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Benzyl Isothiocyanate (BITC) Inhibits Migration and Invasion of Human Colon Cancer HT29 Cells by Inhibiting Matrix Metalloproteinase-2/-9 and Urokinase Plasminogen (uPA) through PKC and MAPK Signaling Pathway

Kuang-Chi Lai,[†,‡] An-Cheng Huang,[§] Shu-Chun Hsu,[∥] Chao-Lin Kuo,[⊥] Jai-Sing Yang,[#] Shin-Hwar Wu,[▽,○] and Jing-Gung Chung*[,◆,ᶻ]

[†]Department of Surgery, China Medical University Beigang Hospital, Yunlin, Taiwan, [‡]School of Medicine, China Medical University, Taichung, Taiwan, [§]Department of Nursing, St. Mary's Medicine Nursing and Management College, Yilan, Taiwan, [∥]Department of Chemical Engineering, Hsiuping Institute of Technology, Taichung, Taiwan, [⊥]School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan, [#]Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan, [▽]Division of Critical Care Medicine, Department of Internal Medicine, Changhua Christian Hospital, Changhua, Taiwan, [○]Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan, [◆]Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, and [ᶻ]Department of Biotechnology, Asia University, Taichung, Taiwan

Benzyl isothiocyanate (BITC), a component of dietary cruciferous vegetables, has antioxidant and anticancer properties. In this study, we show for the first time the antimetastatic effects of BITC in human colon cancer HT29 cells. BITC had an inhibitory effect on cell migration and invasion. Protein levels of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and urokinase-plasminogen activator (u-PA) were reduced by BITC in a concentration-dependent manner. BITC also exerted an inhibitory effect on phosphorylation of c-Jun N-terminal kinase 1 and 2 (JNK1/2), extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) that are upstream of nuclear factor kappa B (NF-κB). BITC inhibited DNA binding activity of NF-κB. Moreover, BITC decreased the levels of c-Fos, c-Jun, Ras, FAK, PI3K and GRB2 in HT29 cells. Reductions in the enzyme activity, protein and mRNA (mRNA) levels of MMP-2 were observed in BITC-treated HT29 cells. BITC also inhibited mRNA levels of MMP-2, -7, and -9 in HT29 cells. Results from zymography showed that BITC treatment decreased MMP-2 expression in a concentration-dependent manner. BITC inhibited PKCδ activity in HT29 cells. Furthermore, inhibitors specific for JNK (SP600125) reduced expression of MMP-2, MMP-9, and u-PA. These results demonstrated that BITC could alter HT29 cell metastasis by reduction of MMP-2, MMP-9, and u-PA expression through the suppression of a PKC, MAPK signaling pathway and inhibition of NF-κB levels. These findings suggest that BITC has potential as an antimetastatic agent.

KEYWORDS: BITC; migration; invasion; MMP2; MMP9; human colon cancer HT29 cells

INTRODUCTION

It is well-known that tumor metastasis plays an important role for causing death in cancer patients, and its blockade has been recognized to enable cancer patients to survive (*1*). Cancer metastasis is a complex multistep process, and tumor invasion into surrounding tissues plays an important role in tumor metastasis (*2*). Tumor invasion is involved first with tumor cell attachment to extracellular matrix components, then local degradation of the matrix by tumor cell-associated proteases, and finally tumor cell locomotion by proteolysis into the region of the matrix that has been modified (*3*). If an agent can inhibit one of these steps, suppression of tumor invasion and metastasis could occur. Thus, it is important to identify promising agents with antimetastatic activity. Matrix metalloproteinases (MMPs) are overexpressed in almost all human cancers (*4*, *5*). The matrix metalloproteinase-2 (MMP-2), MMP-9 and urokinase-PA (u-PA) are responsible for the degradation of extracellular matrix components and play important roles in the process of cancer invasion and metastasis (*6*−*8*). Inhibition of MMP activity has been adopted as an anticancer therapeutic strategy.

Isothiocyanates (ITCs) exist as conjugates in the genus *Brassica* of cruciferous vegetables and the genus *Raphanus*, and they have substantial chemopreventive effects against various types of cancer (*9*−*12*). These compounds inhibit the malignant growth in cancer cells with little or no toxicity toward normal cells (*13*−*15*). Benzyl isothiocyanate (BITC) is an ITC which induces apoptosis and activates caspases in bladder cancer cells (*16*) and suppresses

*Corresponding author. Tel: +886 4 2205 3366, int. 2161. Fax: +886 4 2205 3764. E-mail: jgchung@mail.cmu.edu.tw.

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growth of human breast cancer cells (*9*). Based on the literature review, it also suggests that the mechanism by which BITC causes growth arrest and cell death may be cell line specific. ITCs have been shown to be potent inhibitors of mammary, lung, and liver carcinogenesis induced by environmental and dietary carcinogens in rodent models (*17*−*19*). The *N*-acetylcysteine conjugate of BITC has been shown to inhibit benzo[*a*]pyrene-induced pulmonary tumorigenesis in A/J mice (*20*).

In the present study, we investigated the possible inhibitory effects of BITC on MMP-2, MMP-7 and MMP-9 and migration and invasion in the human colon cancer HT29 cell line respectively. We also determined whether the inhibition of these MMPs and uPA by BITC treatment is mediated through the cellular signaling pathways including PKC, MAPK and NF-κB in HT29 cells.

MATERIALS AND METHODS

Chemicals and Reagents. Benzyl isothiocyanate (BITC), Tris-HCl, Triton X-100, trypan blue, dimethyl sulfoxide (DMSO), propidium iodide, potassium phosphates and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 medium, fetal bovine serum, L-glutamine, penicillin−streptomycin and trypsin−EDTA were purchased from Invitrogen (Carlsbad, CA). Primary antibodies used for Western analysis were uPA, PI3K, PKC, Ras, GRB2, SOS1, JNK, P38, P-ERK, ERK1/2, MMP-2, MMP-9, Rho A, FAK, iNOS, COX-2 and NF-kB which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Human Colon Adenocarcinoma Cell Line. The human colon adenocarcinoma cell line HT29 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 90% RPMI-1640 medium with 2 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), and 1% penicillin−streptomycin (100 units/mL penicillin and 100 μg/mL streptomycin) and were cultured in a humidified incubator (5% CO_2, 95% humidity air at 37 °C).

Cell Viability. HT29 cells (2×10^5 cells/well) were plated in 12-well plates and incubated at 37 °C for 24 h; then each well was individually treated with 0, 0.01, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 μM BITC for 24 h. DMSO (solvent) was used for the control condition. Cells were harvested by centrifugation and then were stained by PI and immediately were used for determining cell viability by using the flow cytometric analysis as previously described (*21*).

