

Inhibition of Invasion and Migration by Newly Synthesized Quinazolinone MJ-29 in Human Oral Cancer CAL 27 Cells through Suppression of MMP-2/9 Expression and Combined Down-regulation of MAPK and AKT Signaling

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Abstract. Anti-metastasis by reducing cellular migration and invasion and by deregulating the expression of matrix metalloproteinases (MMPs) is a therapeutic approach for cancer treatment. The objective of this study focused on the effects of the novel compound 6-pyrrolidinyl-2-(2-hydroxyphenyl)-4-quinazolinone (MJ-29) regarding anti-metastatic actions on human oral squamous cell carcinoma CAL 27 cells and on the verification of the underlying related molecular mechanisms of this event. MJ-29 concentration- and time-dependently caused a suppression of cell adhesive ability utilizing cell adhesion assay; it also inhibited the migration and invasion of CAL 27 cells using scratch wound closure and transwell invasion assays in a concentration-dependent response. Importantly, we confirmed that the applied concentration range of MJ-29 exhibited no dramatic influence of cytotoxicity on CAL 27 cells using the thiazolyl blue tetrazolium bromide assay. MJ-29 also attenuated the enzymatic activity of MMP-2 and MMP-9. Furthermore, we

found that activation of their upstream protein kinases, by MJ-29, potentially exerted an inhibitory effect on the phosphorylated protein levels of extracellular regulated protein kinase 1/2, p38 and c-Jun N-terminal kinase 1/2, as well as serine/threonine kinase AKT by MJ-29 in CAL 27 cells. The expression of RAS and focal adhesion kinase was also down-regulated in MJ-29-treated CAL 27 cells. Collectively, these findings provide further evidence for the molecular signaling basis of the effects of MJ-29 on suppression of migration and invasion which might be useful as a therapeutic strategy to treat human oral cancer.

Oral squamous cell carcinoma (OSCC), a prominent subset of head and neck squamous cell carcinomas (HNSCC), is a prevalent malignancy, globally, as well as a biologically aggressive disease (1-3). In Taiwan, betel quid chewing is a vital etiological factor related to oral cancer (4), from which on average 7.9 individuals per 100,000 died in 2011, according to the report from Department of Health, R.O.C (Taiwan) (5). The clinical treatment options for HNSCC patients consist of multiple-modality therapies with surgery, radiation and multidrug chemotherapy (6), which only control primary tumor growth, with no dramatic increase in the long-term survival over the past 30 years (7, 8). Strikingly, nodal metastasis is the key cause of death in OSCC cases, and the presence of metastatic tumor cells is a major and an increasingly important problem, leading to poor prognosis (9, 10). Strategies for prevention and therapy of metastasis in OSCC remain unsolved (3, 11). It is notable that tumor metastasis can be suppressed and cured, and better therapeutic outcomes are likely to be expected during treatments (3, 12). Accordingly, discovering novel ways for preventing

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metastasis to regional lymph nodes in oral cancer are urgently needed. There is much evidence to demonstrate that the metastatic processes include cell migration, invasion, surface adhesion and degradation of extracellular matrix (ECM) (13-15). The matrix metalloproteinases (MMPs), a family of zinc-dependent proteinases, are implicated in the processes of tumor invasion and metastasis (16); overexpression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) which degrade collagen, lead to increased tumor invasion in OSCC (17, 18).

A series of novel 2-phenyl 6-pyrrolidinyl-4-quinazolinone derivatives, as anti-mitotic agents, have been designed and synthesized, and their biological properties and anticancer actions were studied on tumor cells (19-22). One of these new agents, 6-pyrrolidinyl-2-(2-hydroxyphenyl)-4-quinazolinone (MJ-29) (Figure 1), exhibits the most potent cytotoxicity of this series against many cancer cell lines (19). Our earlier study showed that MJ-29 suppressed proliferation and triggered apoptosis in human leukemia cell lines (20). The purpose of this study was to explore the effects of MJ-29 on the migration and invasion of the human oral cancer CAL 27 cell line.

Materials and Methods

Chemicals and reagents. Dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), Tween-20 and anti- β -Actin were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Cell culture materials [Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA] were purchased from Gibco/Life Technologies (Carlsbad, CA, USA). Anti-MMP-9 (Cat. AB19016), anti-focal adhesion kinase (FAK) (Cat. 05-537) and anti-phospho-serine/threonine kinase AKT (Ser473) (Cat. 05-669) were brought from Merck Millipore Corp. (Billerica, MA, USA). The primary antibodies to MMP-2, RAS, AKT, extracellular regulated protein kinase 1/2 (ERK1/2), p-ERK1/2, p38, p-p38, c-Jun N-terminal kinase 1/2 (JNK1/2), p-JNK1/2 and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and were diluted in phosphate buffered saline (PBS)/0.1% (v/v) Tween-20 prior to use in immunoblotting. MJ-29 was synthesized and kindly provided by Mann-Jen Hour, Ph.D. (School of Pharmacy, China Medical University).

