

Effect of 4-methyl-3-nitrobenzoic acid on non-small cell lung cancer cell migration

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Abstract. Metastasis is a major cause of mortality among lung cancer patients. In a previous high-throughput screening of 20,000 compounds to identify inhibitors of cancer cell migration, 4-methyl-3-nitrobenzoic acid was found to inhibit the migration of nine tumor cell lines. The present study used the human non-small cell lung cancer cell (NSCLC) treatment with 4-methyl-3-nitrobenzoic acid significantly inhibited epithelial growth factor (EGF)-induced chemotaxis and chemokinesis of cancer cells in NSCLC cells. The defect in chemotaxis is likely due to the impairment in EGF-induced cofilin phosphorylation and actin polymerization. Furthermore, EGF-induced cell adhesion, which is another key factor in cell migration, was also inhibited. Taken together, these results suggest that 4-methyl-3-nitrobenzoic acid is a potent inhibitor of cancer cell chemotaxis and may be developed into a novel antimetastasis drug.

Introduction

Lung cancer remains the leading cause of cancer-related mortality in the world. Non-small cell lung cancer (NSCLC), including squamous carcinoma, adenocarcinoma and large cell carcinoma, accounts for approximately 80% of these thoracic malignancies, with 1.2 million new cases diagnosed worldwide each year (1). Despite improved outcomes using various chemotherapeutic agents and regimens in patients with advanced NSCLC, most treatments ultimately fail due to metastasis and intolerable toxicity (2,3).

Metastasis is a complex multistep process that requires the coordination of multiple cell surface receptors, including chemoattractant receptors that direct cell migration, adhesion receptors that modulate interactions of migrating cells with the endothelium and the extracellular matrix, and protease receptors that mediate the interactions of cells with their extracellular environment (4). Increasing evidence suggests that chemotaxis plays a pivotal role in the progress of tumor metastasis (5,6). Chemotaxis involves the ability of a cell to detect an extracellular gradient of chemical stimuli and to move to a higher concentration site (7).

Conventional chemotherapies target cell growth and proliferation. However, their application has always been limited by cytotoxicity and drug resistance. Metastasis accounts for more than 80% of mortality and morbidity. Targeting metastasis may provide a potent approach to containing cancers. In a high-throughput screening of 20,000 compounds, we identified a chemical, 4-methyl-3-nitrobenzoic acid, which inhibits the migration of various cancer cells and demonstrates low cytotoxicity. In the present study, we investigated the role of 4-methyl-3-nitrobenzoic acid and the mechanism by which it inhibits lung cancer cell chemotaxis.

Materials and methods

Cell culture and reagents. The human NSCLC cell line A549 was provided by Dr Dalong Ma of the School of Basic Medical Science, Peking University (Beijing, China). H1299, H-358 were obtained from American Type Culture Collection (Manassas, VA, USA). RPMI-1640 medium and DMEM were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from Gibco (Gibco, USA), and 0.1% fibronectin from Sigma-Aldrich (St. Louis, MO, USA). The microboyden chambers and membranes were obtained from Neuroprobe (Gaithersburg, MD, USA). The recombinant human epithelial growth factor (EGF) was obtained from R&D Systems (Minneapolis, MN, USA). Goat anti-rabbit IgG-FITC, goat anti-mouse IgG-FITC, and antibodies against PKC ζ , P-cofilin, β -actin, and EGF receptor (EGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The MTT assay kit was from Sigma-Aldrich.

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Chemotaxis assay. The chemotaxis assay was performed in a 48-well Boyden chamber as previously described (8). Briefly, cells were suspended in the binding medium at a density of 0.8×10^6 cells/ml and loaded into the upper chambers, while the chemoattractants (EGF) were placed into the lower chambers. The 8- μ m filter membrane was then pretreated with 10 μ g/ml fibronectin in serum-free medium at 4°C overnight, dried in air, and inserted between the upper and lower chambers. The cells in the chambers were then allowed to migrate for 3 h at 37°C in a 5% CO₂ incubator. The filter membrane was rinsed, fixed and stained.

