Dual inhibition of EGFR and c-Met kinase activation by MJ-56 reduces metastasis of HT29 human colorectal cancer cells

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Abstract. Quinazolinone derivatives are known to possess anti-cancer activities on cell metastasis and cell death in different human cancer cell lines. Here, we studied the anti-metastasis activity and the underlying mechanisms of the novel quinazoline derivative MJ-56 (6-pyrrolidinyl-2-(3-bromostyryl) quinazolin-4-one). MJ-56 inhibited cell migration and invasion of HT29 human colorectal cancer cells by wound-healing and Matrigel-coated transwell assays in a concentration-dependent manner. MJ-56-treated cells resulted in the reduced expression of matrix metalloproteinase (MMP)-2, -7, -9 and -10 and the reduced enzymatic activities of MMP-2 and MMP-9. In contrast, MJ-56-treated cells enhanced the expression of the tissue inhibitors of metalloproteinases (TIMPs) TIMP-1 and TIMP-2. Further analyses showed that MJ-56 attenuated the activities of epidermal growth factor receptor (EGFR), c-Met and the downstream ERK-mediated MAPK and PI3K/AKT/ mTOR signaling pathways, which led to decreased protein synthesis by dephosphorylating the translation initiation factors eIF-4B, eIF-4E, eIF-4G and S6 ribosomal protein. In addition, MJ-56 interfered with the NF-κB signaling via impairing PI3K/AKT activation and subsequently reduced the NF-κB-mediated transcription of MMPs. Taken together, the reduced expression of phosphor-EGFR and c-MET is chiefly responsible for all events of blocking metastasis. Our results suggest a potential role of MJ-56 on therapy of colorectal cancer metastasis.

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Introduction

Colorectal cancer is a commonly diagnosed cancer and one of the leading causes of cancer death in the world (1,2). Metastasis is one of the major causes of deaths of colorectal cancer (3). Metastasis occurs through a stepwise process that starts when cancer cells segregate from a primary tumor, migrate across blood vessel walls then into the blood stream and invade into tissue (4-6). Finally, cancer cells disperse throughout new tissues to survive in the new ectopic sites and generate malignant tumors (1-3,6).

Matrix metalloproteinases (MMPs), a group of secreted proteinases, play an important role in a variety of physiological and pathological processing colorectal cancer cell metastasis (7-10). MMPs degrade extracellular matrix (ECM) components in the basement membrane, increase cell invasion and metastasis in colorectal cancer cells (8-12). Many studies have shown that the functions of various metastatic molecules are modulated by MMPs (7,13-15). It has been reported that elevated MMP-2, MMP-7 and MMP-9 protein levels were detected in colorectal cancer. The correlation of MMP-2, MMP-7 and MMP-9 expression with invasion and metastasis has been demonstrated (9,13,14,16,17). It is well established that receptor tyrosine kinase (RTK) activates the phosphatidylinositol 3-kinase (PI3K)/AKT and Ras-Raf-mitogen-activated protein kinase (MAPK) pathways, resulting in the activation of eIF-4B, eIF-4E, eIF-4G and S6 and NF-κB transcription factors (18-23). Those transcription factors are considered to be the regulator of MMP-2, MMP-7 and MMP-9 translation signaling (24,25).

Quinazolinone derivatives have shown anti-malarial, anti-inflammatory, anti-bacterial and antitumor activities in many studies (26,27). Quinazolinone compounds have also been shown to possess antitumor activity to inhibit cell metastasis by downregulating the expression and activities of MMP-2 and MMP-9 in many cancer cell lines (28-30). Our previous study

showed 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) (29). The 2-(3-ethoxyphenyl)-6-pyrrolidinylquinazolinone) (MJ-33) exhibits anti-metastatic effects in human prostate carcinoma DU145 cells via decreased protein levels of MAPKs (mitogen-activated protein kinases), AKT, AP-1 and NF-κB, resulting in the inhibition of MMP-2 and MMP-9 (28). Receptor tyrosine kinases have been indicated as promising molecular targets for cancer therapy (31-34). The c-Met, a receptor tyrosine kinase, is overexpressed and/or mutated in a variety of tumor cells (31,35). Abnormal activation of c-Met signaling can lead to angiogenesis, proliferation, invasion and metastasis (36-38). EGFR, epidermal growth factor receptor, functions with a vital role in colorectal cancer initiation and progression (39,40). Although c-Met and EGFR are recognized as important therapeutic targets for the treatment of malignancies, the inhibitory effect of c-Met and EGFR in human colorectal cancer cells remains unclear. In the present study, we investigated the anti-metastatic activity of MJ-56 (6-pyrrolidinyl-2-(3-bromostyryl)quinazolin-4-one) (Fig. 1), a novel synthesized quinazolinone derivate and the anti-metastatic pathways of MJ-56 in the human colorectal cancer cell line HT29.

Materials and methods

Chemicals and reagents. MJ-56 was designed and synthesized by Mann-Jen Hour from China Medical University, Taichung, Taiwan (Fig. 1). Antibodies against MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, TIMP-1, TIMP-2, p85-PI3K, p110-PI3K, p65 NF-κB, PCNA, p38, GAPDH (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA), EGFR, p-EGFR (Tyr1173), c-Met, p-S6 (Ser240/244), p-Mnk1 (Thr197/202), p-eIF4E (Ser209), p-eIF4G (Ser1108), p-eIF4B (Ser422), p-JNK (Thr183/Tyr185), JNK, p-p38 (Thr183/Tyr185), p-ERK (Thr202/Tyr204), ERK, p-mTOR (Ser2448), mTOR, p-AKT (Ser473), AKT (Cell Signaling; Danvers, MA, USA) were obtained from the indicated vendors. Rabbit anti-mouse IgG (HRP) antibody, goat anti-rabbit IgG (HRP) antibody, donkey anti-goat IgG (HRP) antibody and FITC-conjugated goat anti-mouse antibody were obtained from Santa Cruz Biotechnology, Inc. Propidium iodide (PI) was from Sigma-Aldrich (St. Louis, MO, USA). DMEM medium, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

Cell culture. Human colorectal cancer cell line, HT29, was purchased from Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). HT29 cells were cultured in DMEM medium supplemented with 10% of fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine and incubated at 37˚C in a humidified chamber with 5% CO_2 (24).

