Benzyl isothiocyanate and phenethyl isothiocyanate inhibit murine melanoma B16F10 cell migration and invasion *in vitro*

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Abstract. Benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC) have been demonstrated to induce anticancer function in many human cancer cells and also inhibit cancer cell migration and invasion. However, there are no studies that show BITC and PEITC to inhibit cell migration and invasion in mouse melanoma B16F10 cells. In this study, we investigated anti-metastasis effects of BITC and PEITC in melanoma cancer cells *in vitro*. Under sub-lethal concentrations (from 1, 2.5 up to 5 μM), BITC and PEITC significantly inhibited cell mobility, migration and invasion nature of B16F10 cells. Gelatin zymography assay also showed that BITC and PEITC inhibited matrix metalloproteinase-2 (MMP-2) activity in B16F10 cells. PEITC reduced MAPK signaling associated proteins such as p-ERK1/2, p-p38 and p-JNK1/2 but BITC increased those MAPK signaling associated proteins. BITC and PEITC both suppressed the expression of RhoA, Ras, and SOS-1, however, PEITC increased FAK and GRB2 but BITC increased FAK at 48 h. Furthermore, PEITC decreased the expression of MMP-2 and tissue inhibitors of matrix metalloproteinases (TIMP) but BITC increased them. PEITC inhibited NF-κB protein levels and DNA binding which was confirmed by electrophoretic mobility shift (EMSA) assay. Based on these observations, we suggest that BITC and PEITC can be used in anti-metastasis of melanoma cells in the future.

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Introduction

Melanoma has been recognized to be one of the most malignant tumors with the most aggressive and treatment-resistant form of human skin cancer. Currently its incidence is still increasing worldwide. The treatment for this disease after it spread beyond the primary site is difficult (1). Melanoma has rapid proliferation rate (2), however, the exact mechanisms of the rapid proliferation of melanoma cells was unclear (3). Melanoma cell metastasis is also a cause for difficulty in curing this disease. Although melanoma treatment has shown some breakthroughs in targeted and immunotherapy (4), it is still urgently needed to identify new targets for melanoma treatment.

PEITC, a member of isothiocyanates (ITCs), have been shown to induce cell cycle arrest PC-3 human prostate cancer cells (5), oral cancer cells (6), gastric cancer cells (7) and gastric cancer cells (8). Furthermore, several studies have shown that PEITC induced human cancer cell apoptosis (9-11) and it also inhibited nuclear factor-κB (NF-κB)-regulated gene expression (12) and activation of Atg5-mediated autophagy (13) in human prostate cancer cells. PEITC is also used in clinical trials for lung cancer (14). It was reported that PEITC inhibits the invasion of EGF-stimulated SAS oral cancer cells via targeting EGFR and also to induce its downstream signaling molecules for reducing the expression and enzymatic activities of both matrix metalloproteinase-2 (MMP-2) and MMP-9 (15).

BITC, also one of ITCs, has been shown to induce cell apoptosis in many human cancer cell lines such as bladder cancer cells (16), breast cancer cells (17), ovary cancer cells (18), prostate cancer cells (19) and melanoma A375.S2 cells (20). Furthermore, BITC induced cell cycle arrest and

apoptosis in human leukemia cells through the downregulation of myeloid cell leukemia-1 (Mcl-1) (21) and alters the gene expression with cell cycle regulation and cell death in human brain glioblastoma GBM 8401 cells (22).

Metastasis, a multistep process, which is often resistant to conventional therapies such as chemotherapy and radiation therapy, is involved in cell motility, cellular adhesion and invasiveness, entry to blood circulation, and stays in other tissues for new colonization of a distant site (23). One of the major steps for cancer cell metastasis is the breakdown of connective tissue barriers which is involved with proteolytic enzymes such as MMPs to mediate ECM breakdown and facilitate invasion (24). ITCs inhibit the invasion and migration via blocking FAK/JNK-mediated MMP-9 expression in mouse C6 glioma cells (25). In lung cancer cells, BITC and PEITC inhibit cell metastasis potential *via* the modulation of metastasis-related gene expression and the inhibition of Akt/NF-κB pathway (26). However, there is no available report to show BITC and PEITC suppressing the migration and invasion of mouse melanoma cells. Thus, we investigated the effects of BITC and PEITC on the B16F10 cell metastasis and we found that BITC and PEITC suppressed the migration and invasion of B16F10 cells *in vitro*.

Materials and methods

Test compound, reagents and culture medium. Benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), dimethyl sulfoxide (DMSO), propidium iodide (PI), Tris-HCl, Trypsin and Trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BITC and PEITC were dissolved in DMSO as a carrier solvent and control cultures 0.5% DMSO. DMEM medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture. Murine melanoma B16F10 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in 75 cm^2 flask with DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 humidified incubators at 37˚C.

Cell viability assay. B16F10 cells ($1x10^5$ cells/well) were maintained in 12-well plates with DMEM for 48 h and then PEITC were added to cells at final concentrations (0, 1, 2.5, 5, 10 and 15 μM) and BITC were added to cells at final concentrations (0, 1, 2.5, 5 and 10 μM) for 48 h. After incubation, cells were collected from each treatment, washed with PBS and were stained with PI (5 μg/ml). All samples were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) for percentage of viable cells as previously described (27).