Cell line and culture conditions. Human oral squamous cell carcinoma (OSCC) cell line CAL 27 was kindly provided by Dr. Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan) and originally purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 Units/ml penicillin and 100 μ g/ml streptomycin in a humidified 37°C incubator containing 5% (v/v) CO₂ and 95% (v/v). Cells were grown in T75 tissue culture flasks (Techno Plastic Products AG., Trasadingen, Switzerland) and detached by 0.25% (v/v) trypsin/0.02% (v/v) EDTA and were split every three days to maintain cell growth (23-25).

Cell adhesion assay. CAL 27 cell attachment to a matrix (collagen) was carried out as previously described with some modifications (26, 27). Briefly, cells were treated with or without MJ-29 at 125,

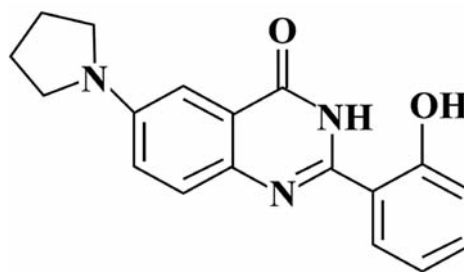


Figure 1. The chemical structure of 6-pyrrolidinyl-2-(2-hydroxyphenyl)-4-quinazolinone (MJ-29).

250 and 500 nM for 24 and 48 h, respectively. Cells were then harvested and placed into 24-well plates (1 \times 10⁵ cells/well), pre-coated with 100 μ l of collagen type I (10 μ g/ml) (Cat. 08-115, Merck Millipore Corp.), for 3 h and consequently incubated in serum-free medium at 37°C for 2 h. Following, the medium with unattached cells was discarded gently, and the plates were washed with PBS twice. The percentage of adherent cells was determined using absorbance by the thiazolyl blue tetrazolium bromide (MTT) assay colorimetric method as previously described (20, 28).

Scratch wound closure assay. CAL 27 cells (5 \times 10⁵ cells/well) were seeded in 6-well plates and allowed to attach to 80% confluency. Cell monolayers were wounded by scratching with 200- μ l pipette tips before being washed twice with PBS to remove floating cells. Cells in the each well were subsequently exposed to serum-free DMEM with or without 125, 250 or 500 nM of MJ-29 for up to 48 h. At the end of treatments, the number of cells in the scratch area of each well was determined, and cells were photographed at \times 100 magnification under a phase-contrast microscope, as described elsewhere (13, 29, 30). The migration of cells was counted in five random fields from each triplicate treatment and expressed related to the control (number of migrated cells to the denuded zone in untreated wells).

Transwell invasion assay. The ability of CAL 27 cells to invade through Matrigel matrix-coated filters was measured by the Boyden chamber assay (30, 31). Polyethylene terephthalate (PET) filters with 8 μ m pore size (Millicell Hanging Cell Culture Inserts, Cat. PIEP12R48, Merck Millipore Corp.) were pre-coated with 30 μ l of Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:9 with PBS, and air dried in a laminar hood for 2 h. In brief, cells in serum-free DMEM were cultured in the upper chamber of the transwell insert (1 \times 10⁴ cells/0.4 ml per well) and treated in the presence of MJ-29 (125, 250 and 500 nM), or incubated with 0.5% (v/v) DMSO as a vehicle control. The lower chambers (600 μ l per well) were filled with 10% (v/v) FBS-medium as chemoattractant, and thereafter these transwell inserts were incubated in a humidified atmosphere with 95% (v/v) air and 5% (v/v) CO₂ at 37°C for 48 h. At the end of incubation, cells had invaded across the Matrigel and invasive cells attached to the lower surface of the filter. Consequently, the cells were fixed with methanol (Sigma-Aldrich Corp.) for 15 min and stained with 2% (w/v) crystal violet (Sigma-Aldrich Corp.) for 10 min, before a cotton swab was applied to gently remove the non-invasive cells on the upper side of the filter. The data are presented as the number of invasive cells penetrating the membrane/filter, counted in five random fields and photographed under a light microscope at \times 200 magnification (29, 31).

MTT assay. Cell viability was determined utilizing the MTT-based *in vitro* toxicology assay. In brief, CAL 27 cells were plated at a density of 1×10^4 cells/well in 96-well plates and treated with DMSO alone [0.5% (v/v) in media served as a vehicle control] and with different concentrations (125, 150 and 500 nM) of MJ-29 for 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, and cells were incubated for 3 h at 37°C. After incubation, violet formazan crystals, produced from MTT, were solubilized by the addition of 200 μ l of DMSO, and the absorbance of the dissolved formazan within the cells was measured at 570 nm by a microplate reader as previously described (20, 28).