Wound healing assay. HT29 cells (cell density: $1x10^6$ cells/well) were seeded 6-well plate and grown to 90% of confluence. The next day, cells were scratched with a yellow tip and treated with various concentrations of MJ-56 (0, 5, 10 and

15 μM) in DMEM serum-free medium for 24 h. The cells were captured and relative cell migration was calculated. All treatments were in duplicate and three independent experiments were performed (4,28,30).

Cell invasion assay. The membrane of transwell insert was rinsed with PBS and coated with Matrigel (BD Matrigel™ Invasion chamber). Cells were seeded into the chamber of the insert at a density of $2.5x10^4$ cells/ml and incubated with 0.5 ml of complete DMEM medium in the transwell. HT29 cells were treated with various concentrations of MJ-56 (0, 5, 10 and 15 μM) for 24 h and cells inside the chamber were removed. Invaded cells were fixed with 4% formaldehyde in PBS and stained with 0.1% of hematoxylin (Sigma-Aldrich), captured and the number of invaded cells were counted and used for the calculation of inhibitory rate (28,30).

Gelatin zymography analysis. HT29 cells (cell density: $1x10^6$ cells/well) were seeded into 6-well plate and grew for 24 h. Cells were treated with various concentrations of MJ-56 (0, 5, 10 and 15 μM) in serum-free DMEM medium for 24 h. Culture medium was collected and spun at 1000 x g for 10 min at 4˚C. Supernatant was collected and 5 μg of total protein was mixed with 2X sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.01% bromophenol blue) and separated in an 8% SDS-polyacrylamide gel with 1% gelatin. Gel was treated with 2.5% Triton-X-100 at room temperature for 30 min to remove SDS, incubated in Zymogen developing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35; Bio-Rad Laboratories; Hercules, CA, USA) at room temperature for 30 min, then refreshed with Zymogen developing buffer and incubated at 37˚C for 24 h. Gel was rinsed with water once and stained with 0.5% of Coomassie blue R-250 (0.5% Coomassie blue R-250, 50% methanol and 10% acetic acid) for 2 h and de-stained in de-staining solution (50% methanol and 10% acetic acid) until clear zones were visualized (28,30).

Preparation of whole cell lysate and nuclear lysate. HT29 cells were treated with various concentrations of MJ-56 for the given time and cells were harvested for the preparation of whole cell lysate using iced-cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) supplemented with the protease inhibitors including phenylmethanesulfonyl fluoride (10 mg/ml), leupeptin (17 mg/ml) and sodium orthovanadate (10 mg/ml). Cells were vortexed briefly and incubated in ice for 30 min and cell lysate was collected by a spin at 12,000 x g at 4˚C for 10 min. Nuclear extracts were obtained by using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific; Rockford, IL, USA). The resulting nuclear pellet was re-suspended in nuclear extraction 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) and incubated in ice for 20 min, then spun at 14,000 x g for 5 min. The supernatant, the soluble nuclear fraction, was collected and used for EMSA analysis of NF-κB and western blot analyses of NF-κB and PCNA (28,30).

Electrophoretic mobility shift assay (EMSA). HT29 cells were seeded at a density of $1x10^6$ cells/ml the day before treatment.

Cells were then treated with 15 μM of MJ-56 for 0 and 4 h. Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) and soluble nuclear fraction was prepared as described above. The protein concentrations were determined by using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Biotin end-labeled oligonucleotide corresponding to consensus NF-κB binding site (5'-GATCCAGGGGACTTTCCCTAGC-3') was prepared with the LightShift Chemiluminescent EMSA kit (Thermo Scientific) and used as the probe. The 5 μg of nuclear extract was incubated with biotin end-labeled duplex DNA, electrophoresed on a 6% polyacrylamide native gel, transferred to a positive nylon membrane, UV cross-linked and incubated with streptavidin-HRP and signals were developed by ECL kit (Millipore) (28,30).

Western blot analysis. Whole cell lysate and nuclear extract were isolated from treated cells as described above, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a nitrocellulose membrane using the iBot Dry Blotting System (Invitrogen Life Technologies). The transferred membranes were incubated in blocking buffer (5% non-fat milk in Tris-buffered saline/Tween-20) for 1 h and incubated with primary antibody in blocking buffer at 4°C overnight. Membranes were washed with Tris-buffered saline/Tween-20 three times for 10 min and incubated with HRP-conjugated secondary antibody for 1 h. Protein signals were revealed by using ECL kit (Millipore) and exposed to Kodak Bio-MAX MR film (Estman Kodak, Rochester, NY, USA) (28,30).

Immuno-fluorescent staining. HT29 cells were seeded onto polylysine-coated slides (cell density: $1x10^5$ cells/ml) the day before staining. On the next day, cells were treated with 15 μM of MJ-56 for 4 h, fixed with 4% formaldehyde in PBS, permeabolized with 0.1% Triton X-100 for 30 min, blocked with 10% normal goat serum for 30 min and probed with primary antibody p65 NF-κB at 4°C overnight. Then, cells were stained for FITC-conjugated goat anti-mouse secondary antibodies for 1 h, washed and mounted in Shandan mounting medium. The images were captured using a fluorescence microscopy (41).

ERK kinase assay. HT29 cells (cell density: $1x10^6$ cells/ml) were treated with 15 μM of MJ-56 for 0-4 h and ERK kinase activity was analyzed using p44/42 MAP Kinase assay kit obtained from Cell Signaling (#9800). The p44/42 MAP Kinase was immuno-precipitated from cell lysate of treated cells with anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody and mixed with Elk-1 fusion protein and ATP in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) and incubated at 37°C for 30 min. The reaction was stopped by boiling in sample buffer and subjected to western blot analysis using anti-phospho-Elk-1 (Ser 383) antibody. The intensity of protein bands was quantified by NIH ImageJ and the intensity of protein band from untreated cells was set as 100% and the intensity of other treatments was calculated accordingly. Shown was the average data from three independent experiments (42,43).
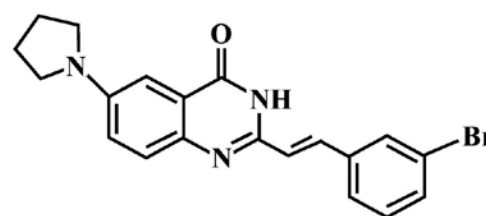
MJ-56

6-pyrrolidinyl-2-(3-bromostyryl)quinazolin-4-one

Figure 1. The chemical structure of MJ-56 (6-pyrrolidinyl-2- (3-bromostyryl) quinazolin-4-one).

