

Monascin accelerates anoikis in circulating tumor cells and prevents breast cancer metastasis

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Abstract. Anoikis resistance has been observed in various types of cancers in which anchorage-independent growth is a crucial step for cancer metastasis. Therefore, agents interfering with this specific cancer cell behavior may be integrated into novel antimetastatic strategies. Monascin (MS), a secondary metabolite found in *Monascus* species, is a known potent chemopreventive compound used for treating metabolic complications; however, the effect of MS on anoikis resistance has not been investigated. In this study, 4T1 breast cells were treated with MS under either suspension or adhesion conditions. The higher cytotoxicity of MS was more potent against suspended cells than against adherent cells. This selective cytotoxicity was due to the induction of anoikis, which was evidenced by changes in cell aggregation, caspase activity, and Annexin V/propidium iodide binding as well as the results of systemic metastasis in an animal model. Furthermore, MS inhibited E-cadherin and β -catenin expression in the cells; the treated cells formed spherical aggregates, which suggested that anchorage-independent growth was prevented by MS. These results provide new insights into the mechanisms underlying the growth-preventing effect of MS on cancer cells and indicate the potential ability of MS to suppress metastasis.

Introduction

Surgery is the treatment of choice for patients with breast cancer. However, the presence of circulating tumor cells (CTCs) after surgery has been reported to be significantly associated with early recurrence (1). The release of tumor cells into

peripheral blood as CTCs is one of the main causes of cancer recurrence (2). Metastasis involves the migration of cancer cells away from primary tumors and their entry into blood circulation. Tumor metastasis is a fundamental cellular process for maintaining tissue homeostasis by removing displaced epithelial or endothelial cells, thus preventing them from seeding to inappropriate sites (3). *In vivo* and *in vitro* studies have demonstrated that the capability of cells to resist anoikis (death after detachment) is critical for successful metastasis (4-6).

Anoikis resistant cancer cells can often survive and grow without adhesion to the basement membrane as aggregated microemboli in the bloodstream and such growth represents a hallmark of the malignant phenotype (7). A previous study has demonstrated that anoikis resistance in CTCs was accompanied by spheroid formation and caspase-3/9/poly (ADP ribose) polymerase (PARP) inactivation (8). Furthermore, E-cadherin and β -catenin are important intracellular signaling molecules associated with cell aggregation (9), which itself is associated with colony formation as cell clusters and indicative of poor prognosis (10). Micrometastases resulting from such tumor cell aggregates by β -catenin/E-cadherin activation are thought to survive within the circulation as small cell clusters or spheroids, thereby effecting suppression of anoikis (11). It has suggested that the disruption of anoikis resistance may serve as a therapeutic strategy for the treatment of malignant cancer (12).

Monascin (MS) is a yellow pigment produced by *Monascus*; it exhibits diverse pharmacological activities, including anti-inflammatory activity and antioxidant activity (13), and induction of cell death in cancer cells (14). However, the effects of MS on CTCs have not been elucidated. In the present study, the ability of MS to induce anoikis was investigated in murine anoikis-resistant 4T1 breast cancer cells. The biochemical mechanisms underlying anoikis induction by MS were also determined. The results of the present study may lead to the development of novel strategies for the CTC metastasis treatment and prevention.

Materials and methods

Reagents. Monascin (cat. no. 52442) and other chemicals and reagents (unless otherwise stated) were purchased from Sigma-Aldrich; Merck KGaA. Media, FBS (cat. no. 26140079) and culture supplements were purchased from Invitrogen;

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Thermo Fisher Scientific, Inc. Anti-PARP (cat. no. 9542), caspase-3 (cat. no. 9665), E-cadherin (cat. no. 3195), β -catenin (cat. no. 9562) and β -actin (cat. no. 4970) were purchased from Cell Signaling Technology, Inc.

