Identification of Hirsutine as an anti-metastatic phytochemical by targeting NF-κB activation

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Abstract. Nuclear factor-κB (NF-κB) activation has been implicated not only in carcinogenesis but also in cancer cell invasion and metastatic process; therefore, targeting the NF-κB pathway is an attractive strategy for controlling metastasis. Amongst 56 chemically defined compounds derived from natural products, we have identified a new phytochemical compound Hirsutine, which strongly suppresses NF-κB activity in murine 4T1 breast cancer cells. In accordance with the NF-κB inhibition, Hirsutine reduced the metastatic potential of 4T1 cells, as seen in the inhibition of the migration and invasion capacity of 4T1 cells. Hirsutine further inhibited the constitutive expression of MMP-2 and MMP-9 in 4T1 cells, and reduced the *in vivo* lung metastatic potential of 4T1 cells in the experimental model. Given that the migration of human breast cancer cells was also inhibited, our present study implies that Hirsutine is an attractive phytochemical compound for reducing metastasis potential of cancer cells by regulating tumor-promoting NF-κB activity.

Introduction

Breast cancer is the most frequently diagnosed cancer and is therefore the leading cause of cancer death in women worldwide (1). While marked progress has been made in the treatment of breast cancer, such as hormonal therapy for estrogen receptor and/or progesterone receptor-positive tumors, other targeted therapies for selected subgroups of patients, such as HER2-positive cancers, have also been successfully developed. Despite these advances, the mortality rate is still high in breast cancer patients, mainly due to metastasis spread (2); therefore the development of metastasis-targeted therapy for breast cancer is clinically important.

Phytochemicals from natural products are a promising source for the development of novel cancer therapeutics. Because of their potential effectiveness and low toxicity profiles (3), many phytochemicals have been successful in clinical development of many diseases (4,5). Nuclear factor-κB (NF-κB), defined as a multi-functional transcription factor, is widely involved in a variety of physiological and pathological processes (6). It has been widely recognized that NF-κB plays a critical role in the initiation, promotion and progression of certain types of cancers through its ability to upregulate genes responsible for cell survival, invasion, angiogenesis and metastasis (7-12). Consequently, the NF-κB pathway is regarded as a potential new drug target in cancer metastasis and progression (13-15).

In this study, we screened 56 phytochemical compounds for their inhibitory activity in NF-κB. Hirsutine was found to be a prominent NF-κB inhibitor and significantly inhibited the metastatic potential of murine 4T1 breast cancer cells both *in vitro* and *in vivo*. Since Hirsutine further reduced the metastatic potential of human breast cancer cells, it is an attractive lead compound targeting metastasis and the progression of breast cancer.

Materials and methods

Reagents. All the compounds (in DMSO) shown in Table I were provided by the Cooperative Research Project I of the Institute of Natural Medicine, University of Toyama, Japan. Antibody against MMP-9 or β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse TNF-α was purchased from Jena Bioscience GmbH (Jena, Germany). Hirsutine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). pGL4.50 [luc2P/CMV-RE/Hygro] and pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and D-Luciferin were obtained from Promega (Sunnyvale, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad,

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CA, USA). Hygromycin B was obtained from Nacalai Tesque (Kyoto, Japan).

Cells. Mouse mammary carcinoma 4T1 cells were maintained in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% bovine serum. MDA-MB-231 and MDA-MB-468 human breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui) supplemented with 10% bovine serum. To establish the luciferase gene expressing 4T1 cells (4T1-luc or 4T1-NFκB-luc), and 4T1 cells ($5x10^5$/well) were seeded in a 6-well plate and pGL4.50 or pGL4.32 vector was transfected using Lipofectamine 2000. The cells were selected with Hygromycin B (100 μg/ml) and cloned by limiting dilution.