***In Vitro* Wound Closure.** HT29 cells (1×10^5 cells/well) were plated in 6-well plates for 24 h wounding by scratching with a pipet tip and then incubated with RPMI-1640 medium containing no FBS and treated with or without BITC (0.01 or 0.25 μM) for 0 and 12 h. Cells were photographed using a phase-contrast microscope (×100) as previously described (*22*).

Cell Invasion and Migration Transwell Assay. HT29 cells were treated with or without BITC and invasion was measured using Matrigel-coated Transwell cell culture chambers (8 μm pore size; Costar, Acton, MA) as described previously (*23*). Cells (1×10^5 cells/well) were placed in serum-free-RPMI-1640 medium for 24 h and then were trypsinized and resuspended in serum-free medium and placed in the upper chamber of the Matrigel-coated (0.8 μg/μL, 37 °C, 2 h; BD Biosciences, San Diego, CA) Transwell insert (5×10^4 cells/well) and with 0.5% DMSO or BITC (0, 0.01, or 0.25 μM). RPMI-1640 medium containing 10% FBS was placed in the lower chamber. Cells were incubated for 24 or 48 h in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. Invading cells in the membrane were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. Noninvasive cells in the upper chamber were removed by wiping the upper side of the membrane with a cotton swab, and cells located on the underside of the filter were counted under a light microscope at ×200 (*23*). For the migration assay of HT29 cells, experimental procedures are the same as in the invasion assay of HT29 cells which as described above except the filter membrane was not coated with Matrigel. Cells located on the underside of the filter were counted under a light microscope at ×200 (*23*).

Western Blotting Analysis. The HT29 cells (2×10^6 cells/well) were plated in 6-well tissue culture plates and grown for 24 h. BITC was added to cells in each well at a final concentration of 2.5 μM, while DMSO (solvent) alone was added to the wells as a vehicle control cell. Cells were incubated with BITC in 90% RPMI-1640 medium with 0.5% FBS at 37 °C for 0, 6, 12, 24, and 48 h. Cells in each well were harvested and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100. The collected cells were sonicated and centrifugated at 13000*g* for 10 min at 4 °C to remove cell debris, and the supernatant was collected for determination of total protein concentration using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin (BSA) as the standard as described previously (*23*). SDS gel electrophoresis and Western blotting were performed as described previously (*24*, *25*) for determining the effects of BITC on protein levels of PI3k, PKC, Ras, GrB2, SOS1, p-ERK, ERK1/2, MMP-2, MMP-9, Rho A, FAK, iNOS, COX-2 and NF-κB p65.

Table 1. The DNA Sequence Was Evaluated Using the Primer Express Software

primer name	primer sequence
homo MMP-2-F	CCCCAGACAGGTGATCTTGAC
homo MMP-2-R	GCTTGCGAGGGAAGAAGTTG
homo MMP-7-F	GGATGGTAGCAGTCTAGGGATTAACT
homo MMP-7-R	AGGTTGGATACATCACTGCATTAGG
homo MMP-9-F	CGCTGGGCTTAGATCATTCC
homo MMP-9-R	AGGTTGGATACATCACTGCATTAGG
homo GAPDH-F	ACACCCACTCCTCCACCTTT
homo GAPDH-R	TAGCCAAATTCGTTGTCATACC

Gelatin Zymography. The activities of MMP-2 and -9 were examined by gelatin zymography. Briefly, the HT29 cells (2×10^6 cells/well) were plated in 6-well tissue culture plates and were incubated in serum-free RPMI-1640 medium in the presence of 2.5 μM BITC for the indicated time; the conditioned medium was then collected. The medium were separated by electrophoresis on 10% SDS−PAGE containing 0.1% gelatin. After electrophoresis, the gels were soaked in 2.5% Triton X-100 in dH_2O twice for a total of 60 min at room temperature and then were incubated in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100, pH 8.0) at 37 °C for 18 h. Bands corresponding to activity of MMP-2 and -9 were visualized by negative staining using 0.3% Coomassie blue in 50% methanol and 10% acetic acid (*26*).

Real-Time PCR. Gene expression was performed by real-time PCR. HT29 cells (2×10^6 cells/well) were cultured in 6-well plates and grown for 24 h. BITC was added to cells in each well for a final concentration of 2.5 μM for 24 h. Cells were then harvested and total RNA was extracted using the Qiagen RNeasy Mini Kit as described previously (*24*, *25*, *27*). RNA samples were reverse-transcribed at 42 °C with High Capacity cDNA Reverse Transcription Kit for 30 min according to the protocol of the supplier (Applied Biosystems). Quantitative PCR conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C; 1 min at 60 °C using 1 μL of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers as shown in **Table 1**. Applied Biosystems 7300 Real-Time PCR system was used for each assay in triplicate, and fold-changes were derived using the comparative C_T method (*26*, *28*).

PKC Activity Assay. The inhibition of PKCδ activity by BITC was determined using the HTScan PKC Kinase Assay Kit from Cell Signaling Technology (Beverly, MA). Briefly, reactions were carried out in 50 μL reaction volumes in 96-well polystyrene plates with final conditions as follows: 25 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM NaVO3, 2 mM dithiothreitol, 200 μM adenosine 5′-triphosphate, 1.5 μM substrate peptide, 2.5 μM BITC, and 50 ng of recombinant human PKCδ enzymes. Reactions were started with enzyme addition and incubated at room temperature for 15 min, and then 50 μL/well stop buffer (50 mM EDTA, pH 8.0) was added to stop the reaction. Twenty-five microliters of each reaction mixture was transferred to a 96-well streptavidin-coated plate containing 75 μL of DDW and incubated at room temperature for 60 min. The plate was washed and incubated with 100 μL/well phosphor-PKC substrate rabbit primary antibody at 37 °C for 120 min and then washed again, and europium labeled secondary antibody was added at room temperature for 30 min. After incubation with

enhancement solution for 5 min, plates were read on a time-resolved plate reader (*29*, *30*).

Electrophoretic Mobility Shift Assay (EMSA). NF-κB binding to DNA was determined by electrophoretic mobility shift assay (EMSA). Cells (2×10^6 cells/well) were incubated with 2.5 μM BITC for 24 and 48 h.

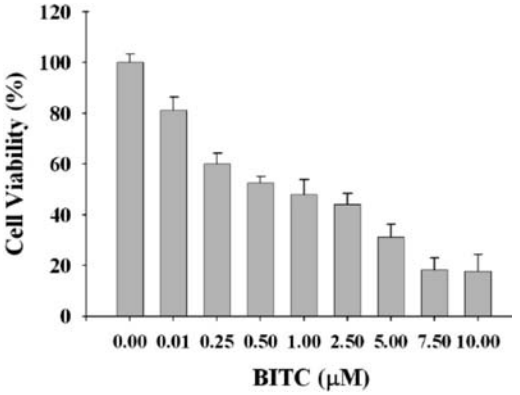

Figure 1. BITC affects the percentage of viable HT29 cells. The HT29 cells were placed in 12-well plates and incubated at 37 °C for 24 h, and then each well was individually cotreated with or without BITC at 0, 0.01, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 μM for 24 h respectively. Cells were harvested by centrifugation and then were stained by PI and immediately were used for determining cell viability by using the flow cytometric protocol as described in Materials and Methods.