Scratch wound healing assay. To investigate the wound healing effect of PEITC and BITC on murine melanoma cells, B16F10 cells ($2x10^5$ cells/well) were placed in 6-well plate for 24 h and after the cells formed a confluent monolayer, they were scratched using a sterile pipette tip to create a wound at confluence and washed in PBS to remove cell debris. Cells in each well were incubated with PEITC and BITC at the final concentrations (0, 1, 2.5 and 5 μM) at 37˚C with 5% CO_2 at time = 0 and 24 h and were photographed by phase contrast microscopy. The relative wound size at each time point of treatment was quantified by ImageJ software. Cell migration inhibition rate (%) = new scratch width/original scratch width x 100% as previously described (28,29).

Cell migration and invasion assay. Matrigel Cell Migration Assay and Invasion System were used for measuring cell migration and invasion *in vitro* as previously described (30). Cell migration was performed with Transwell cell culture chambers (8-mm pore size; Millipore, Temecula, CA, USA). B16F10 cells ($5x10^4$ cells/well) were added in serum-free DMEM and were placed in the upper chamber which was coated with collagen of the Transwell insert and incubated with PEITC and BITC (0, 1, 2.5 and 5 μM). DMEM with 10% FBS was placed in the lower chamber and were incubated for 48 h. The invasive cells (penetrated the filter in the lower surface) were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet and were examined and photographed under light microscopy at x200 followed by counting for the percentage of inhibition (30). Cell invasion experiment was performed similarly to cell migration assay, using matrigel collagen to replace collagen on the filter membrane (30).

Gelatin zymography assay. B16F10 cells ($5x10^5$ cells/well) were maintained in 6-well culture plates for approximately 80% confluency. The serum-free medium with PEITC or BITC was added to each dish for 24 h culture. After incubation, the conditioned medium was collected from each treatment for measuring the total proteins; a 50 μg of protein from each treatment was electrophoresis on 10% SDS-PAGE containing 0.2% gelatin. Gel was washed and soaked in 2.5% Triton X-100 in dH_2O twice at 25˚C for 30 min twice. Gels were soaked in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100, pH 8.0) while shaking for 18 h at 37˚C. Gels were stained with 0.2% Coomassie blue (Bio-Rad, Hercules, CA, USA) in 10% acetic acid and 50% methanol (30,31) and were photographed on a light box. Proteolysis was detected as a white zone (MMP-2 gelatinolytic activities) in a dark blue field.

Protein extraction and western blot analysis. B16F10 cells ($1x10^6$ cells) in 10-cm dish were incubated with PEITC or BITC (0, 1, 2.5 and 5 μM) for 24 and 48 h. After incubation, cells were collected and lysed in ice-cold potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100 for sonication and centrifuged and total protein was measured by Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin (BSA) as the standard as previously described (30). The protein from each treatment of total cells was separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk in TBS-T buffer (10 mmol/l Tris-HCl, 150 mmol/l NaCl, and 0.05% Tween-20, pH 7.8) at room temperature fort 1 h followed by washing in TBS-T buffer. The membrane was incubated with monoclonal antibodies such as anti-p-ERK1/2, anti-p-p38, anti-p-JNK1/2, anti-PKC, anti-phosphatidylinositol 3 kinase (PI3K), anti-p-AKT (Thr308), anti-p-AKT(Ser473), anti-PCAN

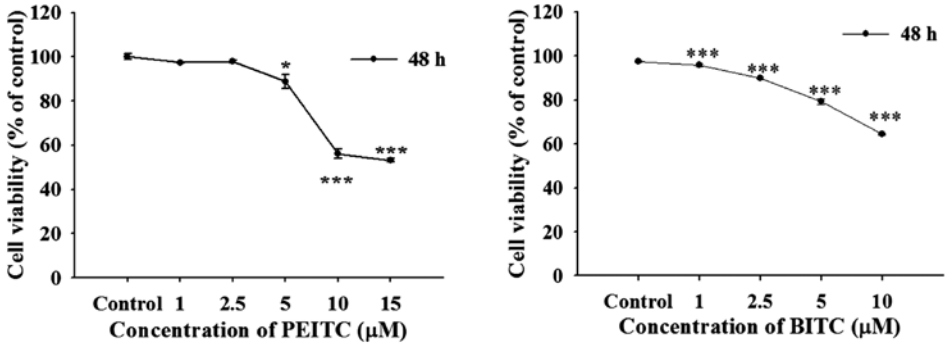

Figure 1. PEITC and BITC affect the cell viability. B16F10 cells ($1x10^5$ cells/well) were treated with various concentrations (0, 1, 2.5, 5, 10 and 15 μM) of PEITC and BITC for 48 h. Cells were harvested for total cell viability as described in Materials and methods. *P<0.05, ***P<0.001 significant difference between PEITC- and BITC-treated groups and the control as analyzed by Student's t-test.

