Arctigenin, a lignan from *Arctium lappa* L., inhibits metastasis of human breast cancer cells through the downregulation of MMP-2/-9 and heparanase in MDA-MB-231 cells

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Abstract. Arctigenin is a bioactive lignan isolated from the seeds of *Arctium lappa* L. which has been widely used as a diuretic and a diaphoretic in Traditional Chinese Medicine. In the present study, the authors investigated the effects of arctigenin on tumor migration and invasion in aggressive human breast cancer cells. The MTT assay results showed that arctigenin did not show a significant cytotoxic effect on the cell viability of MDA-MB-231 cells. However, wound healing migration and Boyden chamber invasion assays demonstrated that arctigenin significantly inhibited *in vitro* migration and invasion of the MDA-MB-231 cells. Furthermore, gelatin zymography results showed that arctigenin reduced the activity of MMP-2 and MMP-9. Western blot analysis results demonstrated that the expression of MMP-2, MMP-9 and heparanase proteins was significantly downregulated following the treatment of arctigenin. Finally, the antiangiogenic activity of arctigenin was also examined by the chick embryo chorioallantoic membrane (CAM) assay. Arctigenin treatment significantly inhibited angiogenesis in the CAM. In conclusion, the results revealed that arctigenin significantly inhibited the migration and invasion of MDA-MB-231 cells by downregulating MMP-2, MMP-9 and heparanase expression. However, further studies are still necessary to investigate the exact mechanisms involved and to explore signal transduction pathways to better understand the biological mechanisms.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012 (1). Metastases to distant sites are common (2). Approximately 65-75% of patients with advanced breast cancer may develop bone metastases (3). Remarkable progress has been made in the treatment of breast cancer. However, despite these advances, the mortality rate is still high, mainly due to metastatic spread (4). Therefore, the development of new agents for breast cancer is important to reduce the mortality caused by this disease.

Natural products are a very important source providing promising leads for the development of novel cancer therapeutics due to their potentially low toxicity profiles and potential effectiveness (5). *Arctium lappa* L., commonly known as burdock or bardana, is a traditional Chinese medicine and perennial plant of the Compositae family. Recent studies demonstrated that *Arctium lappa* L. has numerous pharmacological activities, including anti-inflammatory (6), antitumor (7), antioxidant (8) and antiviral activities (9). However, the information concerning the antitumor constituents in this plant is still limited.

Arctigenin is the main active constituent that is extracted and isolated from the fruit of *Arctium lappa* L. (10). Experimental studies have shown various pharmacological effects of arctigenin including its antitumor activity (11-14). However, the antimetastatic effects of arctigenin on human breast cancer cells remain unknown. In the present study, we investigated the antimetastatic effect of arctigenin in human breast cancer cells. Results of this investigation may provide a scientific explanation for the antimetastatic mechanism of arctigenin.

Materials and methods

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Chemicals and reagents. Arctigenin, isolated from the fruit of *Arctium lappa* L., was kindly gifted by Dr Kunming Qin at Nanjing University of Traditional Chinese Medicine, and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 mM. The purity of arctigenin was above 98% (confirmed by high performance liquid chromatography and spectral analysis). Antibodies against MMP-2 (#4022), MMP-9 (#13667) and β-tubulin (#2128) for western blot analysis were obtained from Cell Signaling Technology (Beverly, MA, USA). An antibody against heparanase (ab42817) was obtained from Abcam (Cambridge, UK). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-
diphenyl-2H-tetrazolium bromide (MTT) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from BD Pharmingen (San Diego, CA, USA).

**Cell culture.** The human breast cancer cell line MDA-MB-231 was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (both from Gibco, Carlsbad, CA, USA), 100 U/ml penicillin G, 2.5 µg/ml amphotericin B and 100 µg/ml streptomycin (complete medium) at 37°C with 5% CO₂ in a humidified atmosphere.