Cells from each treatment then were collected by centrifugation, and nuclear extracts were prepared by using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL). Protein concentrations were quantitated and Biotin end-labeled oligonucleotide sequences 5′-Biotin-GATCCAGGGGACTTTCCCTA-GC-3′ corresponding to the consensus site of NF-κB were used. Five micrograms of nuclear extract proteins was used for EMSA with a LightShift Chemiluminescent EMSA Kit (*31*) following the protocol of the manufacturer. Each nuclear extract was incubated with Biotin end-labeled duplex DNA and was electrophoresed on a 6% polyacrylamide native gel. For the 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−HP conjugate and incubated with the substrate of the ECL kit (*32*).

Statistical Analysis. All data are presented as means ± SD of three independent experiments. Statistical differences were evaluated using the Student's *t*-test and considered significant at $*P < 0.05$.

Effects of BITC on Viability of HT29 Cells. To verify whether BITC inhibits colon cancer cell growth, HT-29 cells were treated with various concentrations of BITC for 24 h, and cells were examined under microscope and then were collected for propidium iodine staining to determine viability. **Figure 1** shows that BITC markedly decreased cell viability in a dose-dependent manner in HT29 cells. A reduction in cell viability by BITC was also confirmed by trypan blue dye exclusion method (data not shown).

Effects of BITC on *in Vitro* Wound Closure in HT29 Cells. We determined effects of BITC on migration of HT29 cells by means of a wound closure assay. As shown in **Figure 2A** and **Figure 2B**, relative wound closure decreased in control cells but was higher when compared with BITC treated cells. Data in **Figure 2B** indicate that inhibition was at 56%

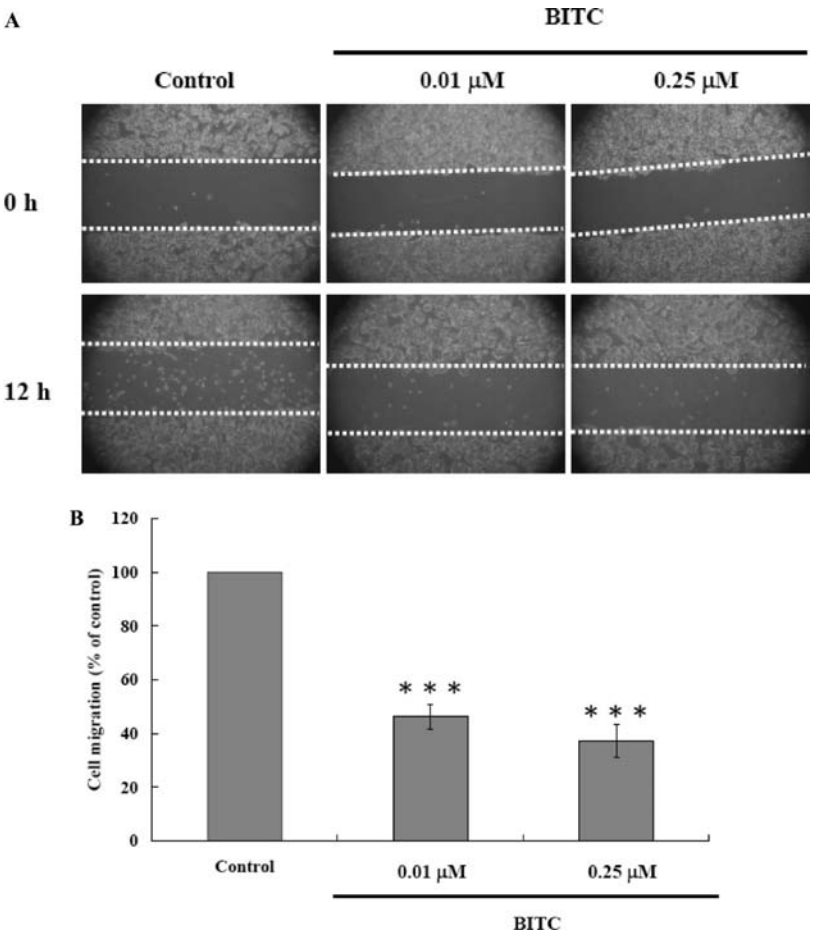

Figure 2. Effects of BITC on the *in vitro* wound closure of HT29 cells. A confluent monolayer of HT29 cells (2×10^5 cells/well) were wounded with a pipet tip and rinsed to remove debris for incubation with or without BITC (0, 0.01, and 0.25 μM) at 12 h. The relative wound closures were monitored and photographed using a Nikon phase-contrast microscope (**A**). Fields shown are representative of the width of quadruplicate wounds, and experiments were made in triplicate cultures. The percentage of inhibition was calculated (**B**). $***P < 0.001$ significant difference between BITC-treated groups and the control.