Zymography assay for MMP-2 and MMP-9. The proteolytic activity of MMP-2 and MMP-9 in condition medium was analyzed using the gelatin zymography assay and by substrate gel electrophoresis, as described elsewhere (13, 30, 32). CAL 27 (5×10^6 cells/well) cells in 6-well plates were maintained in serum-free DMEM with or without MJ-29 (125, 250 and 500 nM) for 48 h, and the supernatants were collected to prepare samples with 2 \times loading buffer as previously described (33). Samples were subjected to 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 0.1% (w/v) gelatin (Sigma-Aldrich Corp.). After electrophoresis, gels were washed with 2.5% (v/v) Triton X-100 twice in distilled water, for 15 min, and incubated at 37°C in developing buffer [50 mM Tris pH 7.6, 10 mM CaCl_2 , 50 mM and 0.05% (w/v) Brij 35] for 24 h. After incubation, bands were visualized, and the gel was stained with 0.3% (w/v) Coomassie Brilliant Blue R250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by destaining with 10% (v/v) acetic acid and 30% (v/v) methanol. Bands of gelatinolytic activity were quantified using the ImageJ 1.45 software for Windows, from the National Institute of Health (NIH) (Bethesda, MD, USA).

Western blot analysis. CAL 27 cells at a density of 5×10^6 cells/well, in 6-well plates, were exposed to 0, 250 and 500 nM of MJ-29 for 12 or 48 h. Cells were then harvested, and whole-cell protein extracts were conducted using the PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea). Equal loading was verified by determining the protein concentration utilizing the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.), and cell lysates (40 μ g) were electrophoresed by 10-12% (w/v) SDS-PAGE. After electrophoresis, proteins from the gels were transferred and blotted onto Immobilon-P transfer membrane (Cat. IPVH00010, Merck Millipore Corp.), as previously described (34, 35). The membranes were blocked with PBS containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat powdered milk and probed first with specific antibodies against MMP-2, MMP-9, AKT, phospho-AKT (Ser473), RAS, FAK, ERK1/2, p-ERK1/2, p38, p-p38, JNK1/2, p-JNK1/2 in blocking buffer at 4°C overnight. Thereafter, bound antibodies were developed using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and were visualized by Immobilon Western Chemiluminescent HRP substrate (Cat. WBLUC0500, Merck Millipore) and X-ray film (GE Healthcare, Piscataway, NJ, USA). To assure equal loading, membranes were stripped and reprobed with an antibody against β -Actin. The density of the immunoreactive bands was quantified utilizing NIH ImageJ (35, 36).

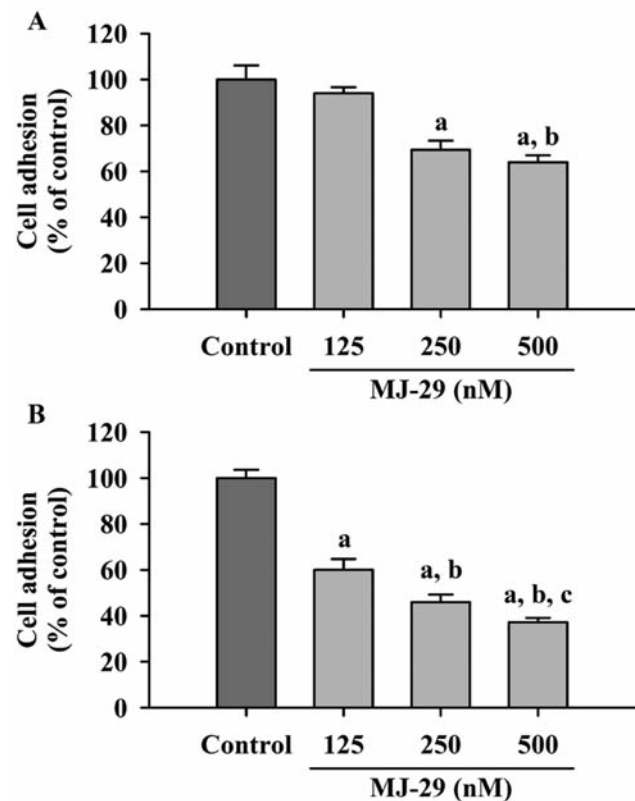


Figure 2. Effects of MJ-29 on adhesion of CAL 27 cells *in vitro*. Cell suspension (1×10^5 cells/well) in complete medium was added to wells pre-coated with collagen type I and cells were incubated at 37°C for 2 h, after treatment with or without 125, 250 and 500 nM of MJ-29 for 24 h (A) and 48 h (B). The adherent ability of CAL 27 cells was determined by the thiazolyl blue tetrazolium bromide (MTT) assay, as described in the Materials and Methods. Data are expressed as the percentage of adhered cells when compared to untreated cells (control) which were set at 100% (% of control). Each bar represents the mean \pm S.D. calculated from three independent experiments. a, significantly different at $p < 0.05$ compared with the 0.5% DMSO-treated control; b and c, significantly different at $p < 0.05$ from treatments with 125 and 250 nM of MJ-29, respectively, by one-way ANOVA followed by Bonferroni's test for multiple comparisons.