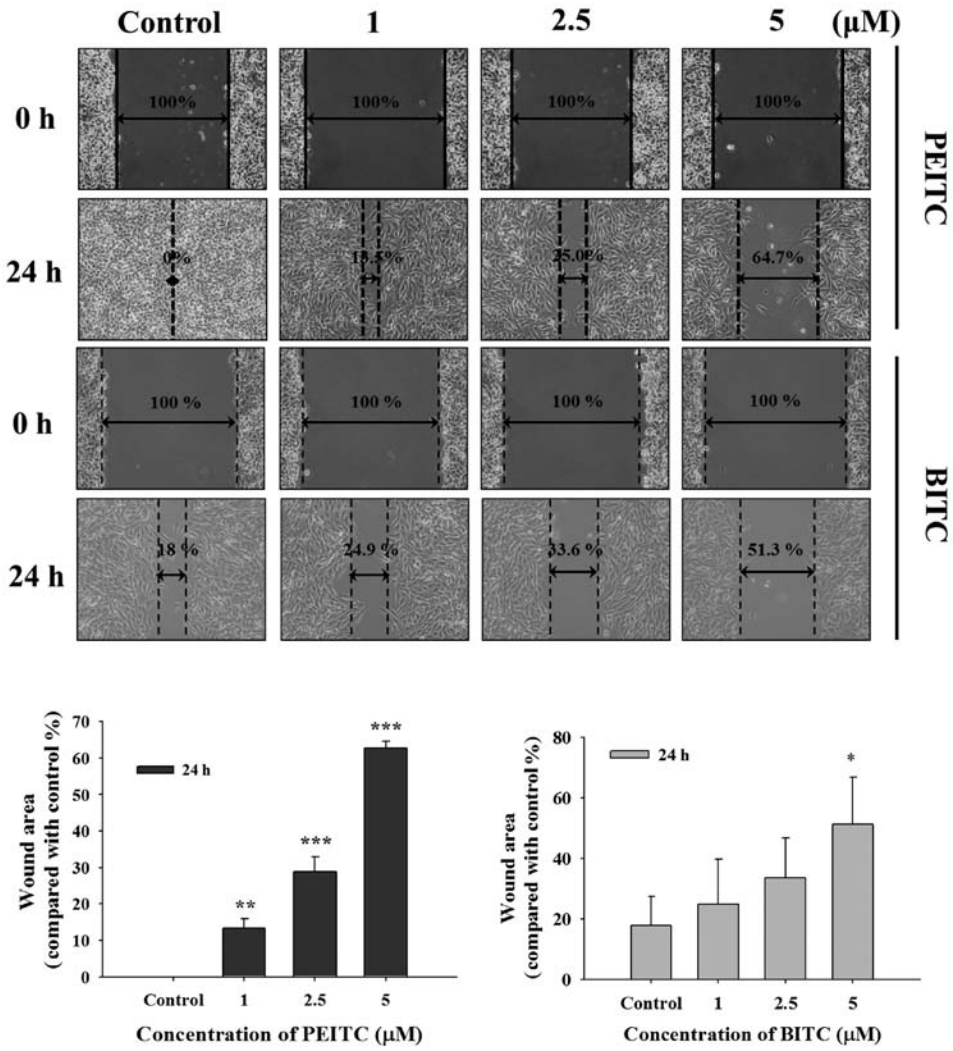

Figure 2. PEITC and BITC affect *in vitro* wound closure of B16F10 cells. Cells ($2x10^5$ cells/well) were placed in a 6-well plate for 24 h and were wounded with a scratch and rinsed to remove debris and then were incubated with or without PEITC and BITC (0, 1, 2.5 and 5 μM) for 24 h. The relative wound closures were monitored and photographed using phase contrast microscopy. The percentage of inhibition was calculated.

anti-p-FAK, anti-RhoA, anti-Ras, anti-GRB2, anti-SOS-1, anti-MMP-2, anti-MMP-9, anti-uPA, anti-TIMP-1, anti-NF-κBp65, anti-NF-κBp50 and anti-E-cadherin. Membranes were washed and incubated with the diluted secondary antibodies (goat anti-mouse immunoglobulin G (IgG), diluted 1:5000, Santa Cruz Biotechnology Inc., Dallas, TX, USA; goat anti-rabbit IgG, diluted, 1:5,000, Santa Cruz Biotechnology Inc.).

Investigated proteins on the membrane were visualized using the enhanced chemiluminescence detection system (ECL®, Millipore, Temecula, CA, USA) (30,32).