**Cytotoxicity assay.** The cytotoxic effect of arctigenin on MDA-MB-231 cells was evaluated by the MTT assay. Briefly, MDA-MB-231 cells were plated into 96-well plates at a final concentration of 1x10⁴ cells/well in complete medium. Cells were treated with a range of concentrations of arctigenin. Twenty microliters of MTT (5 mg/ml) were then added at 24, 48 and 72 h after treatment. The cells were incubated for another 4 h at 37°C in the dark. Formed formazan crystals were dissolved in 100 µl DMSO and the absorbance was measured at 570 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

**Wound healing assay.** MDA-MB-231 cells were plated into a 24-well plate at a density of 2x10⁵ cells/well and allowed to form a confluent monolayer for 24 h. Subsequently, the monolayer was scratched with a sterile pipette tip (1,000 µl), washed with medium to remove floating and detached cells and photographed (time 0 h). The cells were successively treated in medium in the presence of different concentrations of arctigenin (20 and 40 µM) along with vehicle DMSO for 24 h. Scratched areas were photographed (magnification, x40) at 0 h, and then subsequently 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 t₂/wound area at t₀) x 100%, where t₂ is the time after wounding and t₀ is the time immediately after wounding.

**Boyden chamber migration and invasion assays.** MDA-MB-231 cell migration was also investigated by a modified Boyden chamber assay. The method is based on the passage of cells across porous filters separating the upper and lower wells of the migration chamber. Briefly, cells were incubated in the presence or absence of 40 µM arctigenin for 24 h. After trypsinization, 1x10⁵ cells suspended in 0.1% (v/v) BSA medium were placed in the upper chamber of 8-µm pore size Transwells (24-well; Millipore, Billerica, MA, USA) and incubated for 18 h at 37°C under 5% CO₂. For the invasion assay, the upper surface of the Transwell membrane was coated with 1 µg Matrigel. Cells (2x10⁵) (incubated in the presence or absence of 40 µM arctigenin for 24 h) in 0.1% (v/v) BSA medium were placed in the upper part of the Transwell membrane and allowed to migrate for another 24 h. For both the migration and invasion assay, the un migrated 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. The migration and invasion were determined by counting the number of cells with a microscope at a magnification of x100. Five visual fields were chosen randomly and the average number of migrating cells in the five fields was obtained for each group.

**Gelatin zymography.** The enzymatic activities of MMP-2 and MMP-9 were assayed by gelatin zymography in the absence of serum (15). The culture supernatants from compound-treated cultures were collected and centrifuged to remove debris. Subsequently, the medium was concentrated by centrifugal filters (Amicon® Ultra; Millipore). The conditioned medium was then mixed with non-reducing SDS-sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 0.00125% bromo-phenol blue and 2% SDS], and then incubated at 37°C for 30 min. The samples thus prepared were electrophoresed on 7.5% polyacrylamide gel containing 0.1% SDS and 0.1% gelatin at 4°C. After electrophoresis, the gels were washed twice with a rinsing buffer (50 mM Tris-HCl, 2.5% Triton X-100, 5 mM CaCl₂, 1 mM ZnCl₂ and 0.05% NaN₃) at room temperature for 1 h to remove the SDS. Then, the gels were incubated with an incubation buffer (50 mM Tris-HCl, 5 mM CaCl₂, 1 mM ZnCl₂ and 0.05% NaN₃) for 42 h at 37°C and stained with a staining solution (0.1% Coomassie Brilliant Blue, 10% acetic acid and 10% isopropanol). The locations of the gelatinolytic enzymes were visualized as clear bands on the blue background. The bands were scanned by an image scanner and quantified by ImageJ software.

**Western blot analysis.** MDA-MB-231 cells were exposed to arctigenin for 24 h. The 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 MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl 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. The primary antibodies were used at a dilution of 1:1,000. The secondary antibodies were used at a dilution of 1:2,000 and visualized with an enhanced chemiluminescence system (Bio-Rad).

**Chick embryo chorioallantoic membrane (CAM) assay.** The in vivo antiangiogenic activity of arctigenin was evaluated by a CAM assay. Briefly, fertilized chicken eggs were incubated at 37°C with 55-60% humidity. After incubation for seven days, a 2 cm² window was opened at the blunt end of the eggs and the shell membrane was removed to expose the CAM. Then, a sterilized 5 mm diameter gelfoam used as a drug carrier that absorbed 10 µl arctigenin (50 µM) was placed on the CAM. The window was sealed with parafilm and the egg was returned to the incubator for an additional two days with the window upright. Subsequently, the CAM microvessels were observed and photographed with a digital camera (Nikon, Tokyo, Japan).

**Statistical analysis.** All data are expressed as the mean ± SD of at least three independent experiments, and the statistical analysis for single comparison was evaluated by performing...
a Student's t-test. The criterion of statistical significance was p<0.05, p<0.01, p<0.001.