Statistical analysis. Values represent the mean \pm standard deviation (S.D.) of each analysis performed in triplicate. Data were analyzed by one-way ANOVA test followed by Bonferroni's multiple comparison test to compare for differences between the untreated control and MJ-29-treated groups. The differences were considered statistically significant at $p < 0.05$.

Results

MJ-29 suppresses the adhesion of human OSCC CAL 27 cells *in vitro*. Tumor cell adhesion to the ECM is known to have a critical action, fundamental to tumor invasion (13). We investigated the influences of MJ-29 on the adhesive activity of CAL 27 cells. Cells were treated with or without

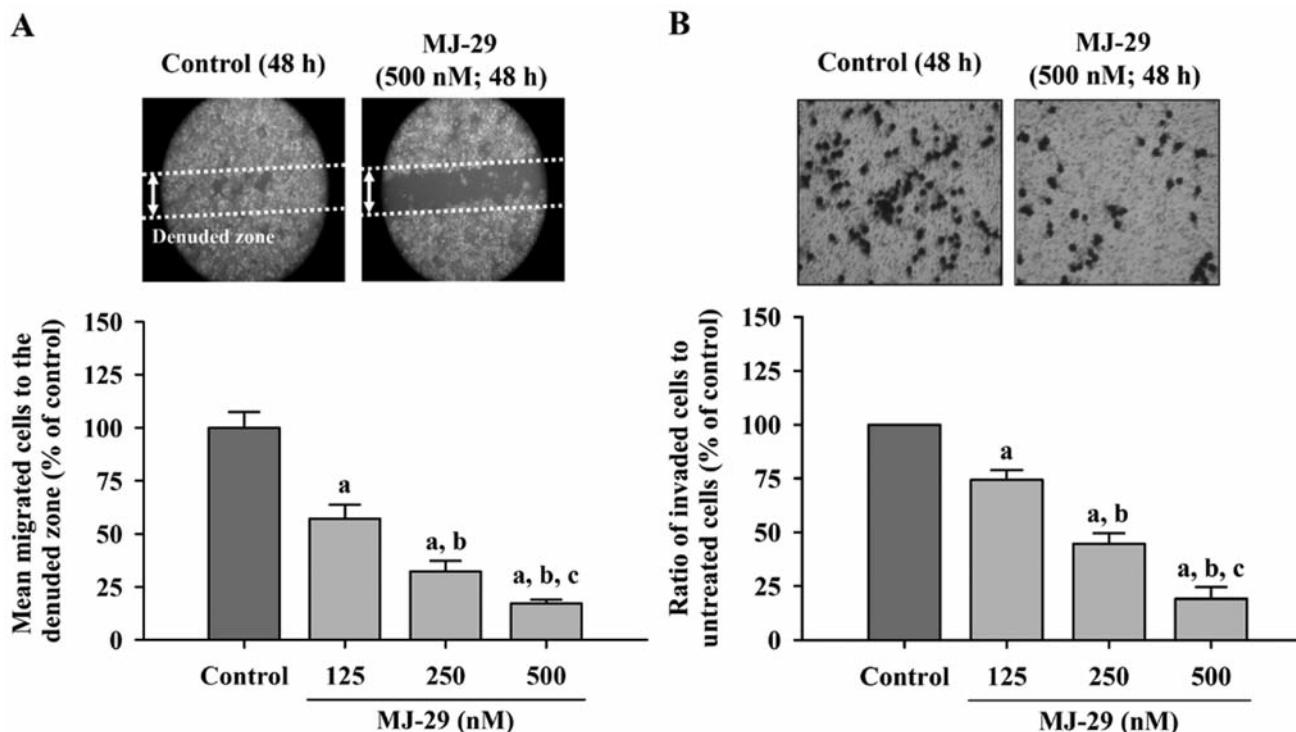


Figure 3. Effects of MJ-29 on motility, migration and invasion of CAL 27 cells. Scratch wound closure and transwell invasion assays were assessed as described in the Materials and Methods. Confluent monolayers of cells in 6-well plates were wounded before incubation in the presence and absence of 125, 250 and 500 nM of MJ-29, and 0.5% DMSO as control. At 48-h exposure after wounding, the relative denuded zone (wound closures) and migrated cells were monitored and photographed using a phase-contrast microscope at $\times 100$ magnification (A). Cells were seeded in the upper chamber of the transwell insert, pre-coated with Matrigel and exposed to MJ-29 (125, 250 and 500 nM). After 48-h incubation, cells invading to lower side of the membrane were stained and subsequently photographed ($\times 200$ magnification) (B). Migration and cell invasion in five random fields were quantified and expressed on the basis of untreated cells (control) representing 100%. The results are indicated as the mean \pm S.D. of three experiments; a, significantly different ($p < 0.05$) from the control; b and c, significantly different ($p < 0.05$) from treatments with 125 and 250 nM of MJ-29, respectively, by one-way ANOVA followed by Bonferroni's test for multiple comparisons. Each experiment was repeated three times with similar results.