AKT kinase assay. HT29 cells (cell density: $1x10^6$ cells/ml) were treated with 15 μM of MJ-56 for 0-4 h and AKT kinase activity was analyzed using AKT Kinase Assay kit from Cell Signaling (#9840). AKT was precipitated from cell lysate of treated cells with anti-AKT antibody and mixed with GSK-3 fusion protein and ATP in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) and incubated at 30°C for 30 min. The reaction was stopped by boiling in sample buffer and subjected to western blot analysis using anti-phospho-GSK3 (Ser219) antibody. The intensity of protein bands was quantified by NIH ImageJ and the intensity of protein band from untreated cells was set as 100% and the intensity of other treatments was calculated accordingly. The average of the data was shown from three independent experiments (44).

Tyrosine kinase assay. HT29 cells (cell density: $1x10^6$ cells/ml) were treated with 15 μM of MJ-56 for 0-4 h and Tyrosine kinase activity was analyzed using Tyrosine Kinase assay kit from Upstate (#17-315) (EMD Millipore Corp., Billerica, MA, USA). Cell lysate was incubated with biotinylated poly (Glu4-Tyr) peptide as the substrate and ATP in a kinase buffer containing Mn^{2+}/Mg^{2+} as the co-factors. Tyrosinated substrate was recognized by anti-phosphotyrosine-HRP and detected by enzyme-linked immunosorbent assay (ELISA). The kinase activity from untreated cells was set as 100% and the kinase activity of other treatments was calculated accordingly. Shown was the average data from three independent experiments (45).

Statistical analysis. The statistical results were expressed as the mean ± SEM of triplicate samples. The difference between groups was analyzed by One-way ANOVA followed by paired two-tailed Student's t-test and *$P<0.05$ was taken as significant.

Results

MJ-56 inhibits migration and invasion of HT29 cells. Quinazolinone derivatives have been shown to possess antitumor activities (26,30,32). To obtain more effective drugs for cancer treatment, we have chemically modified the quinazolinone derivatives. MJ-56, a novel quinazolinone derivative, was synthesized (Fig. 1) and determined its antitumor activity by assaying cell migration and invasion in HT29 colorectal carcinoma cells. HT29 cells were treated with various concen-
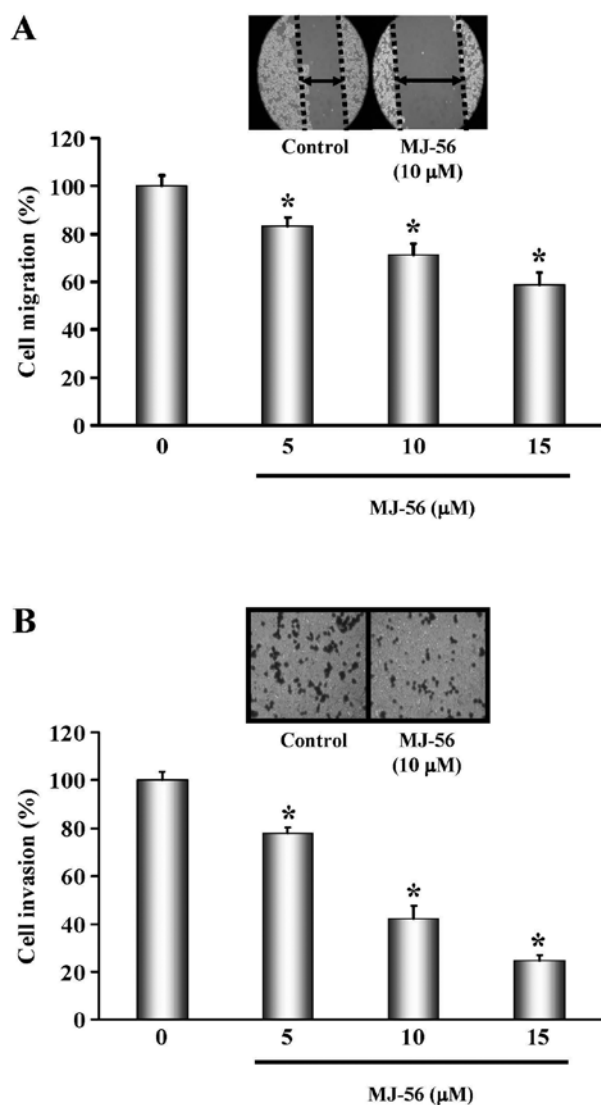
Figure 2. MJ-56 inhibits the migration and invasion of HT29 cells. (A) Migration of cells in a monolayer was measured using the scratch wound healing assay. Cells were seeded in a 6-well plate. After 24 h of growth, cells were scratched with a pipette tip and incubated with FBS free DMEM medium containing with or without MJ-56 (0, 5, 10 and 15 μM) for 24 h. Cell migration was calculated. Representative images are shown in the top panel. (B) Cell invasion was detected by Matrigel. Cells were seeded in a 24-well plate and incubated with FBS free DMEM medium with or without MJ-56 (0, 5, 10 and 15 μM) for 24 h. After 24 h of growth, cells penetrated through the Matrigel to the lower surface of the filter were stained with crystal violet and counted under a light microscope at x200. Representative images are shown in the top panel. Each point is mean ± SD of three experiments. *p<0.05, indicates a significant difference from the control.

trations (0, 5, 10 and 15 μM) of MJ-56 for 24 h. The effect of MJ-56 on the cell migration was determined by the scratch assay. As shown in Fig. 2A, migration of HT29 cells was gradually impeded with an increase in the concentrations of MJ-56. Cell invasion is a critical step during cancer metastasis. To characterize whether MJ-56 inhibits cell invasion, HT29 cells were treated with various concentrations (0, 5, 10 and 15 μM) of MJ-56 and the effects of MJ-56 on cell invasion were examined by the Matrigel-coated transwell assay. MJ-56 inhibited invasion of HT29 cells in a concentration-dependent manner. The inhibitory rate was calculated as the percentage

of 21.88, 54.24 and 75.58% for 5, 10 and 15 μM of MJ-56-treated cells, respectively (Fig. 2B).