Cell culture. The murine breast cancer 4T1 cell line was purchased from the American Type Culture Collection and the normal murine mammary gland (NMuMG) cell line was purchased from Bioresource Collection and Research Center. Each cell line was cultured in DMEM supplemented with 10% FBS and 100 U/ml streptomycin/penicillin. Cells were incubated at 37°C in an atmosphere of 5% CO₂. For obtaining an anchorage-independent culture, cells were cultured in the same complete medium but on a poly [2-hydroxyethyl methacrylate (polyHEMA)]-coated plate. Briefly, polyHEMA was dissolved in 95% ethanol to obtain 12% (w/v) stock solution. Working solution was prepared by further dilution (1:10) of stock solution using 95% ethanol and added to tissue culture dishes or plates (3 ml per 10 cm dish or 0.5 ml per well in a six-well plate). A hydrophobic surface was formed after polyHEMA solution was evaporated at room temperature in a tissue culture hood.

Cell proliferation assay. Both 4T1 and NMuMG cell proliferation rates were detected using the Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). Cells were seeded into a 96-well plate at a density of 5×10^3 cells/well. After culturing for 24 h, 48 h and 72 h at 37°C, 10 μ l of CCK-8 solution was added to the completed medium and cells were further incubated at 37°C for 1 h. Cell viability was determined by measuring absorbance at 450 nm on a microplate reader.

Trypan blue exclusion assay. 4T1 and NMuMG cells were cultured in each well (1×10^6 cells/well) of a 6-well plate with or without a polyHEMA coating. After 24 h incubation, culture medium was discarded and cells were washed with PBS and suspended in trypan blue stock in PBS (final concentration, 4%). Diluted trypan blue solution (0.04%) was subsequently added to the 6-well plate (500 μ l/well). After 3 min incubation at room temperature, stained cells were observed under a light microscope at a magnification of $\times 10$. Dead cells were dyed blue and viable cells were colorless and transparent.

Annexin V-FITC and PI double staining assay. After 48 h treatment with 20 μ M MS, 4T1 cells were harvested via trypsinization and washed with cold PBS. Cells were then centrifuged at 300 \times g for 5 min at 4°C, after which the supernatant was discarded and the pellet was resuspended in 1X binding buffer. Sample solution (60 μ l) was incubated with 3 μ l FITC-conjugated Annexin V (BD Pharmingen; BD Biosciences) and 1 μ g/ml PI (Sigma-Aldrich; Merck KGaA; cat. no. P4170) for 15 min at room temperature in the dark. A total of 240 μ l 1X binding buffer was added to each tube and the samples were counted (20,000 cells) and analyzed using a CytoFLEX™ Flow Cytometer and CytExpert software version 2.4 (Beckman Coulter, Inc.).

Western blot analysis. 4T1 cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer (Merck KGaA; cat. no. 632424) containing protease inhibitor cocktails (EMD Millipore; cat. no. 539134). Protein concentration was

determined using Bicinchoninic Acid Protein Assay Kit (Santa Cruz Biotechnology, Inc.; cat. no. sc-202389). Proteins (20 μ g) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were washed with PBS supplemented with 0.1% Tween-20. After blocking with BlockPRO™ Protein-Free Blocking Buffer (Visual Protein; cat. no. BF01) for 1 h at room temperature, membranes were incubated with primary antibodies against PARP (1:1,000; Cell Signaling Technology, Inc.; cat. no. 9542), caspase-3 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 9665), E-cadherin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 3195), β -catenin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 9562) and β -actin (1:2,000; Cell Signaling Technology, Inc.; cat. no. 4970) primary antibodies overnight at 4°C. Membranes were then incubated with anti-rabbit IgG HRP-linked antibody (1:5,000; Cell Signaling Technology, Inc.; cat. no. 7074) for 1 h at room temperature. Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore; cat. no. WBKLS0500) was used to detect the signal on the membrane that were developed on Hyperfilm™ ECL™ film (GE Healthcare; cat. no. 28906839).

Animal model. All animal care and experimental procedures adhered to the guidelines of the Institutional Animal Care and Use Committee of Wan Fang Hospital, Taipei Medical University (Taipei, Taiwan; approval no. WAN-LAC-106-012). Female 6-week-old BALB/cByJNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were given a standard laboratory diet and distilled water *ad libitum* under a 12-h light/dark cycle at $22 \pm 2^\circ\text{C}$ and humidity (55 \pm 5%) in the animal facility of Wan Fang Hospital. Stable 4T1-Luc cells were established as described previously (15). A total of 20 mice were used (~ 18 g weight). The tail veins of mice were injected with 1×10^6 4T1-Luc cells on day 0. The mice were then divided into four groups on day 1 (n=5 per group): Vehicle control, tumor control, MS (100 mg/kg) and MS (500 mg/kg) groups. MS was fed to the mice via oral gavage once daily, 5 days per week for a total of 4 weeks.