NF-κB luciferase reporter assay. For the detection of luciferase activity, cells were stimulated with or without TNF-α (10 ng/ml) in the screening experiments. Briefly, for screening experiments without stimulation, 4T1-NFκB-luc cells in exponential growth were placed at a final concentration of $2x10^4$ cells/well in a 96-well plate. After 3-h incubation, the cells were treated with compounds or with the vehicle (vehicle control, 0.5% DMSO) for 24 h. Then 20 μl of luciferin (900 μg/ml) was added, and the plates were incubated for another 30 min. Luciferase activity was measured by the Glomax multi detection system (Promega). For the experiments with TNF-α (10 ng/ml) stimulation, 4T1-NFκB-luc cells ($2x10^4$ cells/well) were placed in a 96-well plate for 12 h. After incubation, the cells were treated with compounds and TNF-α (10 ng/ml) for 12 h. Then luciferin was added and luciferase activity was measured.

Cell viability assay. Viability of cells was assessed using a WST-1 Cell Counting kit (Wako Pure Chemical Industries). The experimental conditions for cell viability were similar to the previous luciferase reporter assay. Twenty-four hours after treatment with compounds, 10 μl WST-1 reagent was added and incubated for another 2 h (37˚C, 5% CO_2). The absorbance at 450 nm was measured using a microplate reader.

In vitro wound healing assay. 4T1-NFκB-luc cells were plated in a 24-well plate at a concentration of ($1x10^5$ cells/well) and allowed to form a confluent monolayer for 24 h. The monolayer was then scratched with a sterile pipette tip (1,000 μl), washed with medium to remove floated and detached cells, and photographed (time 0 h). Cells were successively treated in medium in the presence of different concentrations of Hirsutine (12.5 and 25 μM) along with the vehicle DMSO for 24 h. Scratched areas were photographed (magnification, x40) at 0 h and then again 24 h later to assess the degree of wound healing. The percentage of wound closure was estimated by the following equation: wound closure % = 1 - (wound area at t_{24}/wound area at t_0) x 100%, where t_{24} is the time after wounding and t_0 is the time immediately after wounding.

Haptotaxis and hapto-invasion assay. The filters of a Transwell cell culture insert (8 μm pore size; Whatman Japan KK, Tokyo, Japan) were pre-coated with fibronectin (Iwaki, Tokyo, Japan, 1.25 μg/filter) on the lower surfaces. For the hapto-invasion assay, the upper surface of the filters was coated with Matrigel (Becton-Dickinson, Bedford, MA, 1 μg/filter). Cells were pre-incubated with or without Hirsutine (25 μM) for 24 h. After trypsinization, cells in 0.1% (v/v) BSA medium ($1.5x10^4$) were placed in the upper chamber of Transwells. After the subsequent incubation at 37˚C, the residual cells were removed from the upper surface of the membrane and the migrated cells on the lower surface of the membrane were fixed in 100% methanol and stained with hematoxylin and eosin. Migration was determined by counting the cells with a microscope at x100 magnification. Five visual fields were chosen randomly and the average number of migrating cells in the five fields was taken for each group.

Gelatin zymography. Subconfluent monolayers of 4T1-NFκB-luc cells ($5x10^4$/well) pretreated for 24 h with Hirsutine (12.5 and 25 μM) were cultured for another 24 h in serum-free RPMI-1640. After incubation, cell-free supernatants were collected and mixed with sample buffer containing 2% SDS (without 2-mercaptoethalnol) and incubated at 37˚C for 20 min. Comparative gelatin zymography was performed on 10% SDS-PAGE with 0.1% gelatin. Samples were electro-

Table I. Natural compounds.