MJ-56 inhibits the expression and enzyme activity of matrix metalloproteinases and increases the expression of metalloproteinase inhibitors TIMP-1 and TIMP-2. During cancer metastasis, matrix metalloproteinases (MMPs) are indispensible for the degradation of extracellular matrix, an important step for cancer invasion. Due to the inhibitory effects of MJ-56 on cell migration and invasion, we subsequently determined whether MJ-56 may block the expression and enzymatic activity of MMPs. HT29 ells were treated with various concentrations of MJ-56 (0, 5, 10 and 15 μM) for 24 h and cells were harvested for western blot analyses. MJ-56 suppressed the expression of matrix metalloproteinases MMP-2, MMP-7, MMP-9 and MMP-10 in a concentration-dependent manner (Fig. 3A). However, the expression of MMP-1 appeared not to be affected by MJ-56 treatment (top row, Fig. 3A). In contrast, TIMP-1 and TIMP-2 are metalloproteinase inhibitors that can block the enzymatic activities of MMPs, thereby inhibiting cancer invasion. The expression of TIMP-1 and TIMP-2 was elevated with the increase in the concentrations of MJ-56 (0-15 μM) in HT29 cells (Fig. 3B). Concomitantly, MJ-56 inhibited the enzymatic activities of MMP-2 and MMP-9 by gelatin zymography analysis (Fig. 3C). The MMP-9 activity was significantly decreased at 5 μM of MJ-56 and the inhibition of MMP-2 activity was observed at 15 μM of MJ-56. Our data revealed that MJ-56 may upregulate the expression of TIMP-1 and TIMP-2, downregulate the expression of MMP-2, -7, -9, -10 and reduce the enzymatic activities of MMP-2 and MMP-9 in HT29 cells, which might be responsible for the concentration-dependent inhibition of cell invasion by MJ-56.

MJ-56 inhibits tyrosine phosphorylation of EGFR and expression of c-MET. It has been reported that the activation of autophosphorylation on the receptor tyrosine kinase EGFR can activate the downstream signaling events to increase the expression of MMP family proteins such as MMP-2, MMP-7 and MMP-9, contributing to metastasis and invasion of colorectal cancers (46-48). We determined the effects of MJ-56 on the inhibition of tyrosine kinase activity. HT29 cells were treated with 15 μM of MJ-56 for 0-4 h and tyrosine kinase activity was examined by colorimetric detection of protein tyrosine kinase activity. As shown in Fig. 4A, MJ-56 reduced the tyrosine kinase activity of treated cells in a time-dependent manner, with >60% of inhibition after 1 h of treatment. As tyrosine kinase activity was inhibited by MJ-56, we then determined whether MJ-56 acts on the inhibition of receptor tyrosine kinase activity of EGFR, a receptor protein that undergoes auto-phosphorylation via its intrinsic tyrosine kinase activity after activation. HT29 cells were treated with 15 μM of MJ-56 at various time points (0, 0.5 and 1 h) and tyrosine phosphorylation and expression of EGFR was examined by western blotting. As shown in Fig. 4B, MJ-56 inhibited the tyrosine phosphorylation of EGFR and c-Met in a time-dependent fashion, while the protein level of EGFR remained unchanged (second row, Fig. 4B). Given that MJ-56 inhibited the tyrosine kinase activity of receptor tyrosine kinase EGFR, we inferred that MJ-56 would be able to inhibit other receptor tyrosine kinases such as c-Met. MJ-56 also decreased the
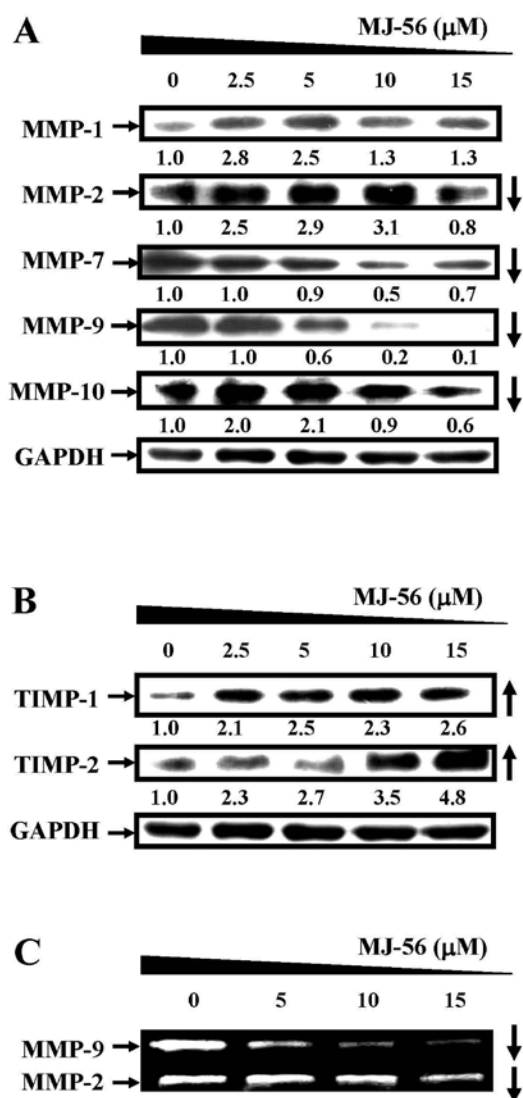
Figure 3. MJ-56 regulates MMPs and TIMPs. (A) The protein expression levels of matrix metalloproteinases MMP-2, MMP-7, MMP-9 and MMP-10 were downregulated in the presence of MJ-56 by western blot analysis. Protein fold changes were normalized to the endogenous GAPDH control. (B) The protein expression levels of metalloproteinase inhibitors TIMP-1 and TIMP-2 were upregulated in the presence of MJ-56 by western blot analysis. Protein fold changes were normalized to the endogenous GAPDH control. (C) Cells were treated with various concentrations (0, 5, 10 and 15 μM) of MJ-56 for 24 h. MMP-2 and MMP-9 activities were quantified by densitomeric analysis. The densitomeric data were expressed as mean ± SD of three independent experiments. *p<0.05, indicates a significant difference from the control.
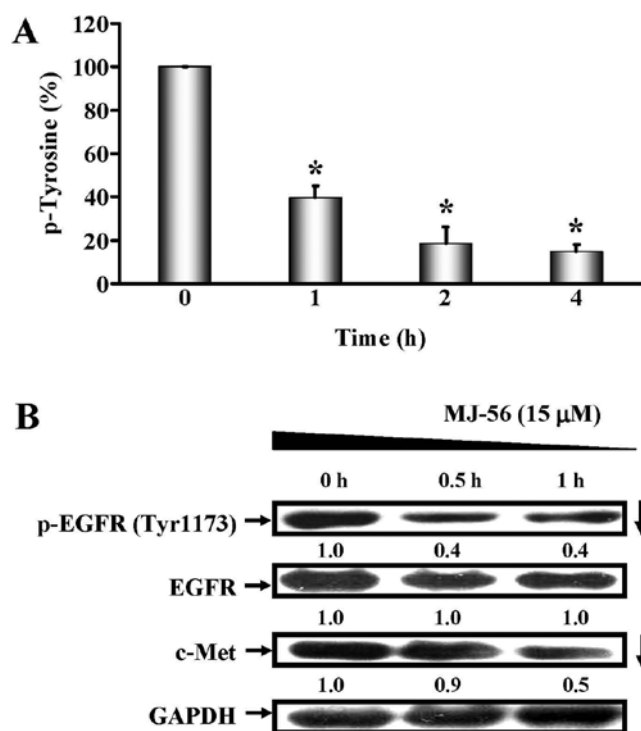