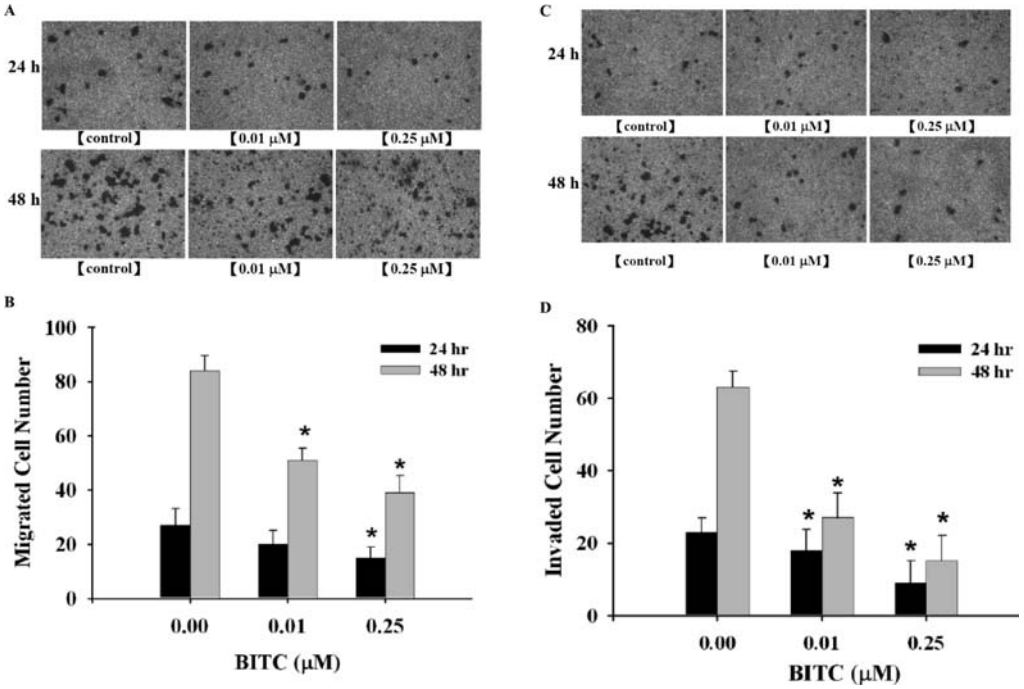

Figure 3. Effects of BITC on the *in vitro* migration and invasion of HT29 cells. The HT29 cells (5×10^4 cells/0.4 mL RPMI-1640) were plated in the upper chamber in the absence or presence of BITC (0, 0.01, and 0.25 μM) and allowed to undergo migration for 24 and 48 h (**A**). Quantification of cells in the lower chamber was performed by counting cells under a Nikon phase-contrast microscope at ×200. *$P < 0.05$, significant difference between BITC-treated groups and the control (**B**). HT29 cells (5×10^4 cells/0.4 mL RPMI-1640) were placed, and cells that penetrated through the matrigel to the lower surface of the filter were stained with crystal violet and were photographed under a light microscope at ×200 (**C**). Quantification of cells in the lower chamber was performed by counting cells under a Nikon phase-contrast microscope at ×200 (**D**). *$P < 0.05$, significant difference between BITC-treated groups and the control.

and 62% when cells were incubated with BITC at 0.01 and 0.25 μM for a 12 h treatment, respectively.

Effects of BITC on Migration and Invasion of HT29 Cells. We determined the effects of BITC on migration and invasion of HT29 cells, and results are shown in **Figure 3**, panels **A**, **B**, **C** and **D**. Results from the migration assay show that BITC had a significant inhibitory effect on cell migration at concentrations between 0.01 and 0.25 μM. Data in **Figure 3B** indicate that the inhibition was at 15−31% and 39−51% when cells were incubated with BITC for 24 and 48 h treatment, respectively. Results from an invasion assay demonstrate that HT29 cells moved from the upper chamber to the lower chamber in the absence of BITC (control group), however, the penetration of the Matrigel-coated filter by HT29 cells was inhibited in the presence of BITC. The percent inhibition at 0.01−0.25 μM BITC were at 18−50% and 56−73% (**Figure 3D**) when cells were incubated with BITC for 24 and 48 h treatment, respectively.

Effects of BITC on Levels of Proteins Associated with Migration and Invasion in HT29 Cells. We determined the effects of BITC on the levels of proteins associated with migration and invasion in HT29 cells, and results are presented in **Figure 4**, panels **A**, **B**, **C** and **D**. It can be seen that BITC reduced protein levels of MKK7, c-Jun, FAK, p-ERK, ERK1/2 and p-JNK, JNK1/2 (**Figure 4A**), Ras, GRB2, Rho A, ROCK1, SOS1, PI3K, AKT, p-AKT and PKCδ (**Figure 4B**), iNOS, NF-κB p65, COX-2 and uPA (**Figure 4C**) and MMP-2, MMP-7, MMP-9 (**Figure 4D**) but did not have an effect on p38 protein abundance.

Effects of BITC on the Activities of MMP-2 from HT29 Cells. Gelatin zymography was used to analyze the effects of BITC on MMP-2 activities for 24 and 48 h treatment of BITC (2.5 and 5 μM). As shown in **Figure 5**, we found that BITC inhibited MMP-2 activities. This is also confirmed by Western blotting which is shown in **Figure 4D**, and the results indicated that BITC inhibited the proteins level of MMP-2.

Effects of BITC on MMP-2, MMP-7 and MMP-9 mRNA Expression in HT29 Cells. In order to investigate whether BITC affected migration- and invasion-associated gene expression in HT29 cells, cells were treated with BITC (2.5 and 5 μM) for 0 and 48 h. Results shown in **Figure 6** indicate that the mRNA expression levels of MMP-2, MMP-7 and MMP-9 mRNA were decreased by BITC in a dose-dependent manner (**Figure 6**).

Effects of BITC on PKCδ and NF-κB Activity in HT29 Cells. **Figure 7** shows that BITC inhibited PKCδ activity after 24 and 48 h treatment and these effects were time-dependent. Activation of NF-κB was reduced following incubation at 24 and 48 h as seen in **Figure 8** by EMSA .

DISCUSSION

BITC has been reported to have anticancer effects in both *in vivo* and *in vitro* experimental models (*8*, *33*, *34*). To date, there have not been any studies on the antimetastasis (inhibition of migration and invasion) effects of BITC on colon cancer cells. The present study demonstrates for the first time that BITC (1) inhibits migration of HT29 colon cancer cells; (2) inhibits invasion of HT29 cells; (3) inhibits secretion of uPA, MMP-2, -7 and -9, FAK and Rho A, iNOS and COX-2; (4) inhibits the activities of MMP-2 and -9; and (5) inhibits NF-κB DNA binding and PKCδ activity.

Cancer cell metastasis is involved in tumor cell adhesion to the ECM, proteolytic cleavage or destruction of the ECM, and cell migration through the resultant defect. In the present study, we demonstrate that BITC decreases invasion and migration of HT29 cells. It was reported that the critical step for invasion and metastasis is the breakdown of the basement membrane, which requires activation of proteolytic enzymes (*35*, *36*) such as MMPs. MMPs play an important role in tumor angiogenesis, metastasis and stimulation of growth factor release from the ECM (*35*). Numerous studies indicate that inhibition of MMP expression or enzyme activity can be used as early targets for preventing cancer metastasis (*37*−*39*). MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) seem to be involved in this initial step as they hydrolyze basal membrane type IV collagen and have been frequently associated with the invasive metastatic potential of tumor cells (*8*, *40*). We clearly show that BITC inhibited proteins levels of MMP-2 and -9 (**Figure 4D**) and demonstrated that BITC inhibits mRNA expression of MMP-2