**Results**

**Effects of arctigenin on MDA-MB-231 cell viability.** The MTT assay was used as an indirect measure to determine the viability of the MDA-MB-231 cells exposed to arctigenin. The MDA-MB-231 cells were treated with arctigenin for 24 h at various concentrations (0-40 µM). As shown in Fig. 1, arctigenin at a concentration below 40 µM showed little effect on the viability of the MDA-MB-231 cells. The cell viability at 24 h was decreased to 89.45±2.85, 84.81±5.96, 83.57±7.02 and 81.77±4.88% compared to the control when the concentration of arctigenin was 5, 10, 20 and 40 µM, respectively. Therefore, these concentrations were selected for further evaluation of the anti-invasion and anti-migration effects of arctigenin.

**Arctigenin suppresses migration of MDA-MB-231 cancer cells in vitro.** To determine whether arctigenin could suppress the migration of MDA-MB-231 cancer cells, mechanical wounds were introduced into confluent monolayers, and wound closure was measured by microscopy. As shown in Fig. 2, arctigenin inhibited wound closure in a dose-dependent manner. When cells were treated with 20 and 40 µM arctigenin (Fig. 2), compared to the control group, the wound closure rate was decreased to 55.56±4.78 and 52.78±3.99%, respectively. Furthermore, a Boyden chamber migration assay also demonstrated the anti-migratory effect of arctigenin. As shown in Fig. 3A, following the treatment of arctigenin (40 µM), the migration rate was decreased to 46.88±4.79%. These results suggest that arctigenin is effective in decreasing the migration of the MDA-MB-231 cells.

**Arctigenin suppresses invasion of MDA-MB-231 cancer cells in vitro.** To further confirm the activity of arctigenin on cell invasion, a Boyden chamber invasion assay was performed to evaluate the anti-invasion effect of arctigenin on MDA-MB-231 cells. As shown in Fig. 3B, the results showed that arctigenin significantly inhibited cell invasion. After treatment with 40 µM of arctigenin, compared to the control group, the invasion rate of the MDA-MB-231 cells through the Matrigel was decreased to 61.70±7.96%. These results suggest that arctigenin is effective in decreasing MDA-MB-231 cell invasion.

**Arctigenin suppresses MMP-2 and MMP-9 enzyme activity in MDA-MB-231 cells.** To determine whether the inhibitory effect of arctigenin on the invasion of MDA-MB-231 cells was related to the activity of MMPs, a gelatin zymography assay was performed to examine the activity of MMP-2 and MMP-9. As shown in Fig. 4, arctigenin-treated MDA-MB-231 cells showed a decrease in the activity of both MMP-2 and MMP-9. When the cells were treated with 20 and 40 µM arctigenin (Fig. 4), compared to the control group, the mean activity of MMP-9 was decreased to 43.05±4.70 and 25.05±4.11%, and the activity of MMP-2 was decreased to 58.89±9.11 and 19.06±5.10%, respectively. These results demonstrated that the decrease of the enzymatic activity of MMP-2 and MMP-9...
proteins secreted from the MDA-MB-231 cells was related to the antimetastatic effect of arctigenin.

**Arctigenin downregulates MMP-2, MMP-9 and heparanase protein expression in MDA-MB-231 cells.** Degradation of extracellular matrix (ECM) proteins is an essential step in the invasion and metastasis of cancer cells and is mainly mediated by MMPs such as MMP-2 and MMP-9 (16). To test whether arctigenin suppresses cancer cell invasion and motility by affecting the expression of matrix metalloproteinases, a western blot analysis assay was performed to examine the expression of MMP-2 and MMP-9 in cancer cells after treatment with arctigenin. As shown in Fig. 5, the expression level of MMP-2 and MMP-9 was significantly downregulated. Heparanase, an endoglycosidase, mediates tumor invasion primarily through the direct cleavage of the heparan sulfate proteoglycan in the ECM (17,18). Recent studies have demonstrated that heparanase plays an important role in tumor metastases (19). In the present study, the effect of arctigenin on heparanase protein expression in MDA-MB-231 cells was also investigated. As shown in Fig. 5, the expression level of heparanase was significantly downregulated following arctigenin treatment.