No.	Compound	No.	Compound
1	Galangin	29	Eleutheroside-B
2	Galangin-7-glucoside	30	Epihesperidin
3	6-Hydroxygalangin-7-glucoside	31	Ergosterol
4	Scutellarin	32	β-Eudesmol
5	Resveratrol	33	E-Ferulic acid
6	Artemisine	34	Geniposide
7	Acoinitine	35	Geniposidic acid
8	Albiflorin	36	Gentiopicroside
9	Alisol A	37	Ginsenoside Rc
10	Alisol B	38	Ginsenoside Rd
11	Alkannin	39	Glabridin
12	Amygdalin	40	Glycyrrhizic acid
13	Arbutin	41	Hirsutine
14	Astragaloside IV	42	Icariin
15	Atractylenolide III	43	Isofraxidine
16	Aucubin	44	Ligustilide
17	Baicalein	45	Loganin
18	Baicalin	46	Magnolol
19	Barbaloin	47	Mesaconitine
20	Bergenin	48	Naringin
21	Catalpol	49	Paeonol
22	E-Cinnamic acid	50	Palmatine chloride
23	Cinobufagin	51	S-Perilladelhyde
24	Cinobufotalin	52	Puerarin
25	Corydaline	53	Rhynchophylline
26	Curcumin	54	Sinomenine
27	Dehydrocostuslactone	55	Swertiamarin
28	Dimethylesculetin	56	Wogonin

phoresed at 10 mA for 4-5 h at 4˚C. Gels were washed with buffer containing 2.5% Triton X-100 and 0.01 M Tris-HCl for 2 h at 4˚C and then washed with 0.01 M Tris-HCl for 40 min at room temperature. Gels were incubated in buffer containing 0.05 M Tris-HCl, 0.5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 48 h at 37˚C. After incubation, gels were stained with Coomassie Brilliant Blue for 6 h and destained with 5% acetic acid and 10% methanol. The bands were scanned by an image scanner and quantified by Image J software.

Western blot analysis. 4T1-NFκB-luc cells were treated with Hirsutine for 24 h. Treated cells were collected, washed with phosphate-buffered saline (PBS), and lysed in lysis buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylemethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 mg/ml aprotinin and 10 mg/ml leupeptin]. The cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8) and 20% (v/v) methanol]. After blocking with Block Ace for 4 h at room temperature, the membrane was incubated overnight with primary antibodies, and then for 60 min with secondary antibodies. Primary antibodies were used at a dilution of 1:1,000. The secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG) were used at a dilution of 1:2,000 and visualized with an enhanced chemiluminescence system (Amersham Biosciences).

Experimental lung metastasis model. Female BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in a temperature-controlled, pathogen-free room. All animals were handled according to the approved protocols and guidelines of the Animal Committee of Toyama University (A2012INM-6). For the experimental metastasis model, 4T1-luc cells were inoculated intravenously (i.v., $5x10^5$) with or without pre-treatment with Magnolol or Hirsutine (24 h, 25 μM). For lung metastasis imaging, mice were injected with D-luciferin 7 days after tumor inoculation, then the lungs were removed to measure luminescence using *in vivo* imaging system (IVIS Lumina II; Caliper Life Sciences, MA, USA).

Statistical analysis. All the data are expressed as the mean ± SD of at least two or three independent experiments unless otherwise stated. Statistical significance was analyzed using Student's t-test. $P<0.05$ was considered significant.

Results

Hirsutine inhibits NF-κB activation in metastatic 4T1 breast cancer cells. In order to identify novel anti-metastatic drug candidates targeting inflammatory signals in cancer cells, we first screened our library of natural product-derived compounds using murine 4T1 breast cancer cells stably expressing NF-κB luciferase reporter (4T1-NFκB-luc cells). 4T1-NFκB-luc cells were incubated with a series of natural product-derived compounds (Table I) for 24 h and luminescence was measured to determine NF-κB activity. To discriminate whether the inhibition of NF-κB activity is related to the reduction of cell viability, all tested compounds were measured for their direct cytotoxicity on 4T1-NFκB-luc cells in parallel with the luciferase reporter assays. As summarized in Fig. 1, 4 out of 56 compounds (resveratrol, curcumin, magnolol, Hirsutine) significantly suppressed NF-κB activity in 4T1-NFκB-luc cells (>80%) with relatively little effect on cell viability (<50%). While the pharmacological effect of

