Formononetin Inhibits Migration and Invasion of MDA-MB-231 and 4T1 Breast Cancer Cells by Suppressing MMP-2 and MMP-9 Through PI3K/AKT Signaling Pathways

Abstract
Formononetin is a naturally existing isoflavone, which can be found in the roots of Astragalus membranaceus, Trifolium pratense, Glycyrrhiza glabra, and Pueraria lobata. It was found to be associated with inhibition of cell proliferation and cell cycle progression, as well as induction of apoptosis in various cancer cell lines. However, the effect of formononetin on breast cancer cell metastasis remains unclear. In this study, we examined the effect of formononetin on the migration and invasion of breast cancer cells MDA-MB-231 and 4T1 in vitro and in vivo. Our data demonstrated that formononetin did not effectively inhibit the cell viability of MDA-MB-231 and 4T1 in 24h with the concentration lower than 160μmol/l. When treated with nontoxic concentration of formononetin, the migration and invasion of MDA-MB-231 and 4T1 cells were markedly suppressed by wound healing assay, chamber invasion assay, and in vivo mouse metastasis model. In vitro, formononetin reduced the expression of matrix metalloproteinase-2 (MMP-2), MMP-9 and increased the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2. Furthermore, the immunofluorescence and immunoblotting assays indicated that formononetin was very effective in suppressing the phosphorylation of Akt and PI3K. Collectively, these results suggest that formononetin inhibited breast cancer cell migration and invasion by reducing the expression of MMP-2 and MMP-9 through the PI3K/AKT signaling pathway. These findings demonstrate a potentially new therapeutic strategy of formononetin as anti-invasive agent for breast cancer.

Introduction
Breast cancer is the most common malignancy among women worldwide. Each year, an estimated nearly 1.4 million patients are diagnosed with breast cancer globally with more than 450 000 deaths each year [1]. Approximately 10–20% of breast cancer cases are in the category of triple-negative phenotype, namely the absence of estrogen receptor-α, progesterone receptor, or amplification of epidermal growth factor receptor [2]. Patients with triple-negative breast cancer have a very poor disease-free survival because these tumors are aggressive and associated with a high rate of metastasis compared with other types [3,4]. Metastasis is a multistep process, which includes detachment of cancer cells from primary tumor, migration, adhesion, and invasion of cancer cells into the blood or lymphatic vessels. The help of matrix metalloproteinases (MMPs) is required for extravasation out of the vessel, leading to the movement of cancer cells to the target tissue. Among all MMPs, MMP-2 and MMP-9, known as key enzymes in the degradation of type IV collagen, are overexpressed in breast cancer cells [5,6] and their elevated expression has been associated with poor prognosis [7]. Thus, MMPs could work as pivotal targets for suppressing breast cancer invasion and metastasis, and the inhibition of MMPs may have considerable advantages in cancer therapy [8]. The discovery of novel compounds with low toxicity and excellent potential for cancer chemoprevention or treatment is an important step of cancer therapy development. Formononetin (7-hydroxy-4′-methoxyisoflavone), an herbal isoflavone, is a major compound in the roots of Astragalus membranaceus, Trifolium pratense, Glycyrrhiza glabra, and Pueraria lobata. It has been proved to have immunomodulatory, antitumorigenic, hypolipidemic, and antiphlogistic properties. Formononetin is a naturally occurring isoflavone, which can be found in the roots of Astragalus membranaceus, Trifolium pratense, Glycyrrhiza glabra, and Pueraria lobata. It was found to be associated with inhibition of cell proliferation and cell cycle progression, as well as induction of apoptosis in various cancer cell lines. However, the effect of formononetin on breast cancer cell metastasis remains unclear. In this study, we examined the effect of formononetin on the migration and invasion of breast cancer cells MDA-MB-231 and 4T1 in vitro and in vivo. Our data demonstrated that formononetin did not effectively inhibit the cell viability of MDA-MB-231 and 4T1 in 24h with the concentration lower than 160μmol/l. When treated with nontoxic concentration of formononetin, the migration and invasion of MDA-MB-231 and 4T1 cells were markedly suppressed by wound healing assay, chamber invasion assay, and in vivo mouse metastasis model. In vitro, formononetin reduced the expression of matrix metalloproteinase-2 (MMP-2), MMP-9 and increased the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2. Furthermore, the immunofluorescence and immunoblotting assays indicated that formononetin was very effective in suppressing the phosphorylation of Akt and PI3K. Collectively, these results suggest that formononetin inhibited breast cancer cell migration and invasion by reducing the expression of MMP-2 and MMP-9 through the PI3K/AKT signaling pathway. These findings demonstrate a potentially new therapeutic strategy of formononetin as anti-invasive agent for breast cancer.