different concentrations (125, 250 and 500 nM) of MJ-29 for 24 and 48 h. Our results, shown in Figure 2, indicate that MJ-29 treatment reduced cell adhesion to collagen; significant inhibitory rates of 30.5% and 36.0% for a 24-h treatment with 250 and 500 nM (Figure 2A), as well as 39.9%, 54.0% and 62.8% for a 48-h exposure to 125, 250 and 500 nM (Figure 2B), respectively, were observed in CAL 27 cells in comparison to the untreated control.

MJ-29 mediates the reduction of CAL 27 cell motility, migration and invasion in vitro. To investigate if MJ-29 reduces tumor migration and invasion, the scratch wound closure and Matrigel invasion assays were performed to determine the effects of MJ-29-affected CAL 27 cells. Our results, shown in Figure 3A, demonstrate that MJ-29 suppressed the migration of CAL 27 cells across the denuded zone (scratch region), and the inhibition of cellular migration was 43.0%, 67.8% and 78.5% on 48-h incubation with 125, 250 and 500 nM of MJ-29,

respectively (Figure 3A). In addition, invasive CAL 27 cells were reduced in the presence of MJ-29 in a concentration-dependent manner as can be seen in Figure 3B. Collectively, these results indicated that MJ-29 concentration-dependently retarded the adhesion, migration and invasion of CAL 27 cells *in vitro*.

Influence of MJ-29 on the viability of CAL 27 cells. To demonstrate whether the inhibitory actions (adhesion, migration and invasion) of MJ-29 in CAL 27 cells were due to non-cytotoxic effects, we investigated the percentage of viable cells *in vitro* by the MTT assay. Figure 4 shows that a 48-h treatment of MJ-29 at different concentrations (125, 250 and 500 nM) exhibited no dramatic cytotoxicity. Even MJ-29 at the highest concentration of 500 nM, did not significantly alter the viability of CAL 27 cells, as compared to that of the untreated control. Based on this evidence, this concentration range (less than 500 nM) was subsequently applied in this study.

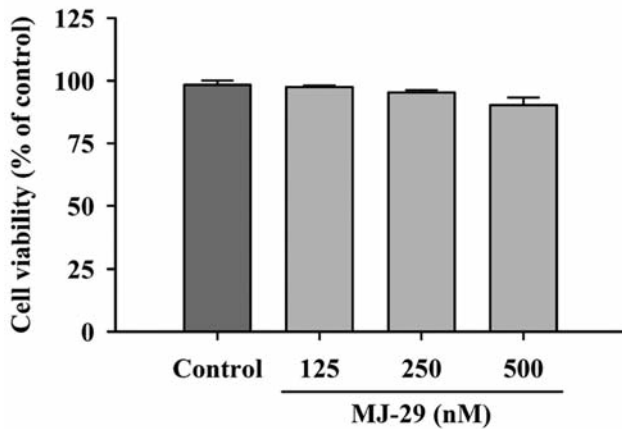


Figure 4. Effects of MJ-29 on cell viability of CAL 27 cells. Cells (1×10^4 cells/100 μ l) were seeded in 96-well plates and treated with or without 125, 250 and 500 nM of MJ-29 for 48 h. 0.5% DMSO in media was used as vehicle control. Cells were counted, and the percentage of viable cells was determined by the MTT-based toxicology assay, as described in the Materials and Methods. Each point is the mean \pm S.D. of three experiments.

Gelatinolytic activities of MMP-2 and MMP-9 in CAL 27 cells were attenuated by MJ-29. It is well known that MMP-2 and MMP-9 are vital for ECM degradation and are required for the cell migration and invasion during metastasis (14, 15). To clarify whether MJ-29 modulates the secretion of MMPs by CAL 27 cells, we employed gelatin zymography. As shown in Figure 5A, the incubation of cells with MJ-29 at 125, 250 and 500 nM for 48 h markedly attenuated the activity of MMP-9 by 74.7%, 83.8% and 91.0% (Figure 5B) and of MMP-2 by 63.7%, 81.8% and 87.3% (Figure 5C) activities in CAL 27 cells after the quantification analysis and these responses are in a concentration-dependent effect. These results suggest that the loss of enzymatic degradation steps (MMP-2/-9) is correlated with the anti-metastatic effects of MJ-29 on CAL 27 cells *in vitro*.