Prior to *in vivo* bioluminescence imaging, mice were anesthetized with isoflurane in an acrylic chamber using a 4% isoflurane/air mixture for induction. Mice were injected intraperitoneally with substrate D-luciferin (Sigma-Aldrich; Merck KGaA; cat. no. L9504) solution (150 mg/kg) in Dulbecco's Phosphate-Buffered Saline (Sigma-Aldrich; Merck KGaA; cat. no. D8537) and maintained under 2% isoflurane. After 5 min, images of the live anesthetized mice were recorded using a bioluminescence IVIS Spectrum System (PerkinElmer, Inc.; part no. 124262), which included a cryogenic cooling unit and a data acquisition computer with Living Image software version 2.5 (Xenogen Corp.). The acquisition and overlay of pseudocolor images were conducted. Images represented the spatial distribution of detected photons emerging from active luciferase within the animals. Bioluminescent signals were quantified using Living Image software 2.5 (Xenogen Corporation) as photons/sec/region of interest on days 7, 14, 21 and 28 of treatment.

Statistical analyses. All statistics were calculated using GraphPad Prism 6 software (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference. Data are presented as mean \pm SD of triplicate experiments. Statistical significance for parametric data were determined

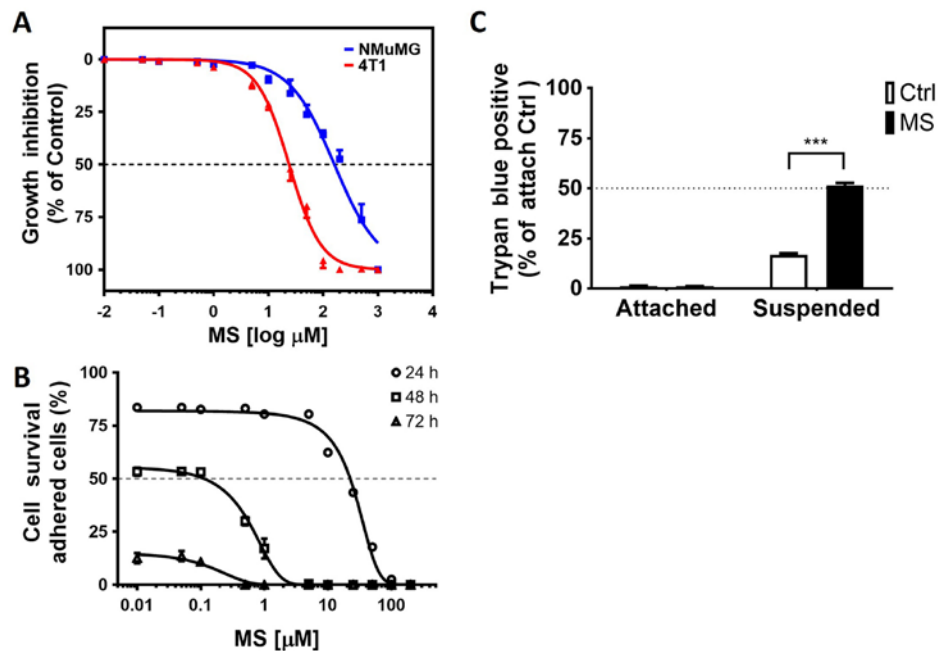


Figure 1. Inhibitory effects of MS on the viability of breast cells. (A) 4T1 breast cancer cells and NMuMG normal breast cells were treated with MS for 48 h. A growth inhibition of 50% was observed at 23.8 and 160.0 μM in 4T1 and NMuMG cells, respectively. (B) Cell survival of 4T1 cells cultured on the polyHEMA-coated plates treated with MS for 24, 48, and 72 h was compared with that of the cells under normal adhesion conditions at the same time points. (C) Trypan blue dye exclusion was used to measure the percentage of dead cells in different culture types treated with and without 20 μM MS for 48 h. The relative percentage of dead cells under the suspension condition was compared with that under the attachment condition. Points indicate the mean \pm standard deviation of at least three independent experiments that were each performed in triplicate. *** $P < 0.001$ vs. control. MS, monascin; Ctrl, control.

using two-way ANOVA with Tukey's multiple comparisons test to compare multiple dependent variables against multiple independent variables. An unpaired two-tailed t-test was used to compare one dependent variable against one independent variable. Error bars represent the standard error of the mean obtained from experiments performed in triplicate.