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Key words
- formononetin
- breast cancer
- migration
- PI3K/AKT
- matrix metalloproteinases

Received 19.09.2013
Accepted 05.05.2014

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DOI http://dx.doi.org/10.1055/s-0034-1376977
Published online: June 30, 2014
Horm Metab Res 2014; 46: 753–760
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 0018-5043

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dem, antioxidant, antiviral, cardioprotective, and estrogenic activities [9–15]. Moreover, formononetin exhibits cytotoxic activity on LNCaP and PC-3 (prostate cancer) [16], MDA-MB-435 and MCF-7 (breast cancer) [17, 18], HuH-7 (liver cancer) [19], SGC-7901 (gastric cancer) [12], as well as HeLa (cervical cancer), and HCT-116 (colon cancer) [20] cancer cell lines. Treatment of human breast cancer cells (MCF-7) with formononetin led to a significant decrease in cyclin D1 protein and gene expression, which was found to be associated with IGF1/Pi3K/Akt pathways [17]. In addition, numerous studies have revealed that formononetin inhibits growth in various cancer types in vivo [17, 20, 21]. However, it remains unclear whether formononetin will be effective in inhibiting breast cancer cell metastasis. MDA-MB-231 triple-negative breast cancer cell, a highly metastatic human breast carcinoma cell line, is derived from a metastatic plural effusion fluid and has been widely used as a model for studying breast cancer metastasis [22]. The 4T1 breast cancer cells with high propensity of metastasis were isolated from a spontaneous mammary tumor of a Balb/cC3H mouse. When these cells were transplanted into mammary pads of Balb/c mice, they formed tumors and metastasize spontaneously to tissues, such as the lung, the liver, and bones, thus providing an excellent model for examining the mechanisms of breast cancer metastasis [23, 24]. Therefore, the objective of the study was to explore whether formononetin inhibited the migration and invasion of MDA-MB-231 and 4T1 breast cancer cells, and to further elucidate the underlying mechanism.

Materials and Methods

Materials

Formononetin was purchased from Phytomarker Ltd. (Tianjin, China). It was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C for further use. Penicillin, streptomycin, phosphate-buffered saline (PBS), trypsin-EDTA, DMEM, RPMI 1640, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). Invasion chamber and matrigel were obtained from BD transduction Laboratories (Burlington, MA, USA). MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 490 nm in an absorbance microplate reader.

Wound-healing assay

Cells were seeded in 1×10⁵ cells/ml and grown to 80–90% confluence in a 12-well plate at 37°C, 5% CO₂ incubator. The monolayers were scratched with a 10 μl sterile pipette tip, washed twice with PBS to remove floating cell debris, and then replaced with complete DMEM. MDA-MB-231-luc and 4T1 cells were treated with formononetin (0, 2.5, 5, 10, 20, and 40 μmol/l) and incubated for 12 h. Cell migration into the wound area was photographed under an inverted microscope. Migrated cells across the blue lines were calculated in 6 random fields from each triplicate treatment, and the data were presented as mean ± SD.

Cell invasion assay

The invasive abilities of MDA-MB-231-luc and 4T1 cells were tested using cell invasion chamber kit. In brief, cells were treated with various concentrations of formononetin. After 12 h, cells were detached by trypsin and resuspended in a serum-free DMEM (5×10⁴ cells/100 μl). The cells were seeded into the upper chamber of Matrigel-coated filter and a DMEM or RPMI 1640 containing 10% FBS of 500 μl was added to the lower chamber. The chamber was incubated 37°C for 6 h. At the end of incubation, the noninvading cells in the upper surface of the filter membrane were carefully removed with a cotton swab. The invading cells on the lower surface of the filter membrane were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet for 10 min and rinsed with water. The invasive cells on the lower surface of filter membrane were counted with a light microscope.