MJ-29 down-regulates the protein expression of MMP-2 and MMP-9 in CAL 27 cells. In an attempt to understand the mechanism by which MJ-29 impacts metastasis *in vitro*, western blot analysis was carried out to explore whether MJ-29 alters the protein levels of MMP-2 and MMP-9 in CAL 27 cells. The results, illustrated in Figure 6A, reveal the noticeable reduction in MMP-2 and MMP-9 protein expression when CAL 27 cells were treated with 250 and 500 nM of MJ-29 for 48 h. MJ-29 is likely to reduce the enzymatic activity (Figure 5) and protein levels (Figure 6A) of MMP-2 and MMP-9 in CAL 27 cells *in vitro*.

MJ-29 inhibits mitogen-activated protein kinase (MAPK) and AKT signaling pathways in CAL 27 cells. Previous reports have demonstrated that the involvement of MAPK and AKT signals

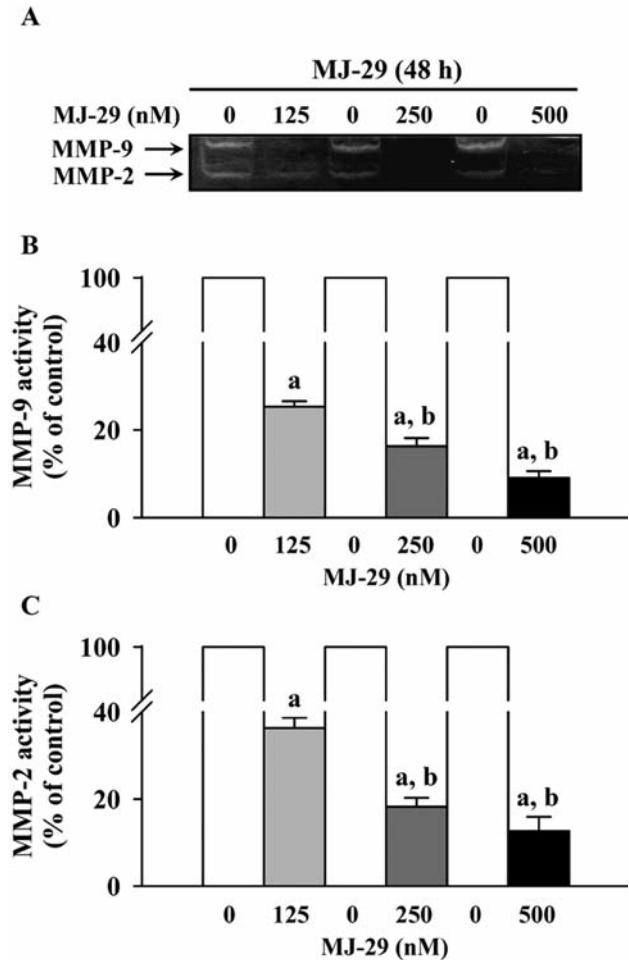


Figure 5. Effects of MJ-29 on the activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 in CAL 27 cells. Cells were incubated with or without 125, 250 and 500 nM of MJ-29 for 48 h and then subjected to gelatin zymography to investigate the MMP-2/9 activity (A). Quantitative results of MMP-9 (B) and MMP-2 (C) enzymatic actions were measured using the NIH ImageJ. The values represented the means \pm S.D. of three independent experiments. a, significantly different at $p < 0.05$ from the control; b, significantly different from treatments with 125 nM of MJ-29 by one-way ANOVA followed by Bonferroni's multiple comparison test. Similar results were obtained from three independent experiments.

not only might be essential for the expression of MMPs but also contribute to altering cell invasion and migration during tumor metastasis (15, 37, 38). We further sought to determine the mechanisms of action for the anti-metastatic effects of MJ-29. After exposure to 250 and 500 nM of MJ-29 for 12 h, CAL 27 cells were collected and assayed utilizing western blotting. We found that treating CAL 27 cells with MJ-29 greatly suppressed the protein expression of RAS, FAK and phospho-AKT (Ser473), but not the one of AKT (Figure 6B). Additionally, no significant alterations were observed in the MAPKs signaling