Figure 4. MJ-56 inhibits tyrosine kinase activity and suppresses protein expression levels of phosphorylated EGFR and c-MET. (A) Treatment of HT-29 with MJ-56 resulted in a decrease in tyrosine kinase activity by colorimetric detection of protein tyrosine kinase activity. The percentage of tyrosine kinase activity in MJ-56 treatment was compared with that in no treatment control. The percentage of tyrosine kinase activity was expressed as mean ± SD of three independent experiments. *p<0.05, indicates a significant difference from the control. (B) The protein expression levels of p-EGFR (Tyr1173) and c-Met were downregulated in the presence of MJ-56 by western blot analysis. Protein fold changes were normalized to the endogenous GAPDH control.

expression of receptor tyrosine kinase Met (third row, Fig. 4B). Our results suggest MJ-56 can inhibit receptor tyrosine kinase by targeting on the tyrosine kinase activity of EGFR and reducing the expression of c-Met receptor tyrosine kinase.

MJ-56 inhibits the ERK-mediated MAPK signaling pathway in HT29 cells. Aforementioned data showed that MJ-56 can inhibit the tyrosine kinase activity of EGFR and the expression of MMPs, thus we investigated whether the inhibition in the tyrosine kinase activity of EGFR and c-Met by MJ-56 results in the inhibition of downstream signaling, thereby reducing the expression of MMPs. We determined the effects

of MJ-56 on the MAPK signaling pathway downstream of EGFR, which includes ERK1/2, c-Jun N-terminal kinase and p38 kinase signaling pathways. HT29 cells were treated with 15 μM of MJ-56 at various time-points (0, 0.5, 1, 3 and 6 h). The expression and phosphorylation status of JNK, p38 and ERK were evaluated by western blot analyses. As shown in Fig. 5A, MJ-56 decreased the phosphorylation of ERK, while the expression of ERK remained largely unaffected. However, MJ-56 did not affect the activation of p38 and JNK. Since phosphorylation of ERK is required for the activation of its kinase activity, we examined whether the MJ-56-mediated inhibition of ERK phosphorylation will modulate ERK kinase activity. HT-29 cells were treated with 15 μM of MJ-56 for 0-4 h and ERK kinase activity was assessed. The results showed that MJ-56 significantly inhibited ERK kinase activity in a time-dependent manner (Fig. 5B). Thus, our data indicated that MJ-56 inhibits the ERK-mediated MAPK signaling pathway.

MJ-56 inhibits the PI3K/AKT/mTOR-dependent signaling pathway in HT29 cells. In addition to MAPK signaling pathway, PI3K/AKT/mTOR-dependent signaling pathway is also a downstream signaling pathway of EGFR. To examine the effects of MJ-56 on the PI3K/AKT/mTOR signaling pathway, HT29 cells were treated with 15 μM of MJ-56 for different time (0-6 h), and western blot analyses were performed. The
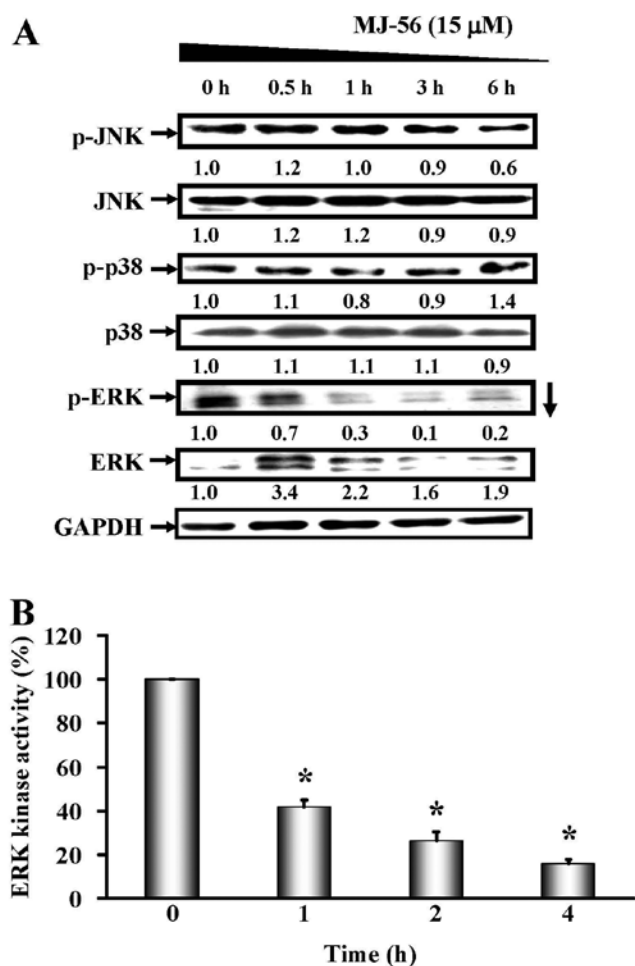
A