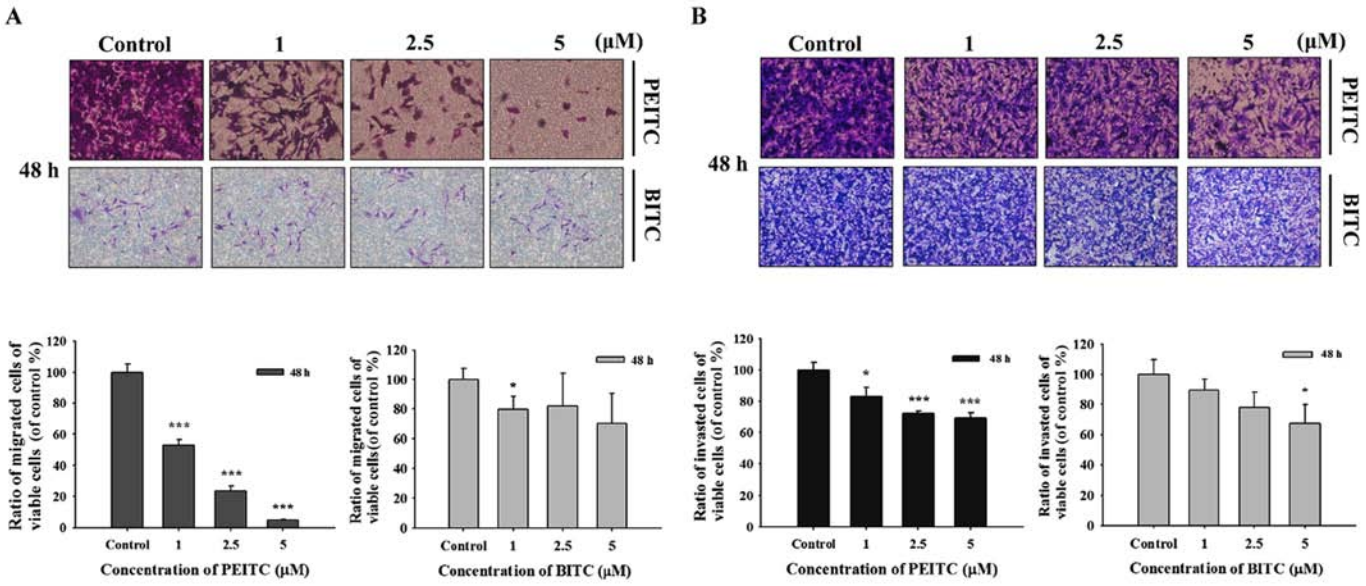

Figure 3. PEITC and BITC suppressed the migration and invasion of B16F10 cells *in vitro*. Cells ($5x10^4$ cells/well) were placed on a filter coated with collagen for migration or Matrigel for invasion and were treated with various concentrations (0, 1, 2.5 and 5 μM) of PEITC and BITC for 48 h. B16F10 cells penetrated through to the lower surface of the filter and were stained with crystal violet and were photographed under a light microscope at x200 (A and B) and cells were counted (A and B) as described in Materials and methods. Results are from three independent experiments. *P<0.05, ***P<0.001, significant difference between PEITC- and BITC-treated groups and the control as analyzed by Student's t-test.

Electrophoretic mobility shift assay (EMSA). A $5x10^5$ cells/well of B16F10 cells were placed in a 12-well and were treated with 0, 1, 2.5 and 5 μM of PEITC and BITC for 48 h. Cells were collected for nuclear extracts by the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL, USA). Nuclear extract protein (5 μg) was performed for EMSA with a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. 5'-Biotin-GATCCAGGGG ACTTTCCCTAGC-3' (biotin end-labeled oligonucleotide sequences) corresponding to the consensus of NF-κB was developed as previously described (33). Both biotin end-labeled duplex DNA were then incubated with a nuclear extract for further electrophoresis in 6% polyacrylamide native gel and then a 100-fold excess of unlabeled double stranded oligonucleotide was added to the reaction for competition. Both samples (DNA) were transferred to a positive nylon membrane. They were UV cross-linked and probed with biotin-HRP conjugate for incubating with the substrate of ECL kit (Millipore) as previously described (33).

Statistical analysis. All data represent at least 3 independent experiments and are expressed as mean ± SD. A significant difference between the PEITC and BITC-treated and control groups were compared by Student's t-test. *P<0.05 and ***P<0.001 were considered as an indication of statistical significance.

Results

PEITC and BITC decrease the viability of B16F10 cells. In order to understand the possible concentrations for inhibiting cell migration and invasion of B16F10 cells, cells were incubated with PEITC (0-15 μM) and BITC (0-10 μM) for 48 h. After incubation, cells from each treatment were collected for measuring cell viability by flow cytometric assay and results

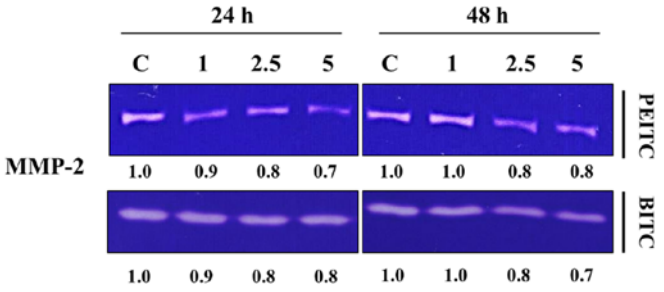

Figure 4. PEITC and BITC affect the activities of MMP-2 in B16F10 cells. Cells ($5x10^4$ cells/well) were incubated with 0, 1, 2.5 and 5 μM of PEITC and BITC for 24 and 48 h and then conditioned medium was collected and gelatin zymography assay was performed as described in Materials and methods. The different activity of MMP-2 was determined by ImageJ software and the results are expressed as a percentage of the control (100%).

are shown in Fig. 1. Results indicated that PEITC and BITC significantly reduced total cell viability from 5 to 15 μM and 1 to 10 μM, respectively in B16F10 cells. Therefore, we selected 1, 2.5, and 5 μM for scratch wound healing assay, cell migration and invasion experiments.