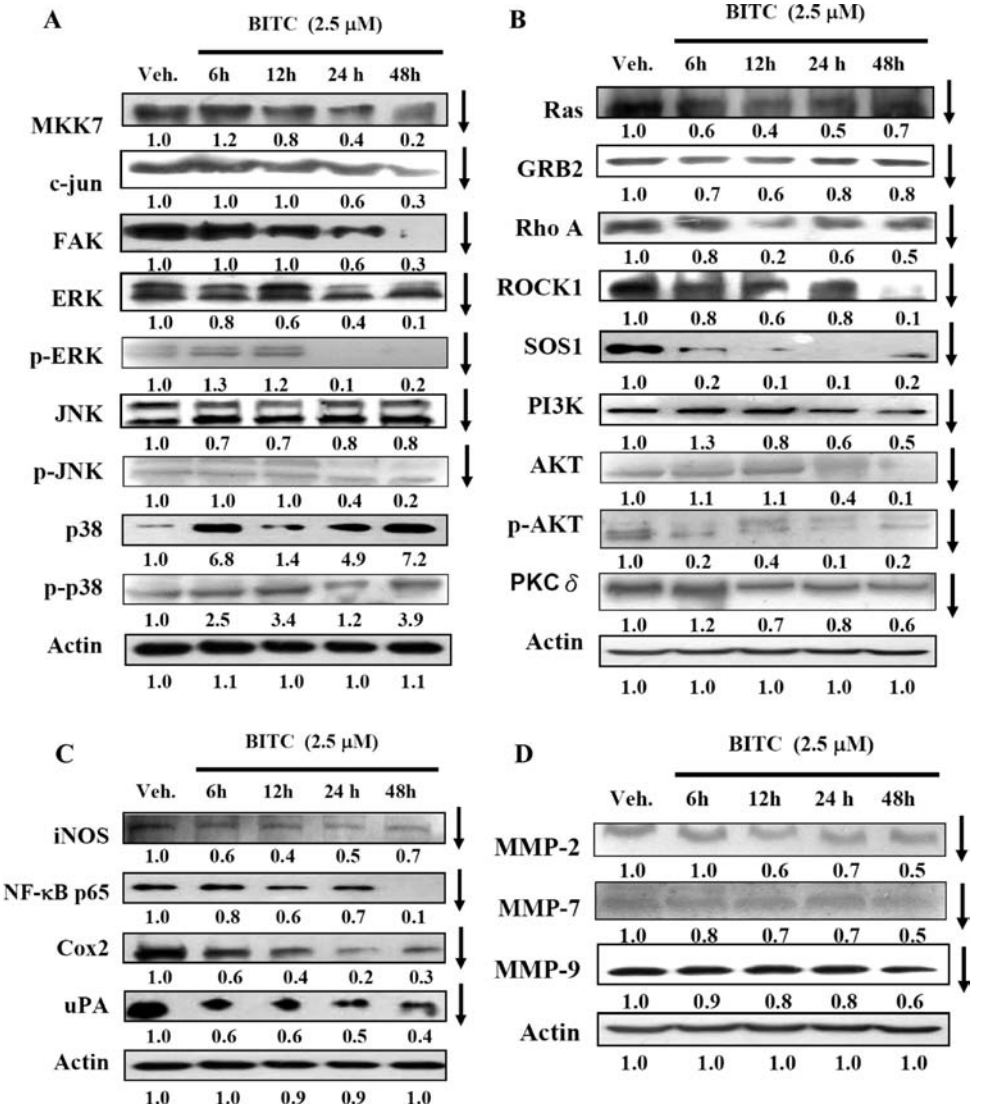

Figure 4. Effects of BITC on the proteins level of associated proteins for metastasis in HT29 cells. The total proteins were collected from HT29 cells treated with 2.5 μM BITC for 6, 12, 24, 48 h before, and the proteins levels (**A**, MKK7, c-Jun, FAK, ERK1/2, p-ERK, JNK1/2 p-JNK1/2 and p38, p-p38; **B**, Ras, GrB2, Rho A, ROCK1, SOS1, PI3K, AKT, p-AKT and PKCδ; **C**, iNOS, NF-κB p65, COX-2 and uPA; **D**, MMP-2, MMP-7, MMP-9) were measured by SDS−PAGE and Western blotting as described in Materials and Methods.

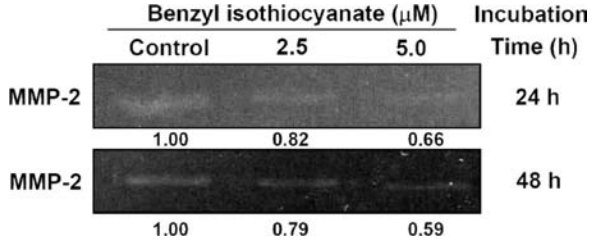

Figure 5. Effects of BITC on MMP-2 activities in HT29 cells. Cells (5×10^4 cells/well) were incubated with 2.5 and 5 μM BITC for 24 and 48 h. Cells were harvested and separated by gelatin zymography as described in Materials and Methods. The ratio of MMP-2 activities was quantitated.

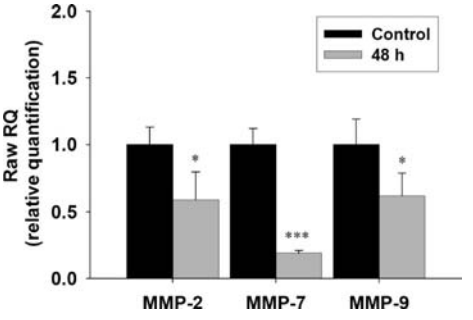

Figure 6. Effects of BITC on MMP-2, MMP-7 and MMP-9 mRNA expression in HT29 cells. The HT29 cells (5×10^5 cells/well) were incubated with 2.5 μM BITC for 48 h. The total RNA was extracted from each treatment of HT29 cells, and RNA samples were reverse-transcribed cDNA then for real time PCR as described in Materials and Methods. The ratios of MMP-2, MMP-7 and MMP-9 mRNA/GAPDH are presented in panel. Data represents mean ± SD of three experiments. *$P < 0.05$, ***$P < 0.001$.

and -9 (**Figure 6**) in HT29 cells. BITC also inhibited protein levels of uPA (**Figure 4C**). uPA is a member of the serine protease family, and it plays a role in tumor invasion, tumor cell proliferation and metastasis (*41*). It was reported that most integrins activate MAPK and PI3K, leading to activation of Akt (*42*). In the present study, BITC also decreased phosphorylation of Akt and JNK. Future studies are needed to explore the relationship between expression of integrins and phosphorylation of Akt and JNK in BITC treated colon cancer cells. We also used the

inhibitor of JNK (SP600125) combined BITC lead to decrease the MMP-2 and uPA (data not shown). These results indicate that inhibition of JNK activity contributes to the decrease in migration of BITC treated HT29 cells. The present results suggest that BITC may have efficacy and prevent the metastasis of colon cancer cells.