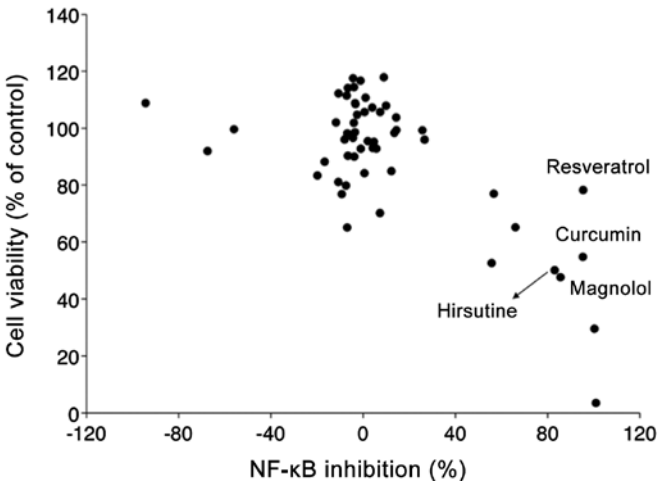

Figure 1. Correlation plot between the cell viability and NF-κB inhibition of 4T1 cells upon treatment with natural compounds. 4T1-NFκB-luc cells in exponential growth were placed at a final concentration of $2x10^4$ cells/well in a 96-well plate and incubated for 3 h. After incubation, the cells were treated with compounds (50 μM) or with the vehicle (vehicle control, 0.5% DMSO) for 24 h. Then 20 μl of luciferin (900 μg/ml) was added and incubated for another 30 min. Luciferase activity was recorded on a Glomax multi detection system. The same condition was used in the WST-1 assay for cell viability.

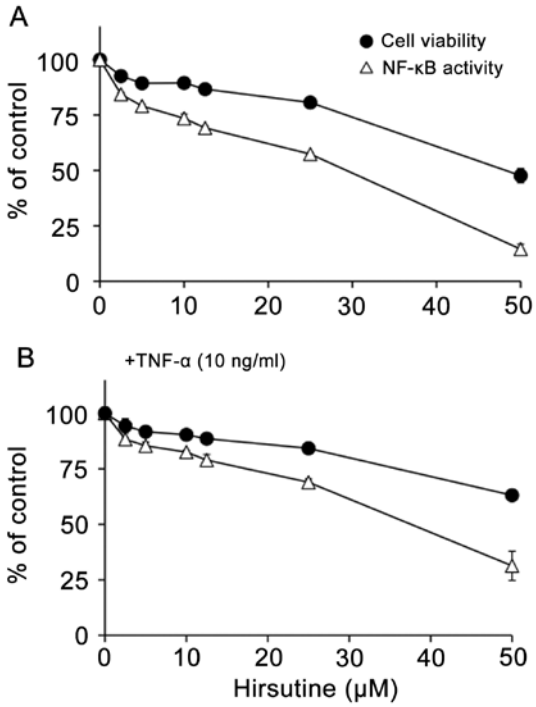

Figure 2. Effect of Hirsutine on cell viability and NF-κB activity in 4T1 cells. (A) Effects of hirsutine on 4T1-NFκB-luc cells. (B) Effects of Hirsutine on 4T1-NFκB-luc cells with TNF-α (10 ng/ml) stimulation. The data are representative results of three independent experiments.