PEITC and BITC inhibit cell mobility in B16F10 cells. Scratch wound healing assay was used to investigate the inhibition of PEITC and BITC on cell mobility of B16F10 cells *in vitro* and results are shown in Fig. 2. One of the representative figures was present and wound healing images (cell mobile capabilities) were undertaken at the same magnification and time (0 and 24 h) after PEITC and BITC treatments in B16F10 cells. PEITC and BITC decreased the closure rate of the scratch, dose-dependently, when compared to the control group at 24 h treatment. Based on the results, it indicated that BITC at 1-2.5 μM has higher inhibition of cell mobility than that of PEITC (Fig. 2).

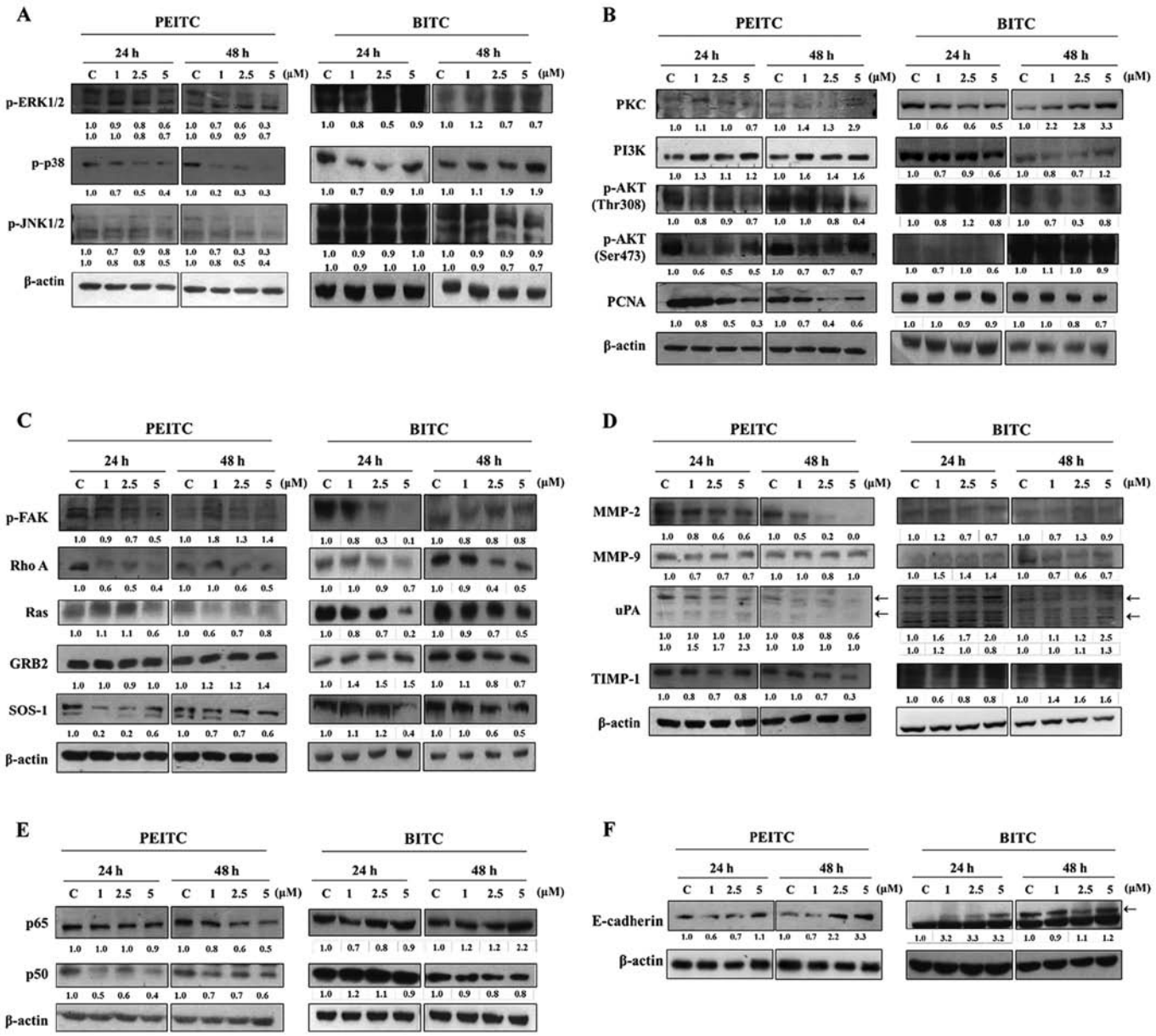

Figure 5. PEITC and BITC affect the levels of associated proteins in migration and invasion of B16F10 cells. Cells ($1x10^6$ cells/dish) were treated with PEITC and BITC (0, 1, 2.5 and 5 μM) for 24 and 48 h. Cells were collected and total protein was determined and for SDS page gel electrophoresis as described in the Materials and methods. The levels of p-ERK1/2, p-p38 and p-JNK1/2 (A), PKC, PI3K, p-AKT (Thr308), p-AKT (Ser473) and PCNA (B), p-FAK, RhoA, Ras, GRB2 and SOS-1 (C), MMP-2, MMP-9, uPA and TIMP-1 (D), NF-κBp65 and NF-κBp50 (E) and E-cadherin (F) expression levels were estimated by western blotting as described in Materials and methods.

PEITC and BITC suppress migration and invasion of B16F10 cells. In order to further investigate PEITC and BITC suppressed cell migration and invasion in B16F10 cells, the Transwell chamber coated with collagen for cell migration and coated with matrigel for cell invasion