EGF activation and Western blotting assay. Western blotting assay was performed as previously described (8). Briefly, the cells were serum-starved for at least 3 h and subsequently activated with 1 ng/ml EGF for 0 sec, 30 sec, 1 min, 2 min and 5 min. The reactions were stopped with ice-cold phosphate-buffered saline (PBS) (pH 6.8), and the cells were lysed with a lysis buffer. Equal amounts of cell lysates (30 μ g total protein per lane) were then loaded and separated by SDS-PAGE, and proteins were transferred onto PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA). The membranes were then probed with antibodies against EGFR (1:1,000), PKC ζ (1:2,000), P-cofilin (1:3,000), cofilin (1:1,000), β -actin (1:5,000) and HRP-conjugated secondary antibody (1:5,000), and visualized by SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Scratch assay. One day prior to the assay, the cells were plated in 35-mm dishes at a density of 1×10^6 cells/ml to form a monolayer. A549 cells were pretreated with 4-methyl-3-nitrobenzoic acid at a concentration of 0.6 and 2 μ M for 24 h: A549 cells treated with drug vehicle were used as a control. An even trace was lined out in the middle of the monolayers using a 10- μ l pipette tip to initiate a scratch (9). The cells were then incubated at 37°C in 5% CO₂, and the wounds were photographed at intervals until they were occluded by migrating cells. The samples were tested three times. Data were expressed as the mean \pm SD.

Adhesion assay. This assay was performed as previously described (8). Briefly, the control cells or 4-methyl-3-nitrobenzoic acid-pretreated cells (2.7×10^5 cells/ml) were suspended in complete medium and incubated at 37°C in 5% CO₂ for 20 min. A 35-mm dish containing dried glass coverslips was then prepared, wherein the coverslips had been pretreated with 10 μ g/ml fibronectin in serum-free medium for 2 h at 37°C and air-dried for 30 min at room temperature. Then, 1.5 ml cells, in the presence or absence of 1 ng/ml EGF, were immediately added into the dish with the dried coverslips. After incubation for 15 min, the cells were gently washed twice with ice-cold PBS and then fixed. The cells attached to the coverslips were counted under a light microscope (magnification, $\times 200$).

Cellular F-actin measurement. Cellular F-actin measurement was carried out as previously described (8). In brief, 1×10^6 cells were pre-incubated for 2 h at 37°C in RPMI-1640 supplemented with 2% (w/v) BSA (Sigma-Aldrich) and 10 mM HEPES (pH 7.2). Following the addition of 1 ng/ml EGF, cells were incubated at 37°C for the indicated periods of time, fixed

with 4% paraformaldehyde (PFA) for 5 min, and permeabilized with 0.1% Triton X-100 for 20 min. These cells were incubated with Alexa Fluor 568 phalloidin (Molecular Probes) for 2 h, and were washed five times. The bounded phalloidin was later extracted by methanol at 4°C for 60 min, while the F-actin content was measured using a microplate fluorescence reader with an excitation wavelength of 578 nm and an emission wavelength of 600 nm. Results were expressed as relative F-actin content, where $F\text{-actin}\Delta t / F\text{-actin}0 = \text{fluorescence}\Delta t / \text{fluorescence}0$. Total protein in the sample was analyzed by a bicinchoninic acid assay (Pierce) and fluorescence signals were normalized against total protein.

MTT assay. A549 cells were trypsinized and made into a single cell suspension. The cells were then plated in a 96-well culture plate at a concentration of 5×10^3 cells per well. Following 24 h of incubation, the medium of the wells in each column was replaced with a medium containing 4-methyl-3-nitrobenzoic acid of 0.01, 0.03, 0.22, 0.67, 1.0, 2.0, 3.0, 4.0, 10.0 and 20.0 μ M, respectively. Blank control wells were added with drug vehicle in the medium. Cells were then cultured for another 24, 48 and 72 h. The supernatants were removed, and 20 μ l MTT was added in each well followed by another 4 h of culture. The supernatants were discarded carefully, and 200 μ l dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO, USA) was added and agitated vigorously to dissolve the purple precipitation formation. The optical density (OD) of each well was tested using a plate reader at 570 nm. The experiment was repeated in triplicate. The inhibitory rate (IR) of cell growth was calculated using the formula: $IR = (ODc - ODt) / ODc \times 100\%$ (ODt: OD of cells in treated groups; ODc: OD of cells in control groups).

Cell cycle assay. Cells were seeded in a 6-well plate and were treated with 4-methyl-3-nitrobenzoic acid at a concentration of 0.67 and 2.0 μ M for 24 h. Drug vehicle-treated cells served as the control. Following the treatment, the media were discarded. The cells were suspended using trypsin and centrifuged at 300 \times g for 5 min at room temperature. The supernatant was discarded, and the precipitation was washed using PBS and centrifuged at 300 \times g for 5 min. The supernatant was discarded again. Cells were fixed overnight in 95% ethanol at 4°C and washed using PBS. Cold PI stain solution at 500 μ l was added to the precipitation and incubated for 30 min in the dark at room temperature. The samples were analyzed by flow cytometry (FCM) (Coulter Epics XL, Miami, FL, USA). The results were analyzed by MultiCycle for Windows.