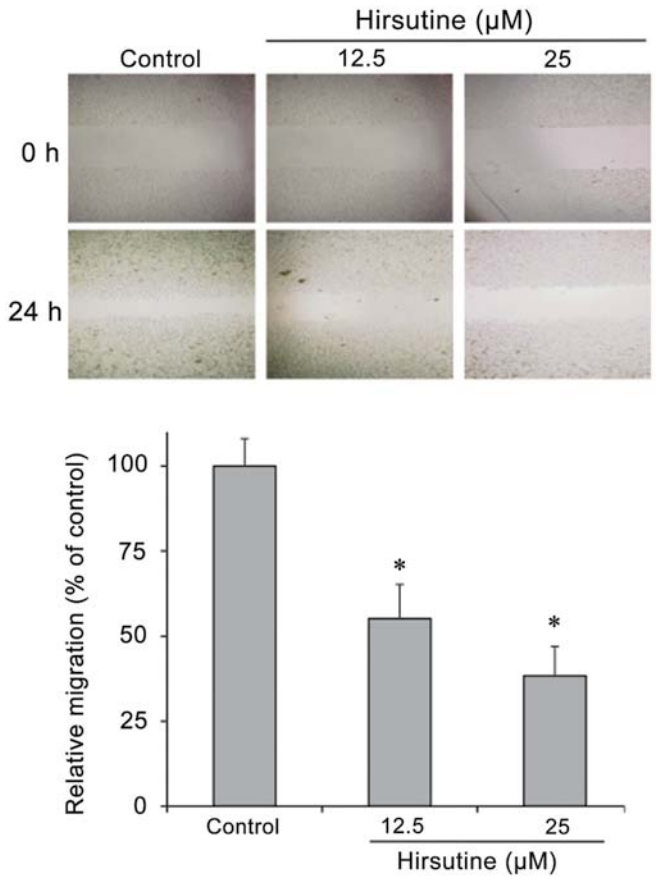

Figure 3. Effect of Hirsutine on cellular migration of 4T1 cells. Cells were treated with Hirsutine for 24 h in the wound healing assay. Briefly, 4T1-NFκB-luc cell monolayers were scraped with a sterile micropipette tip, and the cells were treated for 24 h with various concentrations of Hirsutine (12.5 and 25 μM) or with the vehicle. Scratched areas were photographed (magnification, x40) at 0 h and then again 24 h later to assess the degree of wound healing. The data are representative results of two independent experiments. *P<0.05 compared with the untreated control.

resveratrol (16-18), curcumin (19,20) and magnolol (21-31), in NF-κB inhibition has been previously recognized, there is almost no information of Hirsutine on its effect on NF-κB inhibition in cancer cells; therefore, we decided to further explore the potential of Hirsutine as novel anti-metastatic drug candidate targeting inflammatory signals in cancer cells.

To further determine the specificity of Hirsutine in its inhibition of NF-κB activation, 4T1-NFκB-luc cells were treated with different doses of Hirsutine to evaluate their effect on NF-κB reporter activity and cell viability. Hirsutine specifically inhibited NF-κB activation of 4T1 cells at a concentration that did not largely affect cell viability (Fig. 2A and C) and further inhibited NF-κB activation even in the presence of TNF-α stimulation in 4T1 cells (Fig. 2B and C). Collectively, the presented results strongly support that Hirsutine is a potent inhibitor of NF-κB in metastatic 4T1 breast cancer cells.

Hirsutine reduces metastatic potential of 4T1 cells. We next examined the effect of Hirsutine on the metastatic potential of 4T1 cells accordaning to the inhibition of NF-κB. As shown in Fig. 3, Hirsutine showed dose-dependent inhibition of the cell migration of 4T1 cells, as determined by the wound closure assay. In addition, pre-treatment with Hirsutine inhibited 4T1 cell haptotaxis (Fig. 4A) towards fibronectin in the Transwell chamber assay. Furthermore, Hirsutine pre-treatment showed significant inhibition of the invasion activity of 4T1 cells (Fig. 4B). In order to determine whether the inhibitory effect of Hirsutine on cellular invasion was relative to the proteolytic activity of 4T1 cells, we employed the gelatin zymography assay to examine the effect of Hirsutine treatment on the production of MMP-2 and MMP-9 in 4T1 cells. As shown in Fig. 5, Hirsutine-treated 4T1 cells showed a reduction in the activity of both MMP-2 and MMP-9 (Fig. 5A and C). We further confirmed that the cytoplasmic expression of MMP-9 was also reduced in Hirsutine-treated 4T1 cells as evaluated by western blotting (Fig. 5B). Collectively, these results clearly