were performed and the results are shown in Fig. 3. Fig. 3A indicated that PEITC (1-5 μM) significantly suppressed the migration of B16F10 cells dose-dependently; however, BITC only at 1 μM induced the inhibition of cell migration. PEITC suppressed cell migration was greater than that of BITC. Fig. 3B indicated that PEITC (1-5 μM) significantly suppressed the invasion of B16F10 cells dose-dependently; however, BITC only at 5 μM induced the inhibition of cell invasion. PEITC suppressed cell invasion was greater than that of BITC.

PEITC and BITC inhibit MMP-2 activities in B16F10 cells. B16F10 cells were incubated with various concentrations of PEITC and BITC for 24 and 48 h and were collected for MMP-2 activities by using the gelatin zymography assay and the results are shown in Fig. 4. Results indicated that PEITC and BITC suppressed the activities of MMP-2 in B16F10 cells. The inhibition rate between PEITC and BITC are not significantly different; however both compounds are significantly different when compared to control groups.

PEITC and BITC affect key metastasis-related proteins in B16F10 cells. For further investigating PEITC and BITC suppressed cell invasion and migration involved in the inhibition of metastasis-associated protein expression in B16F10

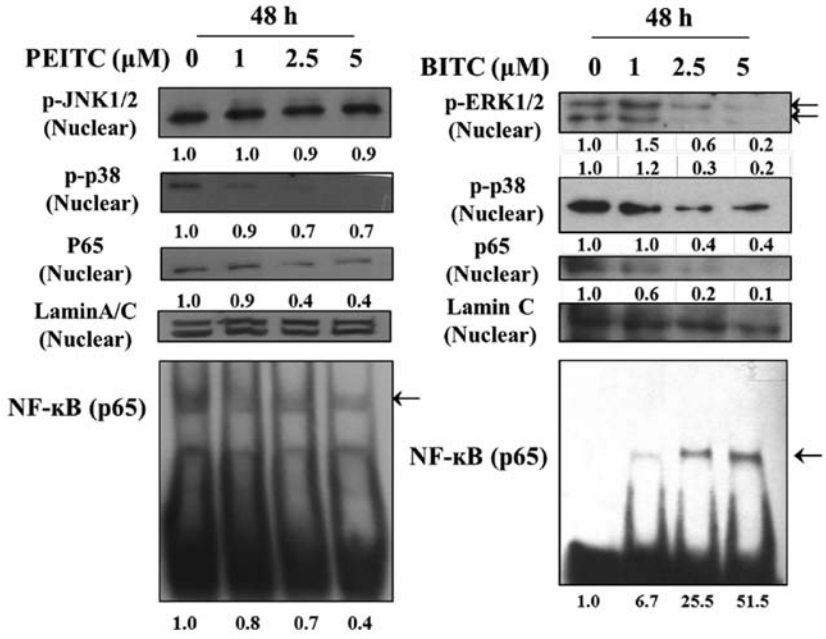

Figure 6. PEITC and BITC affect the binding of NF-κBp65 on DNA in B16F10 cells. For further confirming the effects of PEITC and BITC on the binding of NF-κBp65 on DNA in B16F10 cells, cells were treated with PEITC and BITC (0, 1, 2.5 and 5 μM) for 48 h and then were assayed by using EMSA as described in Materials and methods.