B

Figure 5. MJ-56 blocks ERK-mediated MAPK signaling pathway in HT29 cells. (A) Cells were harvested at 0, 0.5, 1, 3 and 6 h after MJ-56 treatment. The protein levels of p-JNK, JNK, p-p38, p38, p-ERK and ERK were analyzed by western blotting. Protein fold changes were normalized to the endogenous GAPDH control. (B) Cells were harvested at 0, 1, 2 and 4 h after MJ-56 treatment. Cell lysates were immuno-precipitated with anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody and assessed ERK kinase activity by measuring phosphorylation of Elk-1. ERK kinase activity in MJ-56 treatment was compared with that in no treatment control. ERK kinase activity was expressed as mean ± SD of three independent experiments. *p<0.05, indicates a significant difference from the control.
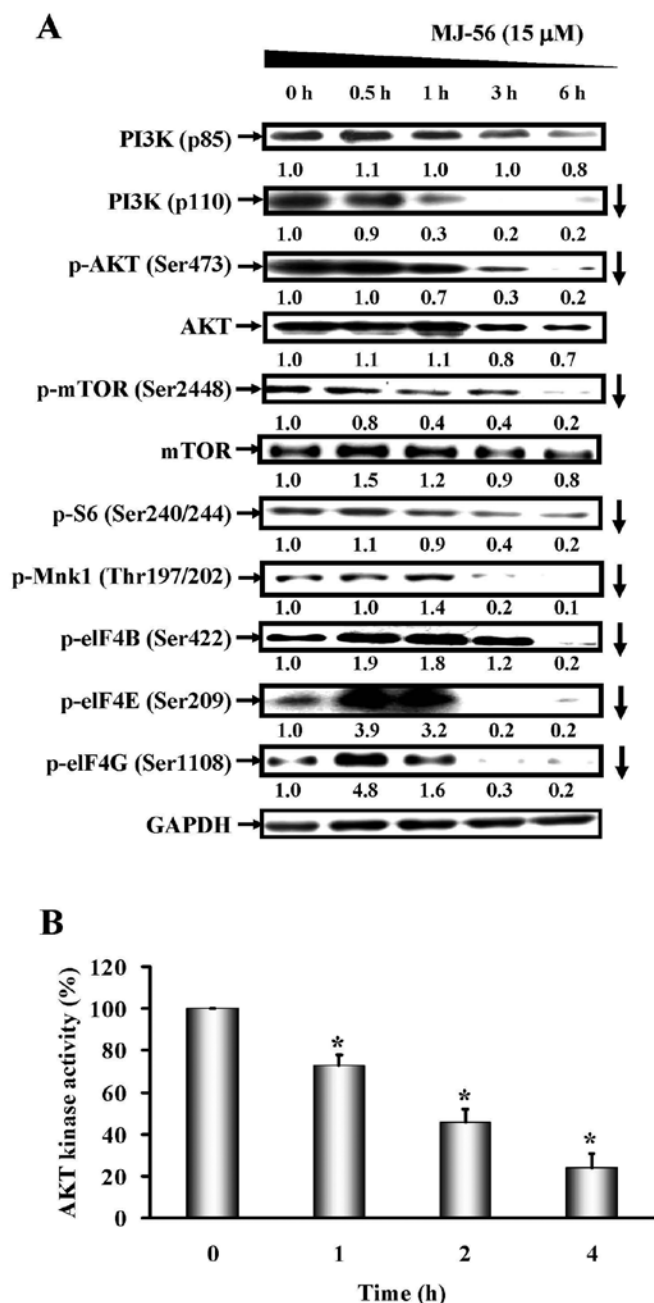
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Figure 6. MJ-56 modulates PI3K/AKT/mTOR-dependent signaling pathway in HT29 cells. (A) Cells were harvested at 0, 0.5, 1, 3 and 6 h after MJ-56 treatment. The protein levels of p110-PI3K, p85-PI3K, p-AKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, p-S6 (Ser240/244), p-Mnk1 (Thr197/202), p-eIF4B (Ser422), p-eIF4E (Ser209) and p-eIF4G (Ser1108) were analyzed by western blotting. Protein fold changes were normalized to the endogenous GAPDH control. (B) Cells were harvested at 0, 1, 2 and 4 h after MJ-56 treatment. Cell lysates were immunoprecipitated with anti-AKT antibody and assessed AKT kinase activity by measuring phosphorylation of GSK-3. AKT kinase activity in MJ-56 treatment was compared with that in no treatment control. AKT kinase activity was expressed as mean ± SD of three independent experiments. *p<0.05, indicates a significant difference from the control.