Statistical analysis. Prism 3.0 software was used for data analysis, and the two-way ANOVA test was used for two-group data. The results were generated from at least three separate experiments.

Results

General effect of 4-methyl-3-nitrobenzoic acid on A549 cells. 4-methyl-3-nitrobenzoic acid was selected from 20,000 chemicals based on five molecular structures by high-flux select migration assay (our unpublished results). The molecular structure of 4-methyl-3-nitrobenzoic acid is shown in Fig. 1A.

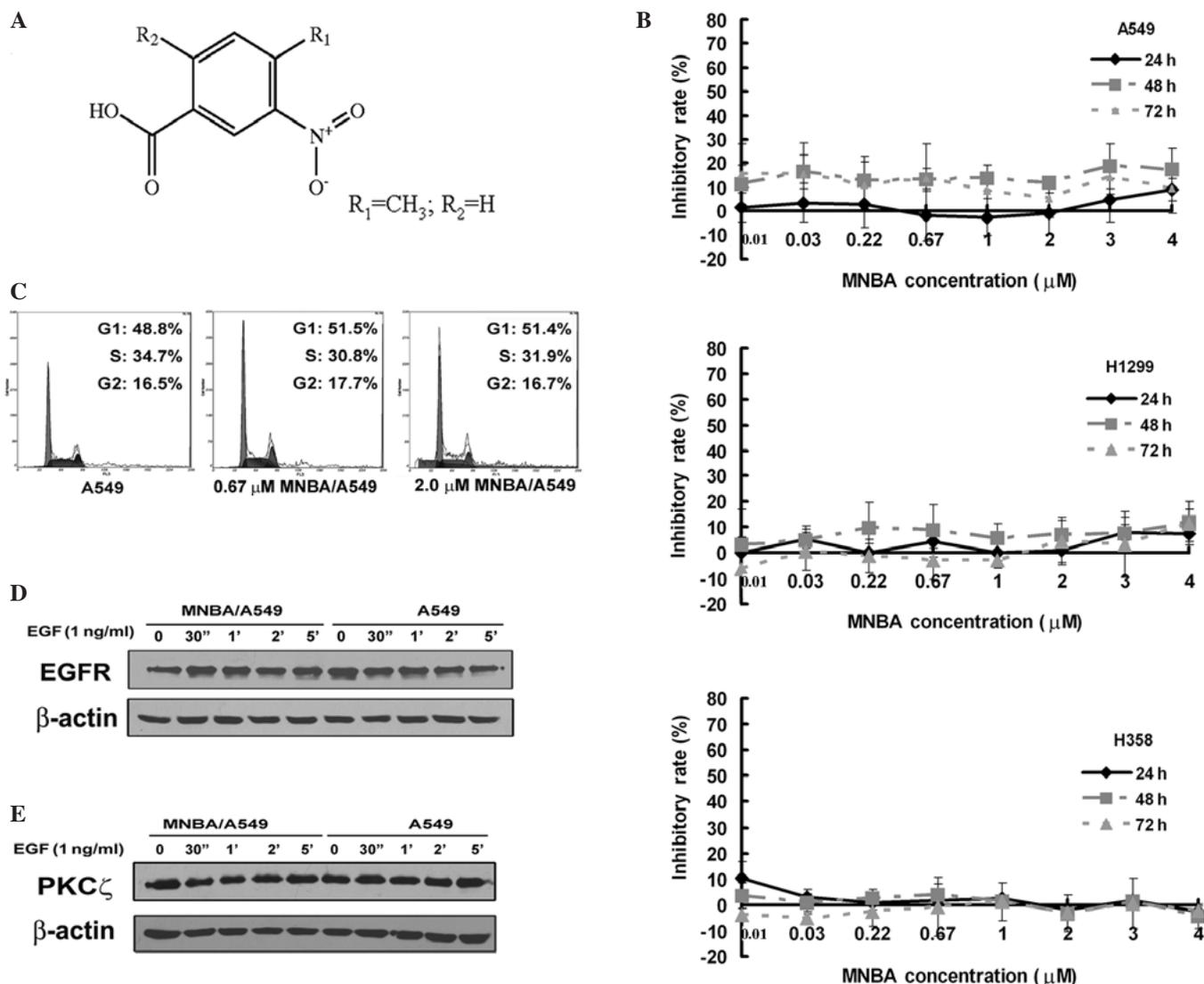


Figure 1. Effect of 4-methyl-3-nitrobenzoic acid on A549 cells. (A) Molecular structure of 4-methyl-3-nitrobenzoic acid. (B) Cytotoxicity assay. Cells were treated with the indicated amounts of 4-methyl-3-nitrobenzoic acid for 3 days followed by the MTT assay. Data were collected by representative image from three repeated experiments. (C) Cell cycle analysis. Cell cycle distribution of A549 cells and A549 cells pretreated with 0.67 and 2.0 μM of 4-methyl-3-nitrobenzoic acid for 24 h. (D and E) Western blotting analysis of EGFR and PKC ζ in total cell lysates from A549 cells treated with drug vehicle (A549) and with 2.0 μM 4-methyl-3-nitrobenzoic acid (MNBA/A549) following stimulation with 1 ng/ml EGF for 0 sec, 30 sec, 1 min, 2 min and 5 min. β -actin was used as a loading control. Data were representative of three independent experiments.