cells, cells after incubation with PEITC and BITC (0, 1, 2.5 and 5 μM) for 24 and 48 h were harvested for western blotting and the results are shown in Fig. 5. The results revealed several depressed key metastasis-related proteins, such as p-ERK1/2, P-p38 and p-JNK1/2 underwent significant reduction at 24 and 48 h treatment by PEITC (Fig. 5A). However, BITC treatment occurred at both time periods and only p-ERK1/2 was significantly reduced (Fig. 5A). At PEITC and BITC treatment at 24 and 48 h, the p-AKT (Thr308), p-AKT (Ser473) and PCNA were significantly reduced when compared to control, however, PKC and PI3K were significantly increased when compared to control in PEITC treatment, and PKC and PI3K were reduced at 24 h treatment of BITC but it increased the expression of PKC and PI3K at 48 h treatment of BITC (Fig. 5B). The RhoA, Ras and SOS-1 were reduced at 24 and 48 h treatment of PEITC, p-FAK was reduced at 24 h treatment of PEITC, GRB2 was increased at 48 h treatment but p-FAK was increased at 48 h treatment of PEITC (Fig. 5C). However, for BITC treatment only GRB2 at 24 h treatment was increased and p-FAK, RhoA, Ras and SOS-1 were decreased at 24 and 48 h treatment including GRB2 at 48 h treatment of BITC (Fig. 5C). The MMP-2, MMP-9, uPA and TIMP were reduced at 24 and 48 h treatment of PEITC (Fig. 5D), however, at BITC treatment for 24 and 48 h, MMP-2 and MMP-9 were reduced, the TIMP-1 was reduced at 24 h treatment of BITC, uPA was increased at both time periods of treatment and TIMP-1 was increased at 48 h treatment of BITC (Fig. 5D). The NF-κBp65 and NF-κBp50 were reduced at 24 and 48 h treatment of PEITC, however, BITC at both treatment periods, NF-κBp65 were increased but NF-κBp50 was reduced at 48 h treatment but 24 h treatment was increased (Fig. 5E). The E-cadherin was increased at 24 and 48 h treatment of PEITC, however BITC treatment at both time periods were increased (Fig. 5F).

PEITC and BITC decreased the binding of NF-κBp65 on DNA in B16F10 cells. In order to understand the effects of PEITC and BITC on the binding of NF-κBp65 on DNA in B16F10 cells, cells were treated with 0, 1, 2.5 and 5 μM of PEITC and BITC for 48 h and were assayed by using EMSA. The results are shown in Fig. 6. The results indicated that NF-κBp65 bind on DNA was decreased in PEITC treatment, however, BITC treatment was increased.

Discussion

Numerous studies have shown that phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) significantly induced cytotoxic effects on many human cancer cell lines. The cytotoxic effects include decreased percentage of viable cell number through cell cycle arrest and apoptosis. However, currently how PEITC and BITC affect mouse melanoma B16F10 cell migration and invasion are unclear. Thus, in the present studies, we investigated PEITC and BITC effect on cell migration and invasion in B16F10 cells *in vitro* and we found that 1) PEITC has lower cytotoxic effects than that of BITC (Fig. 1), thus, we selected 1, 2.5 and 5 μM for further experiments; 2) wound healing assay showed that PEITC have lower inhibition of wound healing (mobility) than that of BITC except at high dose (5 μM) PEITC inhibited cell mobility more than that of BITC (Fig. 2); 3) results obtained from Transwell chamber coated with collagen or matrigel for cell migration and invasion, respectively, PEITC inhibited cell migration more than that of BITC (Fig. 3A) and PEITC inhibited cell invasion more than that of BITC (Fig. 3B); 4) the inhibition of MMP-2 activity was not significantly different between PEITC and BITC (Fig. 4) western blot examination demonstrated that PEITC has reduced MAPK signaling-associated proteins such as p-ERK1/2, p-p38 and p-JNK1/2 (Fig. 5A), but BITC

treatment increased all those MAPK signaling associated proteins (Fig. 5A).

The PEITC increased PI3K but BITC decreased PI3K at both time periods of treatment (Fig. 5B). PEITC and BITC both suppressed the expression of RhoA, Ras, and SOS-1, however, PEITC increased FAK and GRB2, but BITC increased FAK at 48 h (Fig. 5C). PEITC decreased the expression of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) but BITC increased them (Fig. 5D). At 48 h treatment, PEITC decreased NF-κBp65 and NF-κBp50 but BITC increased both (Fig. 5E) and both increased E-cadherin (Fig. 5F); 5) EMSA also confirmed that PEITC inhibited NF-κB binding DNA but BITC increased NF-κB binding to DNA in B16F10 cells (Fig. 6). All these observations are compatible with PEITC and BITC have anti-metastasis capabilities. Thus, our findings may prove that PEITC and BITC have potential as anti-metastatic in melanoma.