It was reported that MMP-2 overexpressed in highly metastatic tumors (*43*). MMP-9 can be stimulated by a Ras oncogene (*44*, *45*) through activation of different intracellular-signaling pathways. The activation of PKC could cause translocation of the protein to membranes and affecting the expression of MMP-9 via modulation of transcription factors including activator protein-1 (AP-1), nuclear factor-kB (NF-kB) or Sp-1 through mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (*46*−*49*). Therefore, the regulation of NF-κB, downstream of the PI3K/Akt and MAPK (ERK1/2, p38 and JNK) pathways, might be involved in BITC suppressed MMP-9 expression and invasion in HT29 cells. Our results also showed that BITC reduced protein levels of PI3K, Akt, MMP-2 and MMP-9. It was reported that PI3K activation stimulated the downstream target Akt, which plays various and important roles including cell invasion (*50*, *51*).

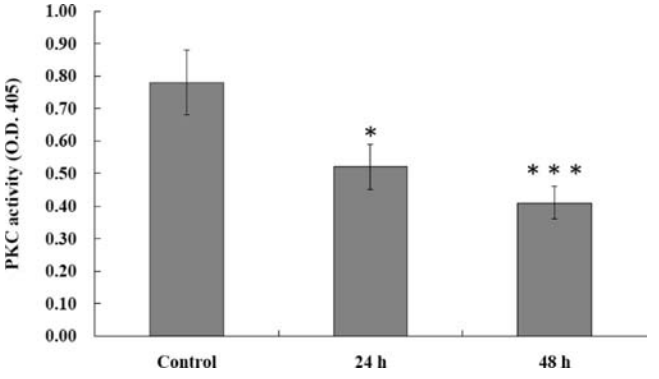

Figure 7. BITC inhibited PKCδ activity in HT29 cells. Cells were incubated with 2.5 μM BITC for 24 and 48 h. Protein extracts were prepared from BITC-treated HT29 cells and PKCδ activity was determined as described in Materials and Methods. *$P < 0.05$, significant difference between BITC-treated groups and the control.

The uPA plays an important role in the decomposition of basement membranes, and the activation of the uPA/uPAR/plasmin proteolytic network plays key roles in tumor invasion and dissemination of various malignancies (*41*, *52*). The levels of uPA and uPAR expression serve as prognostic markers in various malignancies, and high levels of expression are often associated with a poor prognosis (*53*). Our result showed that BITC decreased the levels of uPA in HT29 cells.

In conclusion, the present study assessed the efficacy of BITC on counteracting metastasis and identified molecular mechanisms of such effects in human colon cancer cells (**Figure 9**). It is noteworthy that BITC inhibits migration and invasion through downregulation of PKC and then blocking MAPK and PI3K/Akt signaling pathways, NF-κB, as well as uPA resulting in inhibition of MMP-2 and MMP-9 (**Figure 8**). Future *in vivo* efficacy studies with BITC should be done in animal models. Colon tumor invasion and metastasis present major obstacles in treatment. Thus, inhibition of metastasis of colon cancer cells by BITC could have important preventive and therapeutic benefits against colon cancer.

Figure 9. The proposed mechanisms of BITC inhibited migration and invasion of HT29 cells.

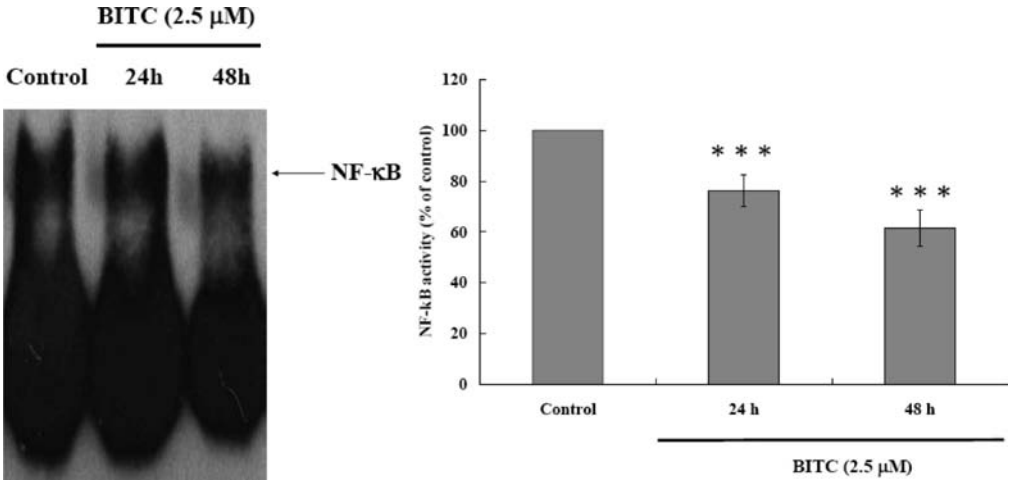

Figure 8. BITC induced NF-κB activity and downstream protein expression change in HT29 cells. Cells were incubated with 2.5 μM BITC for 24 and 48 h. Nuclear extracts were prepared from BITC-treated HT29 cells, and NF-κB activity was determined by electrophoretic mobility shift assay (EMSA) (left). NF-κB activity was calculated (right). ***$P < 0.001$, significant difference between BITC-treated groups and the control.

ABBREVIATIONS USED

BITC, benzyl isothiocyanate; MMP-2, metalloproteinase-2; MMP-9, matrix metalloproteinase- 9; u-PA, urokinase-plasminogen activator; JNK1/2, c-Jun N-terminal kinase 1; ERK1/2, c-Jun N-terminal kinases 1/2; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; NF-κB, nuclear factor-kappa B.

LITERATURE CITED

(1) Chambers, A. F.; MacDonald, I. C.; Schmidt, E. E.; Morris, V. L.; Groom, A. C. Clinical targets for anti-metastasis therapy. *Adv. Cancer Res.* **2000**, *79*, 91–121.

(2) Poste, G.; Fidler, I. J. The pathogenesis of cancer metastasis. *Nature* **1980**, *283* (5743), 139–46.

(3) Liotta, L. A. Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res.* **1986**, *46* (1), 1–7.

(4) Cakarovski, K.; Leung, J. Y.; Restall, C.; Carin-Carlson, A.; Yang, E.; Perlmutter, P.; Anderson, R.; Medcalf, R.; Dear, A. E. Novel inhibitors of urokinase-type plasminogen activator and matrix metalloproteinase expression in metastatic cancer cell lines. *Int. J. Cancer* **2004**, *110* (4), 610–6.

(5) Toda, D.; Ota, T.; Tsukuda, K.; Watanabe, K.; Fujiyama, T.; Murakami, M.; Naito, M.; Shimizu, N. Gefitinib decreases the synthesis of matrix metalloproteinase and the adhesion to extracellular matrix proteins of colon cancer cells. *Anticancer Res.* **2006**, *26* (1A), 129–34.

(6) Yang, S. F.; Hsieh, Y. S.; Lin, C. L.; Hsu, N. Y.; Chiou, H. L.; Chou, F. P.; Chu, S. C. Increased plasma levels of urokinase plasminogen activator and matrix metalloproteinase-9 in nonsmall cell lung cancer patients. *Clin. Chim. Acta* **2005**, *354* (1−2), 91–9.