To test the cytotoxic effect of 4-methyl-3-nitrobenzoic acid, an MTT assay was performed. As shown in Fig. 1B, no significant effect on cell proliferation was detected in A549 cells treated with 4-methyl-3-nitrobenzoic acid until the concentration of 4-methyl-3-nitrobenzoic acid increased to 10 μM , at which point cell proliferation was significantly inhibited. We detected the same effect on cell proliferation in another NSCLC cell lines H1299 and H358. We further examined the effect of 4-methyl-3-nitrobenzoic acid on the cell cycle by FCM. As shown in Fig. 1C, 4-methyl-3-nitrobenzoic acid did not induce a significant G1/S arrest in A549 cells at a concentration of 0.67 and 2.0 μM .

EGF is known to stimulate actin polymerization and lamellipodial extension (10). The overexpression and ligand-induced activation of EGFR, which is a prototypic receptor tyrosine kinase, correlates with the increased invasiveness of tumors *in vivo* and increased migration of cell lines *in vitro* (11). In this study, EGF was used to induce the chemo-

taxis of A549 cells. Thus, we first detected the expression of EGFR by Western blotting assay. As shown in Fig. 1D, 1 ng/ml EGF was used to stimulate the control cells and the cells pretreated with 4-methyl-3-nitrobenzoic acid. Pretreatment with 4-methyl-3-nitrobenzoic acid did not induce any detectable loss of surface EGFR.

Our previous results demonstrated that protein kinase C ζ (PKC ζ) had a pivotal role in NSCLC cell chemotaxis and migration (12). Thus, we investigated the expression of PKC ζ after the cells were pretreated with 2.0 μM 4-methyl-3-nitrobenzoic acid. Our results revealed that pretreatment with 4-methyl-3-nitrobenzoic acid had no effect on the expression of PKC ζ in A549 cells (Fig. 1E).

4-methyl-3-nitrobenzoic acid inhibits NSCLC cell migration. Cell migration is a significant component of tumor cell metastasis (13). We studied the effect of 4-methyl-3-nitrobenzoic acid on NSCLC cell migration. Chemotaxis assay and wound