It is well known that approximately 90% of cancer deaths are caused by metastasis but the exact pathogenesis and mechanisms involved are not completely clear (34). Herein, we used wound healing and Transwell filter to show B16F10 cell suppression of the cell migration and invasion (Figs. 2 and 3). It is well documented that matrix metalloproteinases (MMPs) are involved in cell metastasis (24) and inhibition of MMPs can lead to suppression of cancer cell metastasis (35). Thus, one of the strategies for inhibiting cancer metastasis is to suppress the regulation of MMP proteins. Increased MMP-2 and MMP-9 activities and expression levels are correlated with reduced survival and poor prognosis in human malignancies (36,37) and in many pathological processes including metastatic cancer and tumor-induced angiogenesis (38). In the present study, we used gelatin zymography assay which demonstrated that PEITC and BITC inhibited activities of MMP-2 in B16F10 cells (Fig. 4). PEITC and BITC inhibited MMP-2 production (Fig. 5D) and activity (Fig. 4) is also evident from the inhibition of collagen matrix invasion in B16F10 cells *in vitro*. MMP-2 (gelatinases) involved in tumor invasion and angiogenesis and chemical suppression of MMP-2 may lead to the inhibition of tumor metastasis (39,40). Results from western blotting indicated that PEITC and BITC at 24 and 48 h treatment significantly reduced the protein levels of MMP-2 (Fig. 5D) and PEITC inhibited TIMP-1 at 24 and 48 h treatment but BITC at 48 h treatment led to increased TIMP-1 (Fig. 5D). PEITC suppressed urokinase-type plasminogen activator (uPA) at 24 and 48 h treatment but BITC increased it at both treatment times (Fig. 5D). The uPA protein expression levels have also been considered as promising targets of anticancer drugs (41) because it is involved in cell invasion and metastasis. It was reported that uPA gene transcription is involved in motifs that upstream sequences which correspond to NF-κB, AP-1, and PEA3-binding sites (42,43).

In the current study, the effects of PEITC and BITC on NF-κB transcription activity (DNA binding) were examined by using EMSA assay and results (Fig. 6) indicated that PEIT reduce the binding of NF-κB to DNA in DNA-binding domains but BITC elevated the binding of NF-κB to DNA in DNA-binding domains (Fig. 6). Based on the results from western blotting indicated that PEITC suppressed the expression of MMP-2 and TIMP-1 but BITC increased them (Fig. 5D) and TIMPs act as natural inhibitors of MMPs by tightly binding the MMP in a 1:1 stoichiometric ratio (44). At 48 h treatment of PEITC, it decreased NF-κBp65 and NF-κBp50 but BITC increased both (Fig. 5E) and both increased E-cadherin (Fig. 5F). This reduced binding activity was accompanied by inhibition of the nuclear protein expression of this factor in B16F10 cells that was confirmed by western blotting. These results indicated that NF-κB binding activity suppression was also possibly implicated in the inhibition of MMP or uPA synthesis. It is well known that cancer cells can express high levels of MMPs, cathepsins and uPA, which degrade tissue extracellular matrix (ECM) and facilitate cancer invasion and metastasis (45). NF-κB is a complex family, thus, further investigations are needed in the future. These MMPs have been shown to be present in different types of cancer cells, including melanoma, lung and breast (46).

Numerous studies have shown that in many physiological and pathological settings, the mitogen-activated protein kinase (MAPK) pathway involved in regulating cell death and survival (47,48) plays a central role in regulating the expression of MMP-2 and MMP-9 (49). MMPs are partly mediated by the MAPK pathway (50,51). MAPK includes ERK1/2, c-Jun NH2-terminal kinase, and p38 and other factors. The MAPK pathway regulated various cellular activities such as proliferation, invasion, metastasis, and death (52). It was suggested that agents to inhibit the MAPK pathway might lead to prevent cancer angiogenesis, proliferation, invasion, and metastasis including melanoma (49,53). Results from western blotting indicated that PEITC and BITC treatment at 24 and 48 h significantly reduced the expression of p-ERK1/2, p-p38 and p-JNK1/2 when compared to control (Fig. 5A) which means PEITC and BITC suppressed MAPK signal pathway in B16F10 cells. Fig. 5B indicated that p-AKT(Thr308), p-AKT(Ser473) and PCAN were reduced in PEITC and BITC treatment, however, both agents increased AKT expression in B16F10 cells. Activated PI3K and its downstream target Akt have been recognized to be involved with tumor cell invasion, and oncogenesis (54,55). The downregulation of the PI3k/Akt pathway have been reported to decrease the invasion of melanoma cells (53,56). In melanoma cells, the activation of the PI3K-Akt signaling pathway promoting cell invasion has been shown (57). AKT, downstream of PI3K has been reported to be suppressed by the transcription of the E-cadherin gene (58). Ras regulating RhoA has been recognized to affect tumor cells transmigration through mesothelial monolayer (59). Results (Fig. 5C) indicated that PEITC and BITC significantly reduced the expression of RhoA and Ras in B16F10 cells at both treatment times. Thus, PEITC and BITC inhibited B16F10 cell migration and invasion may also be via the inhibition of RhoA and Ras.

In conclusion, our results indicated that PEITC and BITC reduced the viable cell number of B16F10 cells. We selected the low concentrations (1, 2.5 and 5 μM) of PEITC and BITC bufalin for examining the effects of cancer cell metastasis and we found that PEITC and BITC significantly inhibited cell mobility, migration and invasion of B16F10 cells *in vitro*. We also used western blot assay and found that PEITC and BITC inhibited many metastasis-associated protein molecules including MMP-2, MAPKs, E-cadherin, Ras, RhoA and NF-κB. NF-κB also was confirmed by confocal laser microscopy examination. This finding suggests that PEITC and BITC

are potential candidates for the development of chemotherapeutic treatments for melanoma cells in the future.

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