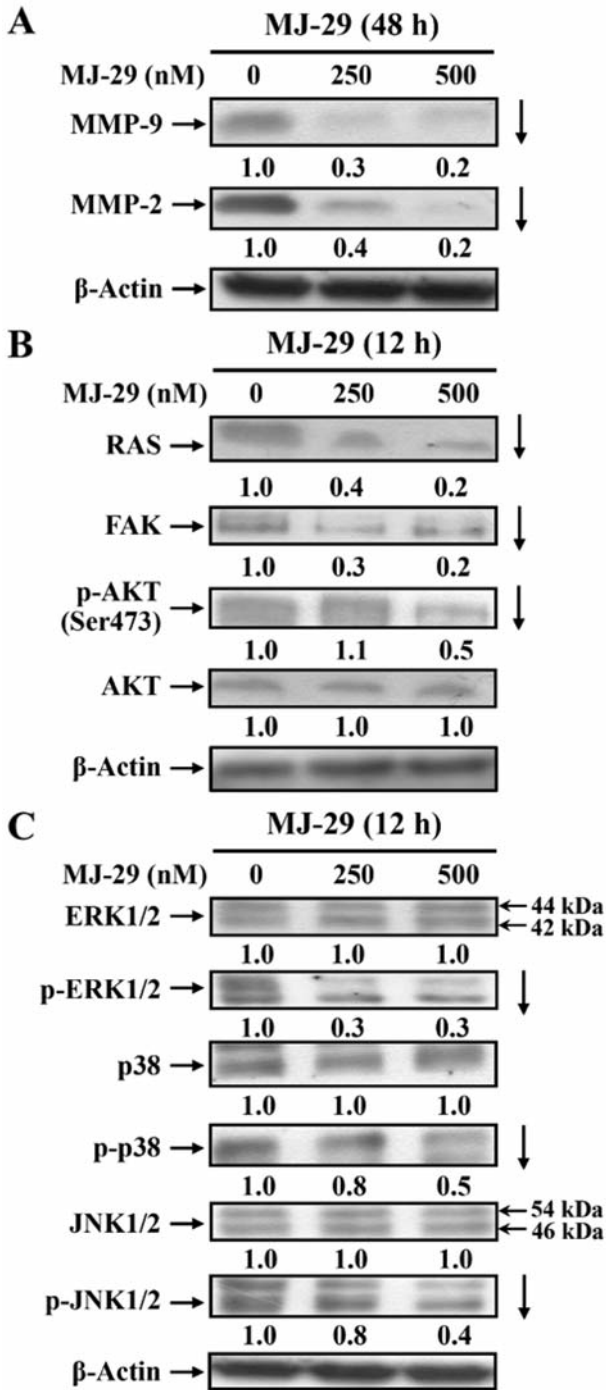


Figure 6. Effects of MJ-29 on the migration and invasion-associated protein expression of CAL 27 cells. Cells were exposed to 0, 250 and 500 nM of MJ-29 for the indicated intervals of time, and cell lysates were thereafter subjected to western blot analysis, as described in the Materials and Methods. The protein expression of matrix metalloproteinase-9 (MMP-9) and MMP-2 (A), RAS, focal adhesion kinase (FAK), p-AKT (Ser473) and AKT (B), and extracellular regulated protein kinase 1/2 (ERK1/2), p-ERK1/2, p38, p-p38, c-Jun N-terminal kinase 1/2 (JNK1/2) and p-JNK1/2 (C) was determined by using specific antibodies, and adjusted for equivalent loading using the β-Actin protein level. Representative data are shown; three separate experiments had similar results.

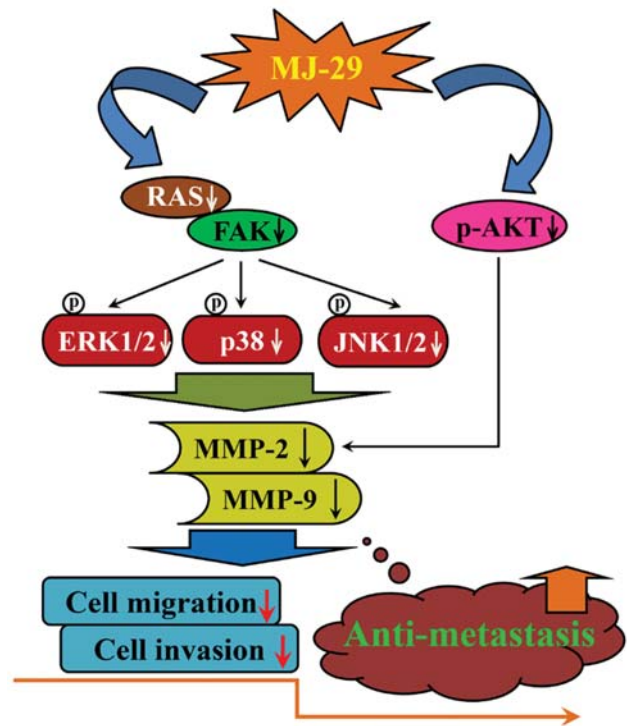


Figure 7. Schematic diagram for proposed signaling pathways in the inhibitory mechanisms of MJ-29 on human oral squamous cell carcinoma CAL 27 cells in vitro. MJ-29 suppresses the levels of RAS, focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAPKs) [phosphorylation of extracellular regulated protein kinase 1/2 (ERK1/2), p38 and c-Jun N-terminal kinase 1/2 (JNK1/2)] and furthermore inhibits matrix metalloproteinase-2 (MMP-2) and MMP-9, leading to reduced CAL 27 cell invasion and migration. Additionally, a decrease of the expression of MMP-2/9 by MJ-29 occurs partially through down-regulating AKT phosphorylation at serine 473 in CAL 27 cells in vitro.

protein levels (ERK1/2, p38 and JNK1/2), while the phosphorylation of ERK1/2, p38 and JNK1/2 proteins were dramatically inhibited by MJ-29 in CAL 27 cells, in comparison to the control (Figure 6C). Hence, the data show that the inhibition of the expression and activity of MMP-2 and MMP-9, by MJ-29 in CAL 27 cells, could be carried out through suppression of ERK, p38 and JNK MAPK-regulated pathways.