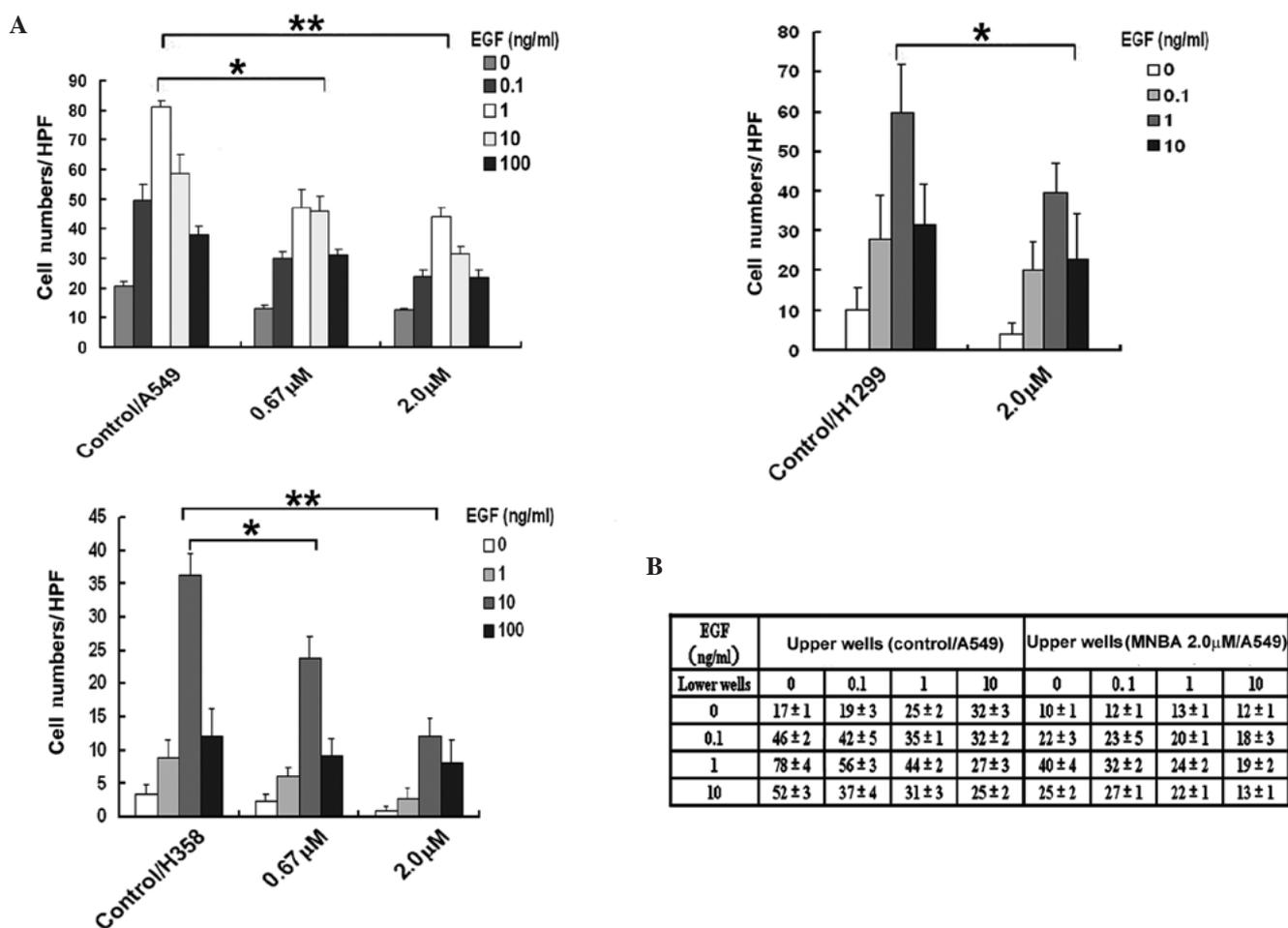


Figure 2. 4-methyl-3-nitrobenzoic acid-impaired chemotaxis and chemokinesis of NSCLC cells. (A) H1299, H358 and A549 cells were pretreated with 0.67 and 2.0 μM 4-methyl-3-nitrobenzoic acid for 24 h in a culture incubator, and drug vehicle-treated cells served as a control. Chemotaxis assay was performed for 3 h. The concentrations of EGF as a chemoattractant are shown. (B) The effect of 4-methyl-3-nitrobenzoic acid on the random cell migration of A549 cells was examined by transfilter chemokinesis assay. Cells were mixed with the indicated concentrations of EGF or binding medium immediately prior to loading the upper chambers. The control was A549 cells treated with drug vehicle, and the MNBA/A549 cells were A549 cells pretreated with 4-methyl-3-nitrobenzoic acid.

healing assay are two types of conventional methods used for studying cell migration. In our Boyden chamber assay, 0.67 and 2.0 μM 4-methyl-3-nitrobenzoic acid was used to treat A549 cells, respectively. As shown in Fig. 2A, compared with the control cells, 4-methyl-3-nitrobenzoic acid treatment at both concentrations inhibited EGF-induced A549 cell chemotaxis significantly ($P < 0.01$). 4-methyl-3-nitrobenzoic acid also impaired EGF-induced chemotaxis of H1299 and H358 cells, two additional NSCLC cell lines. We also tested the effect of 4-methyl-3-nitrobenzoic acid on A549 cell chemokinesis after the addition of the same concentration of EGF to upper and lower wells of the chambers. Fig. 2B shows that random motility decreased in the presence of 4-methyl-3-nitrobenzoic acid compared to that in the control cells.

Scratch assay, an *in vitro* wound-healing assay, is another method for evaluating cell migration capacity. It was observed that control A549 cells migrated into the wound and resulted in wound closure within 24 h, whereas the migration of A549 cells treated with 4-methyl-3-nitrobenzoic acid was significantly reduced (Figs. 3A and B). The control cells and 4-methyl-3-nitrobenzoic acid-treated NSCLC H1299 cells were maintained in a medium supplemented with 0.5% FBS to block cell proliferation, which could otherwise account

for gap closure. Taken together, these results clearly show that 4-methyl-3-nitrobenzoic acid impairs the EGF-induced migration of A549 cells.