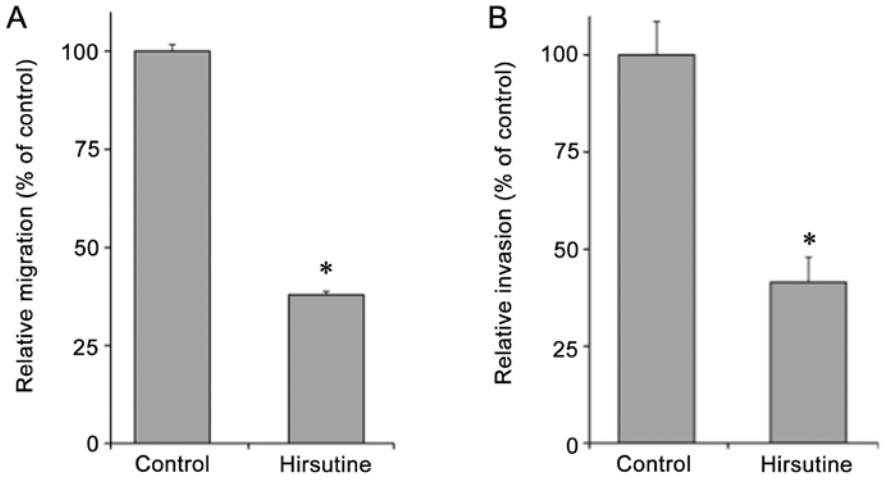

Figure 4. Effect of Hirsutine on haptotaxis or hapto-invasion of 4T1 cells. For the migration assay (A), cells were pretreated with Hirsutine (25 μM) for 24 h. Then an aliquot of cells ($1.5x10^4$) was transferred to each upper well. Cells were allowed to migrate for 6 h. For the invasion assay (B), cells ($7.5x10^4$) pretreated with Hirsutine (25 μM) for 24 h were transferred to each upper well and allowed to migrate for 12 h. For the migration and invasion assays, after incubation, migratory and invasive cells on the bottom of the insert membrane were fixed in 100% methanol and stained with hematoxylin and eosin. Migration and invasion were determined by counting the cells with a microscope at x100 magnification. Five visual fields were chosen randomly and the average number of invasive cells in the five fields was taken for each group. Each experiment was performed in triplicate. The data are presented as the mean ± SD of three replicate experiments. *P<0.05 compared with the untreated control.