Results

Anoikis-sensitization effect of MS in 4T1 breast cancer cells. The cytotoxic effect of MS was evaluated using murine NMuMG normal breast cells and 4T1 breast cancer cells treated with increasing concentrations (0.1–1,000 μM) of MS for 48 h. MS markedly suppressed the survival of 4T1 breast cancer cells without causing toxic damage to normal NMuMG cells (Fig. 1A). To further validate that MS acted as an anoikis sensitizer, adherent and suspended breast cells were treated with increasing concentrations (0.01–100 μM) of MS for 24–72 h. The number of dead cells in the MS-treated samples increased with the MS concentration (10–100 μM); thus, MS exhibited cytotoxicity in a dose- and time-dependent manner (Fig. 1B). The results were further confirmed by using the trypan blue exclusion method to count the number of dead cells. 4T1 cells treated with 20 μM MS for 24 h on polyHEMA-coated culture plates (suspended cells) were more sensitive to anoikis than attached cells (Fig. 1C).

MS induces caspase-dependent apoptosis in a suspension culture of highly metastatic breast cancer cells. 4T1 breast cancer cells were plated on uncoated (attached) or polyHEMA-coated (suspended) tissue culture plates for 48 h. To confirm the occurrence of apoptosis, an

Annexin V-FITC/PI double staining assay was performed. The percentage of apoptotic cells (early and late apoptotic cells) in the polyHEMA-coated plate increased significantly from 9.91 to 44.96% after treatment with 20 μM MS (Fig. 2A). As caspase activation is considered a hallmark of apoptosis (16), western blotting was performed to examine caspase activity. Cleaved caspase-3 expression clearly increased in the cells in the polyHEMA-coated plates after MS treatment. Cleaved PARP was also notably activated in 4T1 cells treated with 20 μM MS under the suspension condition (Fig. 2B).

MS inhibits breast cancer cell aggregation under the suspension condition. In suspension culture, 4T1 cells formed large aggregates, but the cells did not exhibit aggregation after MS treatment (Fig. 3A). These observations suggest that cell aggregation provided growth signals to the tumor cells in suspension. To understand the molecular nature of the survival and growth signals provided by 4T1 cell aggregation, survival pathways were analyzed. The data indicated that MS reduced cell aggregation, which facilitated anoikis and downregulated the expression of E-cadherin and β -catenin (Fig. 3B).

MS treatment reduces lung metastasis in 4T1-Luc animal model. Considering that 4T1 aggregation was inhibited by MS treatment *in vitro* (Fig. 3A), the *in vivo* effect of MS on lung metastasis was determined. 4T1 cells were transfected with the firefly luciferase gene (Luc) in an experimental metastasis model. 4T1-Luc cells were injected into the tail vein of the mice. Non-invasive bioluminescence imaging enabled the early detection of cancer metastasis. Weekly bioluminescence imaging was conducted for 4 weeks, and the radiance antemortem was used as a surrogate measurement of tumor

