in DU145 human prostate cancer cells.

Taken together, these observations suggest that MJ-33 significantly inhibits the invasion, migration and adhesion of DU145 cells. MJ-33 acts as an anti-metastatic agent in prostate cancer cells. Collectively, we have outlined the overall possible signaling pathways for MJ-33-inhibited metastasis in DU145 cells (Fig. 7). We explored for the first time and investigated the roles of AP-1 and NF- κ B in reducing the levels and activities of MMP-2, MMP-9 and u-PA in human prostate cancer cells.

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