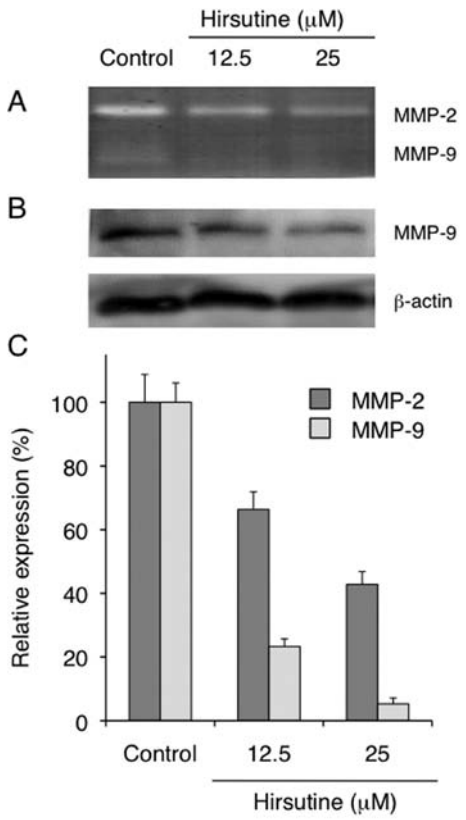

Figure 5. Effect of Hirsutine on MMP activity of 4T1 cells. (A) Gelatin zymography analysis of MMP-9/2 activity in 4T1-NFκB-luc cells. Subconfluent monolayers of 4T1-NFκB-luc cells pretreated for 24 h with Hirsutine (12.5 and 25 μM) were cultured for another 24 h in serum-free RPMI-1640. Culture supernatants from compound-treated cultures were subjected to electrophoresis in a gelatin-embedded SDS-polyacrylamide gel. After electrophoresis, stripes of the gel were incubated with an incubation buffer. After 42 h of incubation, the gel stripes were stained with Coomassie Brilliant Blue, and the locations of gelatinolytic enzymes were visualized as clear bands on the blue background. An image of each gel was scanned. (B) Western blot analysis of MMP-9 expression in 4T1-NFκB-luc cells. Cells were pretreated with Hirsutine (12.5 and 25 μM) for 24 h. Cell lysates were collected and subjected to western blot analysis to detect MMP-9 expression. (C) Intensity of western blotting bands was quantified by densitometry analysis using ImageJ software v1.47. The expression level was quantified as the ratio to β-actin and calculated by setting the value for control as 100%. The data are representative results of two independent experiments. *P<0.05

indicate that Hirsutine reduces the metastatic potential of 4T1 cells *in vitro* by regulating cellular migration or invasion in accordance with the inhibitory activity of NF-κB activation. We then tested the therapeutic potential of Hirsutine in breast cancer using an *in vivo* animal model. In an experimental lung metastasis model of 4T1 breast cancer, we found that pre-treatment with Hirsutine inhibited the metastatic lung colonization of 4T1 cells (Fig. 6).

Hirsutine reduces the migration of human breast cancer cells in vitro. To further explore the clinical relevance of our findings, we tested the effect of Hirsutine on human breast cancer cell lines MDA-MB-231 and MDA-MB-468. Similar to our observation in murine 4T1 cells, both Magnolol and Hirsutine inhibited the haptotaxis of MDA-MB-231 or MDA-MB-468 cells (Fig. 7) at a non-cytotoxic dose (data not shown) implying the efficacy of the compounds in human breast cancer cells *in vitro*.

Discussion

Activation of NF-κB has been observed in many cancers, including breast cancer, melanoma, lung cancer and various types of other cancers (32-34). It is known that NF-κB activation has not only been implicated in carcinogenesis (35,36) but also in cancer cell invasion and the metastatic process (37-40); therefore, targeting the NF-κB-mediated inflammatory signaling pathway is an attractive strategy for controlling metastasis. Amongst 56 chemically defined phytochemicals derived from natural products, we identified a new compound, Hirsutine, that strongly suppressed NF-κB activity in 4T1 breast cancer cells. In accordance with the NF-κB inhibition, Hirsutine reduced the metastatic potential of 4T1 cells, as seen in their inhibition of the migration and invasion of 4T1 cells. Importantly, Hirsutine showed anti-metastasis activity against 4T1 breast cancer cells *in vivo* and therefore could be useful as a lead compound for cancer therapy.

Hirsutine is one of the major alkaloids in *Uncaria* species and its cardioprotective (41), antihypertensive and anti-arrhythmic activity has been reported (42). The presented activity of Hirsutine in the inhibition of NF-κB activation and the reduction