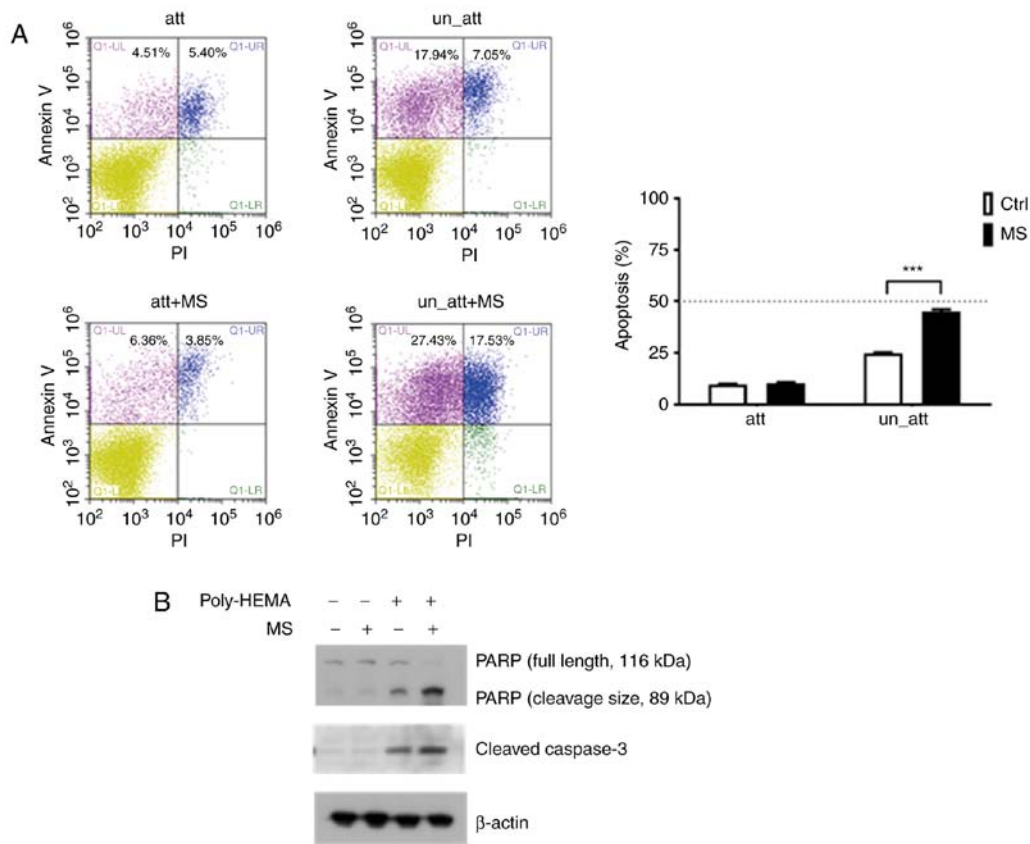


Figure 2. Induction of apoptosis by MS in 4T1 breast cancer cells. (A) Fluorescence-activated cell sorting analysis of Annexin V/PI staining. The results indicated early apoptosis, defined as Annexin V-positive and PI-negative cells (Q1-UL), and late apoptosis, defined as Annexin V-positive and PI-positive cells (Q1-UR). 4T1 cells were cultured on a general culture plate (att) or a polyHEMA-coated plate (un_att), treated with 20 μ M (MS) or without MS for 48 h, and then labeled with Annexin V-FITC and PI. The results are expressed as means \pm SD of three independent experiments. ***P<0.001 vs. control. (B) Western blot analysis for determining the effect of MS treatment for 48 h on PARP and cleavage caspase-3 expression under attachment and suspension conditions. MS, monascin; Ctrl, control; att, attached; un_att, unattached; PI, propidium iodide.

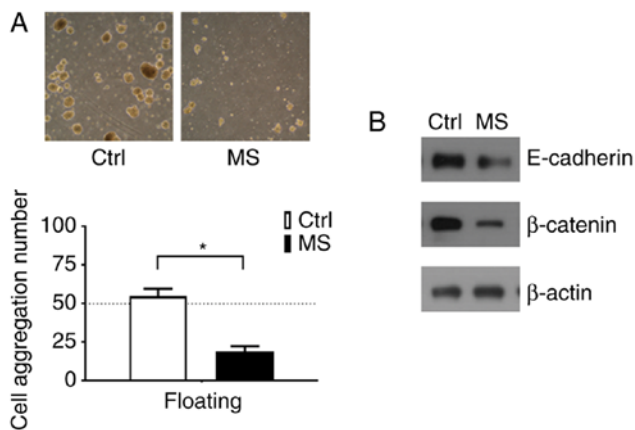


Figure 3. MS inhibits 4T1 breast cancer cell aggregation under the suspension condition. (A) Phase-contrast images of 4T1 cells on the polyHEMA-coated plate treated with or without 20 μ M MS for 48 h. Quantitative counts of clustered 4T1 cells reveal a significant decrease in MS treatment. (B) Western blot analysis of E-cadherin and β-catenin expression to determine the effect of MS treatment for 48 h on the floating cells. *P<0.05 vs. control. MS, monascin; Ctrl, control.

burden. The mice treated with MS (100-500 mg/kg daily) exhibited a notable reduction in both the number and size of pulmonary metastases, compared with the vehicle-treated mice (Fig. 4).