**Arctigenin inhibits angiogenesis in CAM assay.** The CAM assay is the method commonly used for screening anti-angiogenic drugs and studying angiogenic mechanisms in vivo (20). To the best of our knowledge, angiogenesis is required for invasive tumor growth and metastasis and...
constitutes an important point in the control of cancer progression (21). Its inhibition may be a valuable new approach to cancer therapy. Therefore, the chick embryo CAM assay was performed to further evaluate the inhibitory effect of arctigenin on in vivo angiogenesis. Results showed that the formation of new blood vessels was significantly inhibited in arctigenin-treated CAM compared to that in the control (Fig. 6). These results suggest that the anti-angiogenic activity may be one of the antimetastatic mechanisms of arctigenin against breast cancer cells.

Discussion

Metastasis, the spread of malignant cells from a primary tumor to distant sites, poses the biggest problem to cancer treatment and is the main cause of death of cancer patients (22). The mortality rate due to metastatic cancer has been observed to be >90% when compared to the rate attributed to the primary tumors from which the malignant lesions arise (23). Natural products are a very important source providing promising leads for the development of novel cancer therapeutics (5), and a number of them are known for their antimetastatic activity in cancer cells (15,24,25). Arctium lappa L., a traditional Chinese medicine and perennial plant of the Compositae family, is one of these plants which has been attracting much attention recently due to its proven antitumor effects (7,14,26). Arctigenin is one of the main active constituents in Arctium lappa L. Experimental studies have demonstrated its antitumor effects on various cancer cells (27-29). However, the antimetastatic effects of arctigenin on cancer cells remain unknown. In the present study, we investigated the antimetastatic effect of arctigenin on cancer cells. The present study demonstrated that arctigenin significantly suppressed cell metastasis by inhibiting the activities of MMPs.

Matrix metalloproteinases (MMPs), a family of structurally and functionally related zinc-dependent enzymes, significantly contribute to the promotion of metastasis and tumor growth and the mechanisms include proteolytic degradation of ECM components and possibly regulation of tumor cell growth itself (30-32). Among the MMPs, MMP-2 and MMP-9 are involved in facilitating cancer metastasis (33,34).

To determine whether the inhibitory effect of arctigenin on the invasion of MDA-MB-231 cells is related to the activity of MMPs, gelatin zymography and western blot analysis assays were performed to examine the activity of MMP-2 and MMP-9 in MDA-MB-231 cells. Consistent with the aforementioned notions, the results showed that with arctigenin treatment the activity and expression of MMP-2 and MMP-9 was markedly inhibited (Figs. 4 and 5). These results indicated that arctigenin suppressed cell metastasis by inhibiting the activities of MMPs.

Heparanase, an endo-β-D-glucuronidase, has the ability to cleave heparin sulfate (HS) chains of heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) and basement membrane (BM) and plays a critical role in tumor cell invasion, migration and angiogenesis (35). Previous studies have reported the possible mechanisms involving the coordinated actions of MMP-9 and heparanase. Enhanced expression of heparanase promoted the phosphorylation of Akt and Src, following facilitation of gene transcription like VEGF or the promotion of the upregulation of uPA. After activating these signaling molecules, the level of MMP-9 was eventually increased (36,37). Notably, in the present study, after treatment with arctigenin, the expression of heparanase was significantly downregulated. These results could provide an experimental base to explore the coordinated actions of MMPs and heparanase in breast cancer metastasis.

As it is widely known, tumor cell invasion alone is not sufficient to produce distant metastasis; it requires the transport of malignant cells through the blood and/or lymph vessels. Angiogenesis is a crucial factor in tumor growth and metastases and constitutes an important point in the control of cancer progression (21). Its inhibition may be a valuable new approach to cancer therapy. A high level of tumor angiogenic activity often results in rich metastases and poor prognosis. In the present study, arctigenin evidently inhibited the formation of capillaries compared with saline-treated embryos using the chick embryo chorioallantoic membrane assay, indicating the anti-angiogenic effects of arctigenin in vivo. These results suggested that the anti-angiogenic activity may be one of the antimetastatic mechanisms of arctigenin against breast cancer cells.

In conclusion, the authors demonstrated that arctigenin suppressed the metastasis of MDA-MB-231 cancer cells by downregulation of MMP-2, MMP-9 and heparanase.

Figure 6. Effects of arctigenin on angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay. Representative images illustrate the inhibitory effects of arctigenin on the CAM. The images show the inhibition of angiogenesis in the presence of arctigenin (50 µM, 10 µl/egg).
However, more studies are still required to determine the exact mechanisms involved and to explore signal transduction pathways to better understand the biological mechanisms.

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References