(7) Mook, O. R.; Frederiks, W. M.; Van Noorden, C. J. The role of gelatinases in colorectal cancer progression and metastasis. *Biochim. Biophys. Acta* **2004**, *1705* (2), 69–89.

(8) Hwang, E. S.; Lee, H. J. Benzyl isothiocyanate inhibits metalloproteinase-2/-9 expression by suppressing the mitogen-activated protein kinase in SK-Hep1 human hepatoma cells. *Food Chem. Toxicol.* **2008**, *46* (7), 2358–64.

(9) Xiao, D.; Vogel, V.; Singh, S. V. Benzyl isothiocyanate-induced apoptosis in human breast cancer cells is initiated by reactive oxygen species and regulated by Bax and Bak. *Mol. Cancer Ther.* **2006**, *5* (11), 2931–45.

(10) Tang, L.; Zhang, Y. Mitochondria are the primary target in isothiocyanate-induced apoptosis in human bladder cancer cells. *Mol. Cancer Ther.* **2005**, *4* (8), 1250–9.

(11) Verhoeven, D. T.; Goldbohm, R. A.; van Poppel, G.; Verhagen, H.; van den Brandt, P. A. Epidemiological studies on brassica vegetables and cancer risk. *Cancer Epidemiol., Biomarkers Prev.* **1996**, *5* (9), 733–48.

(12) Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18* (1), 1–29.

(13) Srivastava, S. K.; Singh, S. V. Cell cycle arrest, apoptosis induction and inhibition of nuclear factor kappa B activation in anti-proliferative activity of benzyl isothiocyanate against human pancreatic cancer cells. *Carcinogenesis* **2004**, *25* (9), 1701–9.

(14) Xiao, D.; Srivastava, S. K.; Lew, K. L.; Zeng, Y.; Hershberger, P.; Johnson, C. S.; Trump, D. L.; Singh, S. V. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Carcinogenesis* **2003**, *24* (5), 891–7.

(15) Hu, R.; Kim, B. R.; Chen, C.; Hebbar, V.; Kong, A. N. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis* **2003**, *24* (8), 1361–7.

(16) Tang, L.; Zhang, Y. Dietary isothiocyanates inhibit the growth of human bladder carcinoma cells. *J. Nutr.* **2004**, *134* (8), 2004–10.

(17) Sugie, S.; Okumura, A.; Tanaka, T.; Mori, H. Inhibitory effects of benzyl isothiocyanate and benzyl thiocyanate on diethylnitrosamine-induced hepatocarcinogenesis in rats. *Jpn. J. Cancer Res.* **1993**, *84* (8), 865–70.

(18) Lin, J. M.; Amin, S.; Trushin, N.; Hecht, S. S. Effects of isothiocyanates on tumorigenesis by benzo[a]pyrene in murine tumor models. *Cancer Lett.* **1993**, *74* (3), 151–9.

(19) Wattenberg, L. W. Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanate and related compounds. *J. Natl. Cancer Inst.* **1977**, *58* (2), 395–8.

(20) Yang, Y. M.; Conaway, C. C.; Chiao, J. W.; Wang, C. X.; Amin, S.; Whysner, J.; Dai, W.; Reinhardt, J.; Chung, F. L. Inhibition of benzo(a)pyrene-induced lung tumorigenesis in A/J mice by dietary N-acetylcysteine conjugates of benzyl and phenethyl isothiocyanates during the postinitiation phase is associated with activation of mitogen-activated protein kinases and p53 activity and induction of apoptosis. *Cancer Res.* **2002**, *62* (1), 2–7.

(21) Lu, H. F.; Lai, K. C.; Hsu, S. C.; Lin, H. J.; Yang, M. D.; Chen, Y. L.; Fan, M. J.; Yang, J. S.; Cheng, P. Y.; Kuo, C. L.; Chung, J. G. Curcumin induces apoptosis through FAS and FADD, in caspase-3-dependent and -independent pathways in the N18 mouse-rat hybrid retina ganglion cells. *Oncol. Rep.* **2009**, *22* (1), 97–104.

(22) Ho, C. C.; Lin, S. Y.; Yang, J. S.; Liu, K. C.; Tang, Y. J.; Yang, M. D.; Chiang, J. H.; Lu, C. C.; Wu, C. L.; Chiu, T. H.; Chung, J. G. Gallic acid inhibits murine leukemia WEHI-3 cells in vivo and promotes macrophage phagocytosis. *In Vivo* **2009**, *23* (3), 409–13.

(23) Hsu, S. C.; Kuo, C. L.; Lin, J. P.; Lee, J. H.; Lin, C. C.; Su, C. C.; Yang, M. D.; Chung, J. G. Crude extracts of Euchresta formosana radix inhibit invasion and migration of human hepatocellular carcinoma cells. *Anticancer Res.* **2007**, *27* (4B), 2377–84.

(24) Fishman, D. A.; Liu, Y.; Ellerbroek, S. M.; Stack, M. S. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. *Cancer Res.* **2001**, *61* (7), 3194–9.

(25) Pilcher, B. K.; Dumin, J. A.; Sudbeck, B. D.; Krane, S. M.; Welgus, H. G.; Parks, W. C. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J. Cell Biol.* **1997**, *137* (6), 1445–57.

(26) Hsu, S. C.; Yang, J. S.; Kuo, C. L.; Lo, C.; Lin, J. P.; Hsia, T. C.; Lin, J. J.; Lai, K. C.; Kuo, H. M.; Huang, L. J.; Kuo, S. C.; Wood, W. G.; Chung, J. G., Novel quinolone CHM-1 induces apoptosis and inhibits metastasis in a human osterogenic sarcoma cell line. *J. Orthop. Res.* **2009**.

(27) Huang, Y. T.; Hwang, J. J.; Lee, L. T.; Liebow, C.; Lee, P. P.; Ke, F. C.; Lo, T. B.; Schally, A. V.; Lee, M. T. Inhibitory effects of a luteinizing hormone-releasing hormone agonist on basal and epidermal growth factor-induced cell proliferation and metastasis-associated properties in human epidermoid carcinoma A431 cells. *Int. J. Cancer* **2002**, *99* (4), 505–13.

(28) Fujiwara, T.; Saito, S.; Osanai, T.; Kameda, K.; Abe, N.; Higuma, T.; Yokoyama, J.; Hanada, H.; Fukui, K.; Fukuda, I.; Okumura, K. Decreased plasma and cardiac matrix metalloproteinase activities in patients with coronary artery disease and treated with pravastatin. *Eur. J. Pharmacol.* **2008**, *594* (1−3), 146–51.