To summarize, our study proposes that MJ-29 might block the migration and invasion of human oral cancer CAL 27 cells through inhibiting p-AKT, as well as by suppressing MAPK signaling.

Discussion

We previously reported that 2-phenyl-6-pyrrolidinyl-4-quinazolinone and its synthetic 4-quinazolinone derivatives, which were designed and synthesized in our cooperative laboratory were found to possess antimitotic actions and

antitumor activities in colorectal, lung, ovarian, oral, prostate and breast cancer, glioblastoma, osteosarcoma, melanoma and leukemia (19-22). Among them, MJ-29 exhibited more potent tumor suppression activity against various types of cancer cell lines (20-22). Our earlier work demonstrated that MJ-29 suppressed tubulin polymerization, induced mitotic arrest and provoked apoptosis of human leukemia U937 cells, as well as inhibited the growth of U937 cells in a xenograft mouse model (20). In the present study, a high-motility OSCC cell line, CAL 27 (39) was applied as a screening system to study the anti-metastatic effects of MJ-29 *in vitro*.

Metastasis, a characteristic feature of malignant tumor, is known to bring about various alterations that involve marked events of intercellular degradation of the ECM, invasion and migration during progression of the tumor (3, 13, 40). We investigated whether MJ-29 affects these metastatic actions in CAL 27 cells, and our findings revealed that MJ-29 at the concentrations of 125-500 nM induced a significant inhibition of cell adhesion (Figure 2), migration (Figure 3A) and invasion (Figure 3B), and these effects were concentration-dependent. The previous study showed that MJ-29 exerted less cytotoxicity toward normal cells, including peripheral blood mononuclear cells and human umbilical vein endothelial cells (20). Herein, we found that MJ-29 did not significantly affect cell viability of CAL 27 cells at low concentrations (less than 500 nM) (Figure 4). Our results indicated that the inhibitory effects of MJ-29 on CAL 27 cell metastatic processes which we determined, are independent of cellular cytotoxicity. Thus, MJ-29 might have use as a potential treatment for metastasis and it could be a chemotherapeutic agent praiseworthy of further development for treatment of OSCC.

It has been reported that the overexpression and activity of MMPs facilitate the invasion and spread of tumor cells to surrounding or distal tissue (17, 41). MMP-2 and MMP-9 are vital type IV collagenases and gelatinases of basement membrane of type IV collagen, gelatin and fibronectin during tumor invasion and metastasis (14, 42). Our results demonstrated that MJ-29 dramatically inhibited MMP-2 and MMP-9 enzymatic activity (Figure 5) by gelatin zymography and their protein expression (Figure 6A) by western blot in CAL 27 cells. Additionally, previous evidence showed that serine/threonine kinase AKT and MAPK signaling pathways regulate a variety of genes whose products are correlated with cell growth, invasion, inflammation, apoptosis and autophagy (43, 44). Increased serum levels of MMP-2 and MMP-9 occur in patients with oral cancer (45), and overexpression of MMP-2 and MMP-9 in oral tumor tissues is related to invasion and infiltration of OSCC cells (17, 41). Modulating the activation of transcription factors such as p70^{S6K} and activator protein 1 (AP-1) through RAS/MAPK and AKT signal pathways can regulate MMP-2 and MMP-9 gene expression. The p70^{S6K} and AP-1 elements of MMP-2 and MMP-9 promoter are

centrally involved in the induction of genes associated with the invasion of tumor cells (46-48). In our study, data shown in Figures 6B and C indicate that the levels of RAS, FAK and phospho-AKT (Ser473) were decreased (Figure 6B), and these of p-ERK1/2, p-p38 and p-JNK1/2 were also observed to be inhibited (Figure 6C) in MJ-29-treated CAL 27 cells. On the basis of these results, we suggest that MJ-29 inhibits CAL 27 cell invasion and migration through suppressing AKT^{Ser473} phosphorylation and MAPK signaling pathways.

In conclusion, this is the first study to demonstrate that MJ-29 is capable of inhibiting cell adhesion, migration and invasion through the down-regulation of MMP-2 and MMP-9 in human OSCC. We further showed that the phosphorylation of AKT and MAPK signaling pathway proteins may be coordinately involved in the inhibitory effects on these essential steps of metastasis by MJ-29. Our proposed signaling route in the key regulatory mechanisms of CAL 27 cells, affected by MJ-29, can be seen in Figure 7. Further study of MJ-29 and its on OSCC in an animal model is needed to clarify the exact mechanism(s) involved in its anti-metastatic effects.

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