Western blot analysis

After treatment with various doses of formononetin for 12 h, the MDA-MB-231-luc and 4T1 cells were lysed with RIPA buffer containing protease and phosphatase inhibitors. The protein concentrations were measured with a BCA kit (Beyotime, China). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with a solution containing 5% nonfat dry milk TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h. The indicated primary antibodies were incubated overnight at 4°C, washed, and monitored by immuno-blottedting using a DyLight 800-conjugated secondary antibody.
The membrane was scanned using a LI-COR Infrared Imaging Odyssey (Gene Company Limited).

Real-time quantitative PCR
MDA-MB-231-luc and 4T1 cells were treated with different concentrations of formononetin for 12 h and total RNA was isolated using the Trizol reagent according to manufacturer's protocol. The RNA concentrations were quantified with the Qubit Flurometer. Real Time PCR (RT-PCR) was carried out using 1 ng of total RNA, which was reverse transcribed into complementary DNA using the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions. Quantitative RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit. The PCR protocol conditions were as follows: HotStar Taq DNA polymerase was activated at 95 °C for 2 min, followed by 40 cycles at various temperatures/times (i.e., 94 °C for 15 s, 60° for 30 s, and 72 °C for 30 s). At the end of the amplification period, melting curve analysis was done to confirm the specificity of the amplicon. Fold-changes of genes after treatment with different concentrations of formononetin were calculated by normalizing the Ct values to the GAPDH internal control. The sequences of the primer pairs in this experiment were as follows: human GAPDH, 5′-GGCATCTTGCGTACTACT-3′ (forward), 5′-GCCAGTTGGGATAGG-3′ (reverse); human MMP-9, 5′-GGAGCCACAGACATCTCATC-3′ (forward), 5′-TCGTCAGTCGAATGCGG-3′ (reverse); human MMP-2, 5′-CTTCAAGTCTGACCTGATG-3′ (forward), 5′-TACCGCTAAAGGGTATCCATC-3′ (reverse); human TIMP-1, 5′-CTTCTGGAATCCAGACCTCGT-3′ (forward), 5′-CCTAAGGCTTGAAACCTTT-3′ (reverse); human TIMP-2, 5′-AACGGCTGCTGAGAAGGAAG-3′ (forward), 5′-CACACACTACCGAGGAGG-3′ (reverse). Mouse GAPDH, 5′-GGCTTCTCGTTCATCTAC-3′ (forward), 5′-TGTCATGTCGATAGTT-3′ (reverse); mouse MMP-9, 5′-TGTCATGTCGATAGTGAG-3′ (forward), 5′-ATCTGCTGCAAGTGCTG-3′ (reverse); mouse MMP-2, 5′-AATCTGCTGCAAGTGCTG-3′ (reverse); mouse MMP-1, 5′-GGCAGATCCAGTCGACTG-3′ (forward), 5′-AGTGTCATGTCGATAGTG-3′ (reverse); mouse TIMP-1, 5′-CAGTAGTGTCGATAGTG-3′ (forward), 5′-AGTGTCATGTCGATAGTG-3′ (reverse); mouse TIMP-2, 5′-GGGAGTACCATGATGGAACC-3′ (forward), 5′-GGCCCGTGTAGATAACCTCGAT-3′ (reverse).

Immunofluorescence analysis
MDA-MB-231-luc cells on coverslips were washed twice with PBS and fixed by 4% parafomaldehyde at 4 °C for 10 min. Permeabilization of the cells was performed by incubating the cells with 0.1% saponin and 1% fetal bovine serum in PBS at 4 °C for 10 min. The cells were blocked with 3% BSA at room temperature for 30 min and incubated with primary antibody against p-AKT at 4 °C overnight (0.1% saponin and 1% fetal bovine serum in PBS). Cells were washed twice in PBS, then incubated simultaneously with fluorescein isothiocyanate-labeled secondary antibody for 1 h at room temperature. Negative controls were prepared by incubation of the cells with anti-IgG antibody. Nucleus was stained with DAPI for 2 min. After staining, cells were rinsed 4 times with PBS and prepared for microscopic analysis. The images were captured using an immunofluorescence microscopy (Olympus).