Discussion

Anchorage independence is a well-known characteristic of CTCs that allows floating cancer cell metastasis (1). Based on this assumption, anoikis resistance may be a crucial early characteristic of malignant cancer, because cancer cells are either deprived of extracellular matrix or exposed to foreign matrix components during metastasis (17). Moreover, anoikis resistance is associated with a high degree of tumor metastasis and advanced stage of cancers (18). Therefore, targeting anoikis-resistance pathways represents a promising strategy for antimetastatic therapy. In previous report, we have described that the count of CTCs is a significant predictor for liver metastasis within six months of surgery (19). Due to the difficulty in establishing the primary tumor circulating cells, the 4T1 breast cancer cell line was used to develop CTC-like model. Similarly, in another study performed by Park *et al* (20), stable CTC-like cells derived from human breast cancer MDA-MB-468 cell line were established. Therefore, the effects of MS on 4T1 cells aggregation was examined via poly-HEMA coating culture. The present results indicated the suspended cells, forced to grow under anchorage-independent conditions, exhibited upregulated β-catenin/E-cadherin expression and downregulated expression of the intrinsic apoptosis pathway, via inhibiting PARP/caspase-3 cleaved as a mechanism for suppressing anoikis. To the best of our

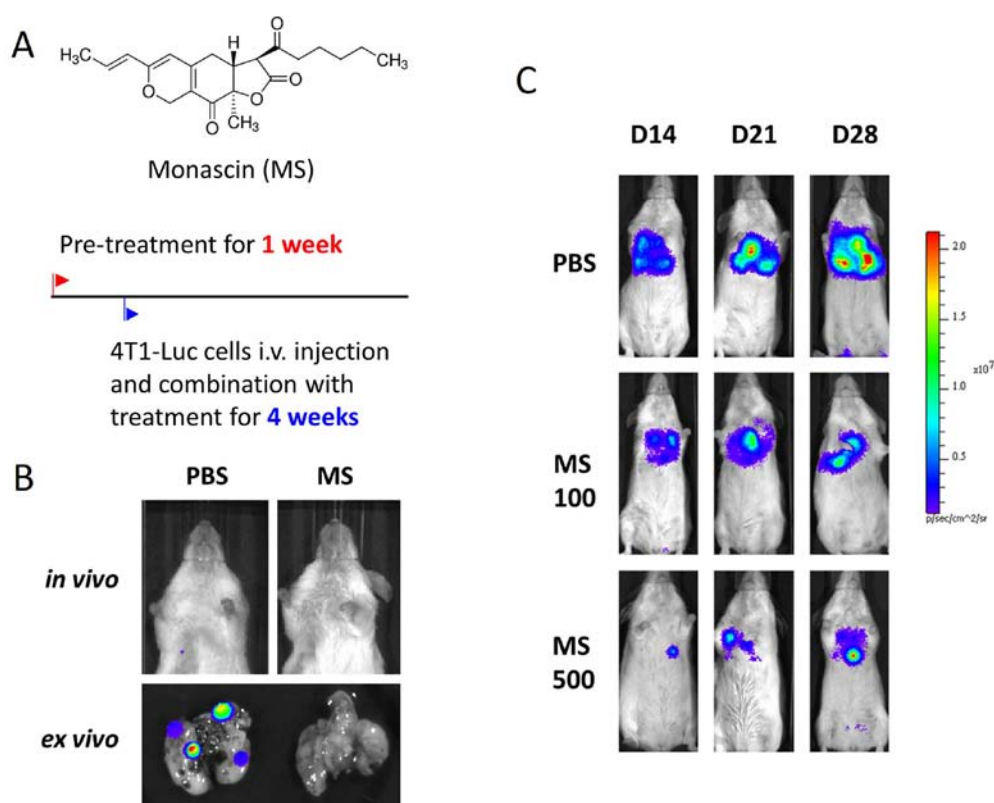


Figure 4. MS treatment reduces tumor growth in a tail vein-injected mouse model. (A) Before injection of 4T1 cells expressing the firefly luciferase gene (4T1-Luc), female BALB/c mice were treated (oral gavage) with 100 or 500 mg/kg MS (or vehicle PBS control) for 1 week. Then, the mice were intravenously injected with 4T1-Luc cells (1×10^5) through the tail vein, and the treatment (MS or vehicle PBS) continued for 4 weeks. (B) For 1 week after tumor injection, bioluminescence signals were not detected in any of the living mice. Pulmonary metastasis susceptibility was observed via detection of bioluminescence signals in the vehicle PBS-treated mice after sacrifice; however, no signal was detected in the MS-treated groups. (C) Bioluminescence signals [mean photon flux (photons/s)] were assessed in the areas over the lungs on days 14, 21 and 28. $n=5$ mice per group. MS, monascin.

knowledge, this was the first report of MS resensitizing breast cancer cells to anoikis and reducing colony-forming ability under a suspension condition.

The secondary metabolite MS produced by *Monascus* species has been reported to exhibit multiple biological effects, including anti-inflammatory effects (21). In the present study, the effects of MS on tumor growth and metastasis in 4T1 metastatic tumor-bearing mice were investigated, and the effects on cell adhesion, anoikis resistance and cell migration in 4T1 breast cancer cells were examined. In the current study, it was revealed that MS restored anoikis sensitivity in suspended 4T1 cancer cells. Notably, MS inhibited the proliferation of 4T1 cells but was not cytotoxic to normal NMuMG cells. Also, MS selectively induced cell death in suspended 4T1 cells and MS-induced cell death was demonstrated to be due to apoptosis, as demonstrated by the high levels of proapoptotic caspase activation and PARP cleavage. Finally, MS treatment prevented the growth of floating 4T1-Luc cells injected into the tail vein of the experimental mice and inhibited the migration of the cells to the lungs.