4-methyl-3-nitrobenzoic acid inhibits F-actin polymerization and cell adhesion of NSCLC cells. Cell migration and invasion is dependent on the actin cytoskeleton. Rearrangements of the actin cytoskeleton play a role in the various steps of the migration process (14). Cell migration involves the polymerization of monomeric G-actin into filamentous F-actin, which is a prerequisite for filopodia and lamellipodia formation and plays a crucial role in the chemotactic responses of cells (15). To gain a better understanding of the underlying mechanism of 4-methyl-3-nitrobenzoic acid on cell migration, studies were performed to evaluate actin polymerization and cytoskeleton rearrangement. The cells were stained with phalloidin-Alexa568, and the levels of F-actin polymerization were detected using a microplate fluorescence reader. As shown in Fig. 4A, in normal A549 cells, EGF is capable of inducing transient F-actin polymerization. However, in cells pretreated with 4-methyl-3-nitrobenzoic acid, we detected a decrease in the F-actin content induced by EGF. Cofilin, the substrate of LIMK1, is essential for the regulation of actin

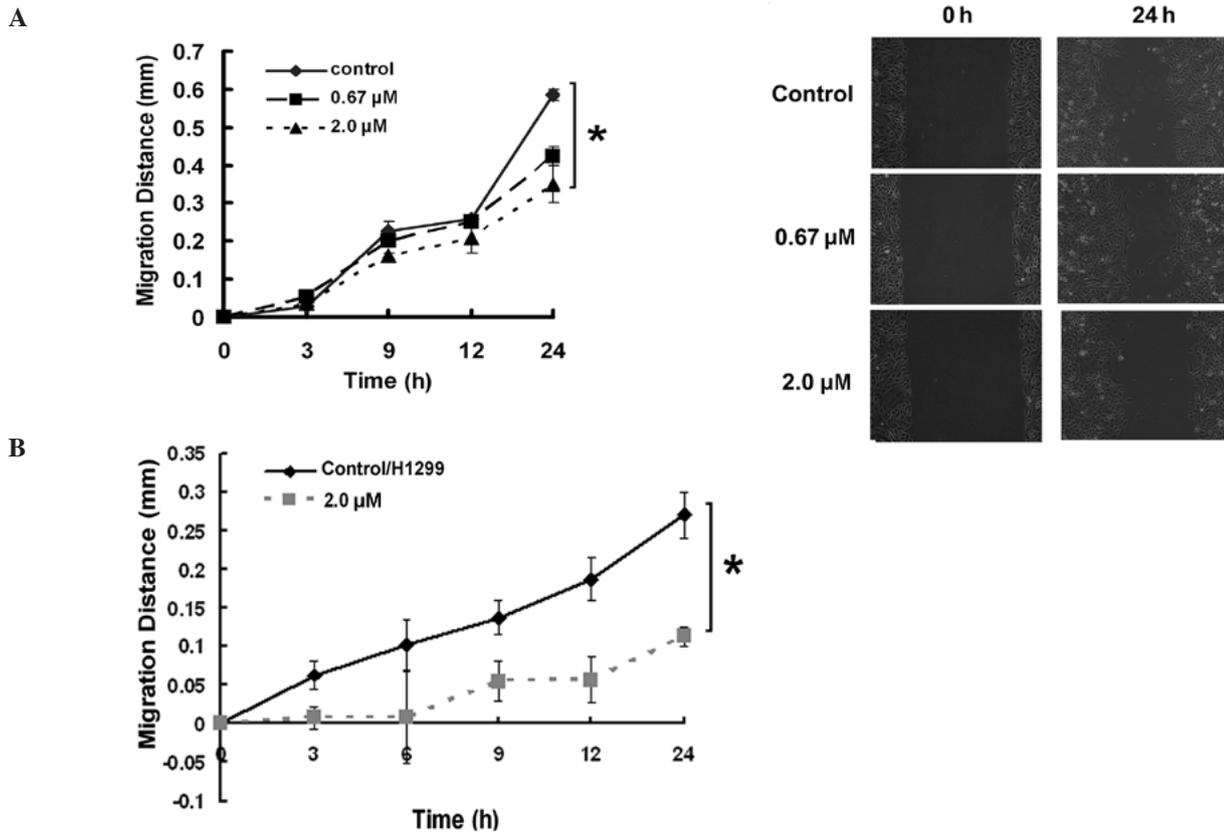


Figure 3. 4-methyl-3-nitrobenzoic acid-impaired A549 cell migration in scratch assay. (A) Drug vehicle-treated A549 cells (control) and 4-methyl-3-nitrobenzoic acid-treated H1299 cells (2.0 μ M) were grown in a 6-well plate until confluence was achieved, and the monolayers were wounded with a 10- μ l pipette tip. The images were photographed at 0 and 24 h (magnification, x100). (B) Quantification of *in vitro* scratch assay on drug vehicle-treated H1299 cells (control) and 4-methyl-3-nitrobenzoic acid-treated A549 cells (0.67 μ M and 2.0 μ M). The distance of cell migration during incubation times was measured (bar: SD of three data points).