results showed that the expression of PI3K (p85), the regulatory subunit of PI3K, appeared not to be affected (top row, Fig. 6A). MJ-56 decreased the expression of PI3K (p110), the catalytic subunit of PI3K kinase (second row, Fig. 6A). In addition, MJ-56 reduced the phosphorylation of AKT and mTOR, while the expression of AKT and mTOR seemed not to be altered (Fig. 6A). As phosphorylation of AKT is important for its kinase activity, the reduction in phosphorylation of AKT by MJ-56 implies that the kinase activity of AKT will be affected. As shown in Fig. 6B, MJ-56 indeed inhibited the enzymatic activity of AKT kinase in a time-dependent manner. Therefore, our results suggested that MJ-56 is able to inactivate the PI3K/AKT/mTOR-dependent signaling pathway in HT29 cells. To evaluate whether MJ-56 inhibits the downstream proteins of ERK-mediated MAPK and PI3K/AKT/mTOR signaling pathways, HT29 cells were treated with 15 μM of MJ-56 for 0 to 6 h and the phosphorylation of S6, Mnk1, eIF-4B, eIF-4E and

eIF-4G was examined by western blot analyses. As shown in Fig. 6A, MJ-56 treatment led to a time-dependent inhibition in the phosphorylation of S6, Mnk1, eIF-4B, eIF-4E and eIF-4G, although MJ-56 treatment resulted in different inhibition kinetics for each protein. Our data showed that MJ-56 inhibits the phosphorylation of Mnk1 and eIF-4E, downstream targets
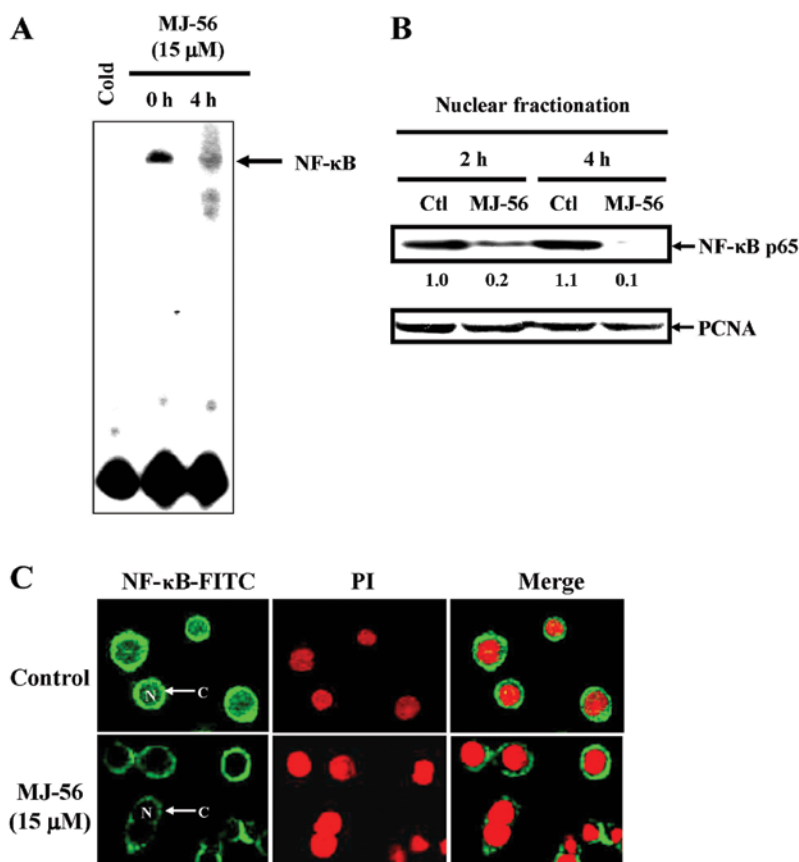
Figure 7. MJ-56 regulates the NF-κB signaling pathway in HT29 cells. (A) MJ-56 affected the DNA binding activities of NF-κB in HT29 cells. Cells were treated with 15 μM of MJ-56 for 4 h. Nuclear extracts were prepared and analyzed for NF-κB DNA binding activity using biotin labeled oligonucleotides containing the NF-κB consensus sequences. EMSA assay was performed as described in Materials and methods. Lane 1, unlabeled consensus oligonucle-otides; lane 2, control; lane 3, 15 μM of MJ-56 for 4 h. Excess free probe is indicated at the bottom. Results from three repeated and separate experiments were similar. (B) NF-κB expression was examined by western blotting. Cells were treated with 15 μM of MJ-56 for 2 and 4 h. Protein fold changes were normalized to the endogenous PCNA control. (C) NF-κB localization was examined by immunostaining. Cells treated with 15 μM of MJ-56 for 4 h were immunostained with FITC-conjugated anti-p65 antibody and propidium iodide (PI). N represents nucleus and C represents cytoplasm.

of ERK-mediated MAPK signaling pathway and S6, elF-4B, elF-4G, downstream targets of PI3K/AKT/mTOR signaling pathway, thereby disturbing translation initiation and protein synthesis at the ribosomes and hence reduced the expression of MMPs.

MJ-56 inhibits the NF-κB signaling pathway in HT29 cells. It is known that PI3K/AKT signaling activates NF-κB. Activated NF-κB mobilizes to the nucleus, binds to the cognate site on the target promoters such as MMPs and turns on their gene expression. To clarify the effects of MJ-56 on the NF-κB signaling pathway, HT-29 cells were treated with 15 μM of MJ-56 for 0 and 4 h. Translocation of NF-κB into the nucleus resulting in binding at NF-κB-responsive elements was accessed by electrophoretic mobility shift assay (EMSA). After HT-29 cells were treated with 15 μM of MJ-56 for 4 h, the amount of NF-κB that binds DNA dramatically blocked due to reduced nuclear translocation of NF-κB (Fig. 7A). Nuclear extract was obtained from MJ-56-treated cells and assessed for western blot analyses of NF-κB p65 (Fig. 7B). The results showed that the mobilization of NF-κB into the nucleus was greatly reduced after 2 h of MJ-56 treatment, as compared to that of control treatment. Consistently, immuno-fluorescent staining also showed that MJ-56 obstructs the translocation of NF-κB
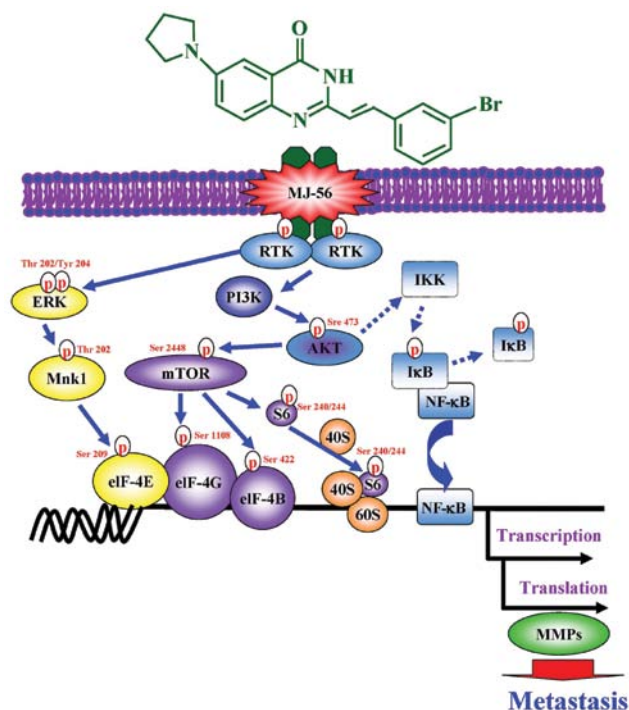