In vivo mouse metastasis models
All animal work was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. Four to six weeks old female Balb/c nude mice, purchased from Sino-British Sippr/BK Lab Animal Co, Ltd (Shanghai, China), were used for animal study. For lung metastasis study, 2 × 10^6 viable MDA-MB-231-luc breast cancer cells were resuspended in PBS and subsequently injected into the lateral tail vein in a volume of 0.1 ml. Mice were randomly divided into 3 groups: (1) vehicle (1% DMSO in PBS) group (control), (2) low-dose formononetin group (10 mg/kg), (3) high-dose formononetin group (20 mg/kg). There were 10 mice per group. Formononetin was administrated intraperitoneally (i.p.) every 2 days. Bioluminescent imaging was performed by anesthetizing and injecting mice retro-orbitally with 1.5 mg of D-luciferin (15 mg/ml in PBS), and images were acquired at 5 min after injection using the Xenogen IVIS 200 system before analysis using the Living Image software (Xenogen).

Statistical analysis
All data were presented as the means ± standard deviation (S.D.) of 3 independent experiments done in triplicate. Statistical analysis was performed by Student’s t-test or one-way analysis of variance (ANOVA). Survival curves were analyzed according to the Kaplan-Meier method, and p-value was calculated for the comparisons between curves by the log-rank test. In all cases, p < 0.05 was considered statistically significant.

Results

Cytotoxicity of formononetin to MDA-MB-231-luc cells
Chemical structure of formononetin was shown in Fig. 1a. To verify the effect of formononetin on cell viability, cells were treated with formononetin at indicated concentrations for 24 h and tested by MTT assay. Compared with untreated control cells, the viabilities of MDA-MB-231-luc and 4T1 cells were not significantly affected by formononetin at a concentration between 0 and 80 μmol/l (Fig. 1b). Furthermore, the effect of formononetin (0–80 μmol/l) occurred in the absence of evident cytotoxicity, as verified by cell morphology (Fig. 1c). Thus, noncytotoxic concentration of formononetin was used in the subsequent experiments.

Formononetin inhibited breast cancer cell migration and invasion
To investigate the inhibitory effect of formononetin on migration of MDA-MB-231-luc and 4T1 cells, the wound-healing assay was performed. After incubation with formononetin for 12 h, the number of cells migrated to the denuded zone was analyzed. The results demonstrated that formononetin suppressed migration of breast cancer cells in a dose-dependent manner. Treatment with formononetin (2.5, 5, 10, 20, and 40 μmol/l) was inhibited by 10.6, 15.9, 38.7, 57.8, and 75.9% of MDA-MB-231 cell migration, and by 13.0, 18.8, 35.4, 64.8, and 75.1% of 4T1 cell migration, respectively (Fig. 2a, b). To further examine the effect of formononetin on the invasive ability of breast cancer cells, a BD chamber coated with matrigel was used. Our data showed that the number of cells invading the lower chamber was significantly reduced by formononetin and a 79.2% or 78.5% reduction in cell invasion of MDA-MB-231 or 4T1 breast cancer cell was observed for treatment with 40 μmol/l formononetin (Fig. 2c, d). These results suggest that formononetin is effective in preventing breast cancer cell migration and invasion.
Effects of formononetin on MMPs expression
Since the expression of MMPs is crucial to extracellular matrix (ECM) degradation, which is required for cell invasion, it is vital to determine whether MMPs are involved in the inhibition of migration and invasion by formononetin. The effects of formononetin on MMPs were investigated by Western blot and real time-PCR. As shown in Fig. 3a, b, formononetin treatment led to a dose-dependent reduction in MMP-9 and MMP-2 expression. The results also demonstrated that formononetin elevated the expressions of TIMP-1 and TIMP-2, which were known to be...
negative regulators of MMPs. These results suggest that formononetin can affect expressions of proteins and genes involved in proteolytic activation.