polymerization and depolymerization during cell migration (16). EGF-induced activation of PI3K results in the phosphorylation of cofilin, whereas cofilin activation promotes F-actin polymerization. Thus, we investigated the effect of 4-methyl-3-nitrobenzoic acid on the phosphorylation of cofilin induced by EGF in A549 cells. As shown in Fig. 4B, EGF at 1 ng/ml significantly induced the phosphorylation of cofilin. Treatment of A549 cells with 4-methyl-3-nitrobenzoic acid markedly reduced the phosphorylation of cofilin induced by EGF, which was consistent with the reduction in F-actin polymerization. Taken together, these results indicate that 4-methyl-3-nitrobenzoic acid inhibits EGF-induced NSCLC cell migration by inhibiting actin polymerization and cytoskeleton rearrangement.

Cell adhesion is essential for cell movement. Excessive adhesion may result in disabled detachment and movement (17-18). We further examined the effect of 4-methyl-3-nitrobenzoic acid on cell adhesion in A549 cells. EGF at 1 ng/ml induced a marked increase of cell adhesion to fibronectin-coated glass coverslips for A549 cells (Fig. 4C), which is consistent with our findings in A549 and H1299 cells (12). However, treatment with 4-methyl-3-nitrobenzoic acid significantly impaired the EGF-stimulated rapid cell adhesion of A549 cells. Specifically, there was more than a 2-fold decrease in the number of adhesive cells in A549 cells pretreated with 2.0 μ M 4-methyl-3-nitrobenzoic acid compared with normal A549 cells following EGF stimulation ($P < 0.05$). Although

the number of adherent cells in A549 cells pretreated with 0.67 μ M 4-methyl-3-nitrobenzoic acid also decreased, there was no statistically significant difference between the two groups. These results indicate that 4-methyl-3-nitrobenzoic acid significantly inhibits A549 cell adhesion.

Discussion

Distant metastasis is a leading cause of cancer-related deaths in patients with NSCLC (19). It appears that the malignant tumor acquires the metastatic phenotype through the process of clonal evolution occurring during the multistep tumor progression (20). In the present study, a new chemical, 4-methyl-3-nitrobenzoic acid, was found, which has low cytotoxicity but is capable of inhibiting NSCLC cell chemotaxis, migration and adhesion. The results demonstrate that pretreatment of 4-methyl-3-nitrobenzoic acid in A549 cells impairs F-actin polymerization, which is essential for cytoskeletal rearrangement in cell migration and chemotaxis (21). The actin cytoskeleton controls the overall structure of cells and is highly polarized in chemotaxing cells, with F-actin assembled predominantly in the anterior leading edge and in the cell's posterior to a lesser degree (22). Cofilin, a LIM kinase 1 (LIMK1) substrate, is directly responsible for severing actin filaments and regulating actin polymerization and depolymerization during cell migration. Additionally, the phosphorylation of cofilin by LIMK1 is critical to its role in actin reorganization