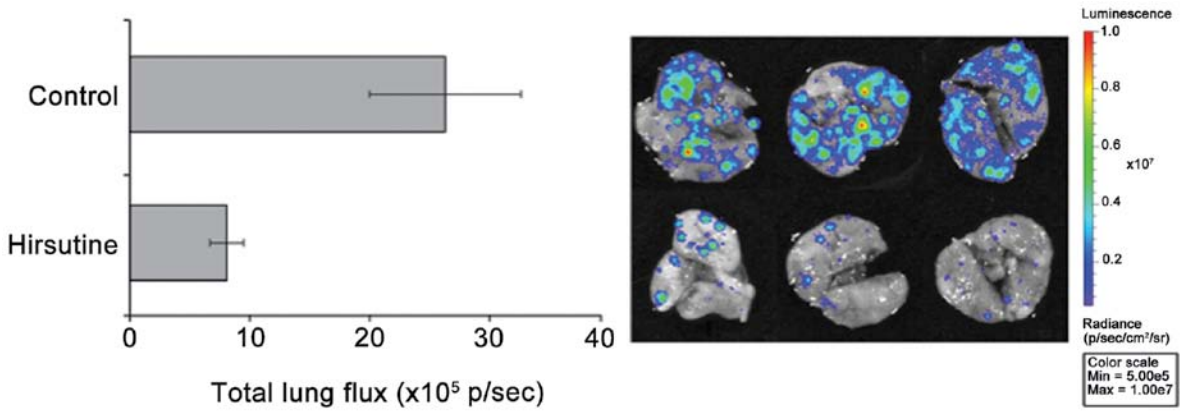

Figure 6. Anti-metastatic effect of Hirsutine in experimental lung metastasis model. Mice were given an intravenous injection of $5x10^5$ 4T1-luc cells pre-incubated with or without 25 μM Hirsutine for 24 h. Seven days after tumor inoculation, mice were sacrificed and lung metastases were measured by the IVIS system. The data are representative results of two independent experiments and are presented as the mean luminescence ± SEM (n=6). *P<0.05.

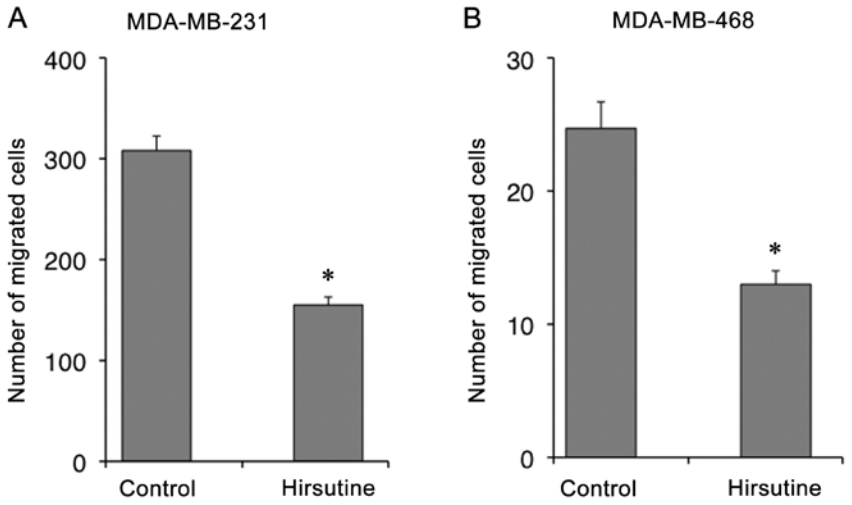

Figure 7. Effect of Hirsutine on haptotaxis of human breast cancer cells. MDA-MB-231 (A) or MDA-MB-468 (B) cells were pretreated with Hirsutine (25 μM) for 24 h. Then an aliquot of $1.5x10^4$ cells was transferred to each upper well and incubated for 6 h (A) or 12 h (B). After incubation, migratory cells on the bottom of the insert membrane were fixed in 100% methanol and stained with hematoxylin and eosin. Migration was determined by counting the cells with a microscope at x100 magnification. Five visual fields were chosen randomly and the average number of invasive cells in the five fields was taken for each group. Each experiment was performed in triplicate. The data are presented as the mean ± SD of three replicate experiments. *P<0.05.

of the metastatic potential of cancer cells is unexplored. While the underlying mechanisms that account for the multifaceted and differential role of NF-κB in cancer metastasis are presently unknown, the NF-κB-mediated inflammatory signaling pathway is an attractive clinical target for controlling metastasis in humans. Importantly, our present observation is applicable clinically because we observed the inhibitory effect of Hirsutine on NF-κB activation in human breast cancer cell lines as seen in the inhibition of p-65 phosphorylation (data not shown) and further in the inhibition of their migration (Fig. 7). Further studies are still required to determine the exact mechanisms of action of Hirsutine in its anti-metastatic activity by clarifying the potential involvement of other signaling pathways and/or transcriptional factors. Considering that the effective doses of Hirsutine shown in this study were relatively high, we also need to consider either chemical or biological modification of Hirsutine to achieve higher potency. Nevertheless, our current study clearly indicates that Hirsutine could be an attractive lead compound for reducing the metastasis potential of cancer cells by regulating NF-κB tumor-promoting activity.

Acknowledgements

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