(29) Guo, K.; Liu, Y.; Zhou, H.; Dai, Z.; Zhang, J.; Sun, R.; Chen, J.; Sun, Q.; Lu, W.; Kang, X.; Chen, P. Involvement of protein kinase C beta-extracellular signal-regulating kinase 1/2/p38 mitogen-activated protein kinase-heat shock protein 27 activation in hepatocellular carcinoma cell motility and invasion. *Cancer Sci.* **2008**, *99* (3), 486–96.

(30) Obara, K.; Ito, Y.; Shimada, H.; Nakayama, K. The relaxant effect of okadaic acid on canine basilar artery involves activation of PKCalpha and phosphorylation of the myosin light chain at Thr-9. *Eur. J. Pharmacol.* **2008**, *598* (1−3), 87–93.

(31) Chadwick, D. W.; Anhut, H.; Greiner, M. J.; Alexander, J.; Murray, G. H.; Garofalo, E. A.; Pierce, M. W. A double-blind trial of gabapentin monotherapy for newly diagnosed partial seizures. International Gabapentin Monotherapy Study Group 945−77. *Neurology* **1998**, *51* (5), 1282–8.

(32) Lin, M. W.; Tsao, L. T.; Chang, L. C.; Chen, Y. L.; Huang, L. J.; Kuo, S. C.; Tzeng, C. C.; Lee, M. R.; Wang, J. P. Inhibition of lipopolysaccharide-stimulated NO production by a novel synthetic compound CYL-4d in RAW 264.7 macrophages involving the blockade of MEK4/JNK/AP-1 pathway. *Biochem. Pharmacol.* **2007**, *73* (11), 1796–806.

(33) Hecht, S. S. Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism. *J. Nutr.* **1999**, *129* (3), 768S–774S.
(34) Nakamura, Y. Chemoprevention by isothiocyanates: molecular basis of apoptosis induction. *Forum Nutr.* **2009**, *61*, 170–81.
(35) Coussens, L. M.; Werb, Z. Matrix metalloproteinases and the development of cancer. *Chem. Biol.* **1996**, *3* (11), 895–904.
(36) Liotta, L. A.; Stetler-Stevenson, W. G. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res.* **1991**, *51* (18 Suppl), 5054s–5059s.
(37) Guruvayoorappan, C.; Kuttan, G. Amentoflavone inhibits experimental tumor metastasis through a regulatory mechanism involving MMP-2, MMP-9, prolyl hydroxylase, lysyl oxidase, VEGF, ERK-1, ERK-2, STAT-1, nm23 and cytokines in lung tissues of C57BL/6 mice. *Immunopharmacol. Immunotoxicol.* **2008**, *30* (4), 711–27.
(38) Waas, E. T.; Wobbes, T.; Lomme, R. M.; DeGroot, J.; Ruers, T.; Hendriks, T. Matrix metalloproteinase 2 and 9 activity in patients with colorectal cancer liver metastasis. *Br. J. Surg.* **2003**, *90* (12), 1556–64.
(39) Okada, N.; Ishida, H.; Murata, N.; Hashimoto, D.; Seyama, Y.; Kubota, S. Matrix metalloproteinase-2 and -9 in bile as a marker of liver metastasis in colorectal cancer. *Biochem. Biophys. Res. Commun.* **2001**, *288* (1), 212–6.
(40) Zhang, L.; Shi, J.; Feng, J.; Klocker, H.; Lee, C.; Zhang, J. Type IV collagenase (matrix metalloproteinase-2 and -9) in prostate cancer. *Prostate Cancer Prostatic Dis.* **2004**, *7* (4), 327–32.
(41) Kwaan, H. C. The plasminogen-plasmin system in malignancy. *Cancer Metastasis Rev.* **1992**, *11* (3−4), 291–311.
(42) Eccles, S. A. Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention. *Int. J. Dev. Biol.* **2004**, *48* (5−6), 583–98.
(43) Nee, L.; Tuite, N.; Ryan, M. P.; McMorrow, T. TNF-alpha and IL-1 beta-mediated regulation of MMP-9 and TIMP-1 in human glomerular mesangial cells. *Nephron Exp. Nephrol.* **2007**, *107* (2), e73–86.
(44) Lee, S. O.; Jeong, Y. J.; Kim, M.; Kim, C. H.; Lee, I. S. Suppression of PMA-induced tumor cell invasion by capillarisin via the inhibition of NF-kappaB-dependent MMP-9 expression. *Biochem. Biophys. Res. Commun.* **2008**, *366* (4), 1019–24.
(45) Cho, H. J.; Kang, J. H.; Kwak, J. Y.; Lee, T. S.; Lee, I. S.; Park, N. G.; Nakajima, H.; Magae, J.; Chang, Y. C. Ascofuranone suppresses PMA-mediated matrix metalloproteinase-9 gene activation through the Ras/Raf/MEK/ERK- and Ap1-dependent mechanisms. *Carcinogenesis* **2007**, *28* (5), 1104–10.
(46) Zhang, S.; Li, Z.; Wu, X.; Huang, Q.; Shen, H. M.; Ong, C. N. Methyl-3-indolylacetate inhibits cancer cell invasion by targeting the MEK1/2-ERK1/2 signaling pathway. *Mol. Cancer Ther.* **2006**, *5* (12), 3285–93.
(47) Chung, T. W.; Lee, Y. C.; Kim, C. H. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERK and PI-3K/AKT pathways: involvement of invasive potential. *FASEB J.* **2004**, *18* (10), 1123–5.
(48) Chakraborti, S.; Mandal, M.; Das, S.; Mandal, A.; Chakraborti, T. Regulation of matrix metalloproteinases: an overview. *Mol. Cell. Biochem.* **2003**, *253* (1−2), 269–85.
(49) Simon, C.; Simon, M.; Vucelic, G.; Hicks, M. J.; Plinkert, P. K.; Koitschev, A.; Zenner, H. P. The p38 SAPK pathway regulates the expression of the MMP-9 collagenase via AP-1-dependent promoter activation. *Exp. Cell Res.* **2001**, *271* (2), 344–55.
(50) Hollborn, M.; Stathopoulos, C.; Steffen, A.; Wiedemann, P.; Kohen, L.; Bringmann, A. Positive feedback regulation between MMP-9 and VEGF in human RPE cells. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48* (9), 4360–7.
(51) Carpenter, C. L.; Cantley, L. C. Phosphoinositide kinases. *Curr. Opin. Cell Biol.* **1996**, *8* (2), 153–8.
(52) Andreasen, P. A.; Kjoller, L.; Christensen, L.; Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* **1997**, *72* (1), 1–22.
(53) Duffy, M. J.; Maguire, T. M.; McDermott, E. W.; O'Higgins, N. Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. *J. Surg. Oncol.* **1999**, *71* (2), 130–5.

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