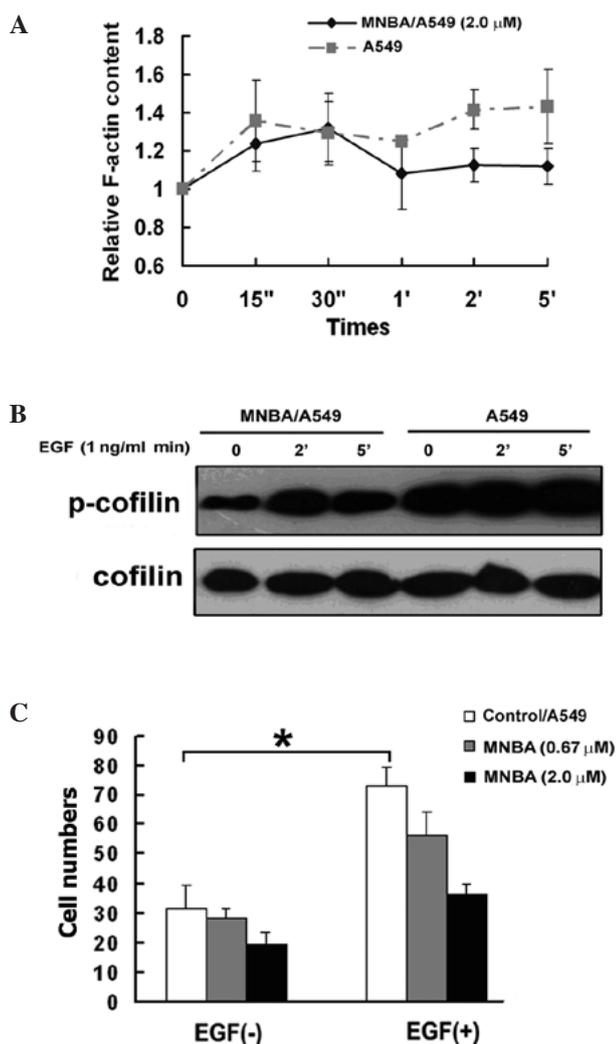


Figure 4. Effect of 4-methyl-3-nitrobenzoic acid on EGF-induced A549 cell actin polymerization and adhesion. Cells were pretreated with 2 μ M 4-methyl-3-nitrobenzoic acid for 24 h and the drug vehicle-treated A549 cells served as a control. (A) Time course of relative F-actin content. A549 cells treated with drug vehicle (A549) and with 2 μ M 4-methyl-3-nitrobenzoic acid (MNBA/A549) were treated with 1 ng/ml EGF for various periods of time prior to being fixed and stained with 568 phalloidin. Relative F-actin content was obtained as described in Materials and methods. Data were collected from three independent experiments. (B) Western blotting analysis of phosphorylated cofilin. A549 cells treated with drug vehicle (A549) or with 2 μ M 4-methyl-3-nitrobenzoic acid (MNBA/A549) were stimulated with 1 ng/ml EGF for 0 sec, 2 min and 5 min. Total cell lysates were collected and the phosphorylation of cofilin (P-cofilin) was analyzed as described in Materials and methods. Cofilin was used as a loading control. (C) 4-methyl-3-nitrobenzoic acid-impaired cell adhesion of A549 cells induced by EGF. A549 cells treated with drug vehicle (control) or with 0.67 or 2 μ M 4-methyl-3-nitrobenzoic acid (MNBA) were seeded on fibronectin-coated coverslips and then treated with 1 ng/ml EGF for 5 min to induce cell adhesion. Cell numbers in each high-power field (HPF) were counted in five fields on every coverslip under a microscope (magnification, \times 200). Data collected from three independent experiments were summarized as mean \pm SEM ($n=3$).

(23). As shown in the present results, 4-methyl-3-nitrobenzoic acid down-regulates the phosphorylation of cofilin in A549 cells. Taken together, these findings indicate that 4-methyl-3-nitrobenzoic acid treatment in NSCLC cells results in a major cytoskeletal reorganization defect in response to EGF, possibly through the regulation of cofilin phosphorylation. The detailed molecular mechanism by which 4-methyl-3-nitrobenzoic acid acts on tumor cell migration, including the manner

in which it identifies its targeting molecule(s), requires further exploration in future studies.

The process of tumor metastasis constitutes a number of stages that include tumor cell invasion from the primary tumor, intravasation, arrest and extravasation of the circulation system, followed by angiogenesis and growth at a distant location. In the metastatic process, not all components of each stage are of comparable therapeutic benefit. Numerous molecules that promote tumorigenesis also promote metastasis and are key targets for the development of therapeutics in the treatment of the two aspects of cancer progression. Our data show that 4-methyl-3-nitrobenzoic acid has a relatively minor effect on A549 cell proliferation (Fig. 1), suggesting that the target(s) of this drug is not related to tumorigenesis. The compound targets the dissemination of cancer cells, which may affect cancer metastasis. Whether 4-methyl-3-nitrobenzoic acid possesses other antimetastatic functions such as anti-angiogenesis is under investigation. The *in vivo* effect of 4-methyl-3-nitrobenzoic acid is also currently under investigation in several models of metastasis.

The development of drugs for cancer treatment is ongoing. However, mortality has not been reduced significantly for a variety of cancer types. The development of anticancer agents mainly focuses on targeting tumorigenesis; however, the majority of cancer patients succumb to tumor metastasis. Neoadjuvant and adjuvant cancer therapy is a promising area of investigation that aims to increase survival in the early stages of NSCLC; however, this treatment modality has shown low efficacy with considerable side effects (24). Gene therapeutic approaches have been developed in NSCLC treatment, but remain in an early pre-clinical stage (25). Few therapies have currently been developed to specifically target metastasis development in an adjuvant setting. Our current findings on the capacity of 4-methyl-3-nitrobenzoic acid to inhibit the spread of cancer cells provides a promising candidate to target cancer metastasis. We hypothesize that, in combination with other therapeutic regimens, the inhibition of cancer cell metastasis to distant locations may provide a new approach in cancer treatment.

Acknowledgements

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