Figure 8. A model for the inhibitory effects of MJ-56 on invasion and migration in HT29.

into the nucleus (Fig. 7C). These data suggest that MJ-56 interferes with the NF-κB signaling and therefore NF-κB target genes such MMPs fail to be expressed.

Discussion

Previous studies demonstrated that quinazolinone derivatives not only exerts anticancer activity against many cancer cell lines *in vitro* and *in vivo*, but also induced cell death through apoptosis or autophagy and inhibited cell metastasis in cancer cells (28,49-52). In our laboratory, a series of quinazolinone derivatives have been designed and synthesized and which are established to have anti-mitotic functions and anticancer activities in colorectal, lung, ovarian, oral, prostate and breast cancer, as well as in glioblastoma, osteosarcoma, melanoma and leukemia (28-30,49,50,53-55). The MJ-56 (6-pyrrolidinyl-2-(3-bromostyryl)quinazolin-4-one), one of the quinazolinone derivatives, exhibits the most potent cytotoxicity against colorectal cancer cell lines including HT29, COLO 205, SW480, SW620 and HCT116 (data not shown). In this study, our results provide detailed evidence that MJ-56 could modulate anti-cell migration and invasion effects and trigger MMPs activities by EGFR and c-Met pathways in HT29 human colorectal cancer cells. MMPs are known to be an accelerator of colorectal cancer cell invasion and metastasis and there are associated with the progression of tumorigenesis (8,9,11,14,24,39,47,48). Our results demonstrated that MJ-56 can inhibit migration and invasion of HT29 cells (Fig. 2). MJ-56 reduced the protein levels of MMP-2, MMP-7, MMP-9, MMP-10 (Fig. 3A) as well as increased the protein levels of metalloproteinase inhibitors TIMP-1 and TIMP-2 by western blot analyses (Fig. 3B). MJ-56 also reduced the enzymatic activities of MMP-2 and MMP-9 by gelatin zymography assays (Fig. 3C).

Interference with receptor tyrosine kinase provides a novel approach in cancer therapy agents (56-59). Activation of the EGFR promotes processes responsible for cancer cell proliferation, angiogenesis, invasion, metastasis and inhibition of cell death (60-63). The agents targeting members of the human epidermal growth factor receptor (EGFR) have shown hopeful therapeutic efficacy (58,64). Cetuximab (Erbitux) is a clinical success of selective EGFR inhibitor. Cetuximab improves the effectiveness of treatment for metastatic colorectal cancer (64-66). In this study, we demonstrated that MJ-56 is an EGFR and c-Met receptor tyrosine kinase inhibition agent using western blot and kinase assay analyses (Fig. 4). EGFR and c-Met are phosphorylated, which turn on downstream intracellular signaling cascades such as MAPK, PI3K/AKT/mTOR and NF-κB pathways. Our results suggested that inhibition of EGFR and c-Met receptor tyrosine kinase activity by MJ-56 might be suitable for novel targeted therapy of colorectal cancer.

MMP promoters have several regulatory motifs recognized by various proteins such as NF-κB, S6, eIF-4B, eIF-4E and eIF-4G. The NF-κB protein binding to the MMP-2, MMP-7 and MMP-9 promoter is centrally involved in the induction of *MMP-2, MMP-7* and *MMP-9* gene expression associated with cell invasion (24,28,30,46). On the other hand, the S6, eIF-4B, eIF-4E and eIF-4G binding to the MMP-7 promoter are centrally involved in the induction of

MMP-7 gene expression (24). Multiple pathways leading to activation of those transcription factors in cancer cells may contribute to MMP transcription and metastatic enhancement (67-72). MJ-56 reduced the protein levels of MMPs associated with inactivation of ERK (Fig. 5) and AKT (Fig. 6) as well as displayed inhibitory effects on NF-κB, S6, eIF-4B, eIF-4E and eIF-4G (Fig. 6). The inhibitory effect on nuclear entry of NF-κB was consistent with less DNA binding activity of NF-κB (Figs. 7B and C). ERK is intricately involved in the expression of the components that are associated with MMP promoter induction through eIF-4E and its relation with Mnk1 phosphorylation (73-76). Activation of mTOR through the PI3K/AKT pathway leads to the phosphorylation of 4EBP1 and S6K1. The 4EBP1 phosphorylation inhibits the translation by interrupting the binding of eIF4E with eIF4G to form an eIF4F translation initiation complex that consists of eIF4A, 4G and 4E. Activated S6K1 causes phosphorylation of S6 and eIF4B, which, in turn, result in an increase in eIF4A RNA helicase activity (24,77-82). We suggested that PI3K/AKT/mTOR and ERK1/2 are the master regulators of translation initiation in MMPs. Inhibition of the ERK and PI3K/AKT pathways might have the potential of preventing cancer cell invasion and migration.

A model was proposed to elucidate molecular mechanisms by which MJ-56 suppresses cell migration, invasion and MMP activity in HT-29 human colorectal cancer cells (Fig. 8). In conclusion, MJ-56 might inhibit invasion and migration of HT29 cells through targeting the receptor tyrosine kinases, EGFR and c-Met. Thereafter, ERK and PI3K/AKT/mTOR signaling pathways are inactive. NF-κB signaling pathway is blocked, which leads to blocking of the transcription and translation of matrix metalloproteinases.

Acknowledgements

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