However, there were a few limitations to the present study. The current results were not derived from isolated primary circulating tumor cells, and clinical data was not available to support the *in vitro* and *in vivo* findings. Further studies are required to investigate the effect of MS treatment on clinical breast cancer metastasis, and this should be confirmed in a clinical trial. Multiple animal models have been developed recently,

but these models are not designed appropriately to study the step-by-step progression of metastasis (22). In this complicated metastasis process, the present study only describes the anoikis resistance on circulating tumor cells. Despite having certain limitations, such as the step of intravasation from primary site into the bloodstream, the poly-HEMA coating culture-induced cell cluster formation may be an *in vitro* experimental model for anoikis resistance investigation.

Previous studies on tumor metastasis and the epithelial-mesenchymal transition (EMT) have reported that EMT enables cancer cells to migrate away from the primary tumor (23-25). However, whether cell detachment additionally triggers EMT remains unclear. In the present study, high levels of β -catenin expression following detachment may be associated with cell aggregation and proliferation. β -Catenin is a versatile protein that serves multiple fundamental functions in cells, such as controlling intercellular junction integrity and regulating transcriptional processes as a co-transcription factor, mediating the canonical Wnt signaling pathway (26). Thus, targeting β -catenin activity and reducing anoikis resistance may represent strategies for preventing the development and progression of breast cancer. A previous study has revealed that disruption of E-cadherin-mediated adhesion sensitizes multicellular spheroids of tumor cells to treatment with chemotherapeutic drugs (27). MS, with the function to downregulate E-cadherin, may therefore have potential as a neoadjuvant for clinical metastatic prevention after surgery.

In conclusion, the current results demonstrate that MS targeting anti-anoikis may increase the killing of breast tumor cells persisting in the circulation as anchorage-independent micrometastasis. Future studies are needed on MS, including evaluation of anoikis resistance as a clinical application.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KYC, YTT and BYH conceived and designed the study. KYC, JAL and BYH performed animal experiments. HYY and ACH assisted in the animal experiments. YTT and BYH directed the project, analyzed and interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care and experimental procedures adhered to the guidelines of the Institutional Animal Care and Use Committee of Wan Fang Hospital, Taipei Medical University (approval no. WAN-LAC-106-012, Taipei, Taiwan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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