**Effects of formononetin on the PI3K/AKT signaling pathway**

It has been reported that MMP-2 and MMP-9 expressions are critically mediated by the PI3K/Akt pathway. To investigate the effect of formononetin on the AKT in MDA-MB-231-luc cells, immunofluorescence analyses were performed. The results demonstrated that formononetin inhibited the expression of the p-AKT in a concentration-dependent manner (Fig. 3c). To confirm the data generated by immunofluorescence staining, we performed Western blot analysis of phosphorylated and total PI3K, AKT, JNK, and ERK. Our results showed that formononetin could inhibit the expressions of p-PI3K and p-AKT in a dose-dependent manner, but the levels of phospho-ERK and phospho-JNK were not affected by formononetin (Fig. 3d). Taken together, all of our data suggest that formononetin suppresses the PI3K/AKT signaling pathway in breast cancer cells.

**Effects of formononetin on lung metastasis in vivo**

The mouse tail vein injection tumor metastasis model was used to evaluate the inhibitory effect of formononetin on MDA-MB-231-luc metastasis. After tail vein injection, vehicle control or formononetin at 10 mg/kg/day or 20 mg/kg/day was administered. Treatment continued for 35 days after MDA-MB-231-luc cells injection, and the treatment effect of formononetin was examined by bioluminescence imaging. As shown in Fig. 4a, b, formononetin dramatically reduced the development of lung metastases in a dose- and time-dependent manner. Photon flux in the lungs of mice treated at this dose was dramatically reduced (p < 0.05 or p < 0.01) compared with control (Fig. 4b). Furthermore, survival analysis showed that mice receiving a
treatment of formononetin had a statistically significantly longer survival than the mice with vehicle control treatment (Fig. 4c).

Discussion

Recently, considerable emphases have been given to identify new anticancer agents from natural sources. Formononetin, a naturally existing isoflavone, has been reported to have a wide range of pharmacologic effects, such as inhibition of cell proliferation and cell cycle progression, and induction of apoptosis in various cancer cell lines [12, 16–20, 25]. Metastatic spread of breast cancer is responsible for 90% of human cancer-related deaths and thus remains one of the important negative predictors of breast cancer prognosis [26, 27]. However, whether formononetin exerts an inhibitory effect on breast cancer metastasis has not been elucidated previously.

In the present study, we have demonstrated that formononetin strikingly inhibited the migratory and invasive abilities of breast cancer cells at noncytotoxic concentrations in vitro (Fig. 2). These results indicated that formononetin inhibited breast cancer cell migration and invasion, and the effect was not attributed to its cytotoxicity. In addition, we also found that formononetin inhibited breast cancer cell metastasis and prolonged animal survival time in vivo (Fig. 4). These results indicated that formononetin might play a beneficial role in breast cancer metastasis.

A vital step in cancer metastasis processes is the proteolytic degradation of the ECM by proteolytic enzymes, such as MMPs [28]. MMPs are a family of zinc-containing endopeptidases, of which MMP-2 and MMP-9 are highly expressed in aggressive breast tumors and are associated with poor clinical outcome [5, 6, 29, 30]. The inhibition of MMP-2 and MMP-9 expressions is a critical step in the prevention of cancer metastasis [27, 31–33]. TIMPs, the regulators of MMPs, are also involved in tumor progression, invasion, metastasis and angiogenesis [34]. Furthermore, increased expressions of TIMP-1 or TIMP-2 have been shown to suppress cell invasion [35–37]. In our study, it was found that the inhibition of MMP-2 and MMP-9 expressions and the increase in TIMP-1 and TIMP-2 expressions were attributable to the anti-invasive effect of formononetin (Fig. 3a, b).

Many have reported that MMP-2 and MMP-9 expression were mediated by the PI3K/Akt pathway [38–43]. PI3K is a lipid kinase that controls multiple cellular processes through AKT activation. Furthermore, AKT activation can lead to cancer invasion and metastasis by stimulating the secretion of MMPs [39, 44, 45]. The presence of phosphatidylinositol 3-kinase γ (PI3Kγ) overexpression is characteristic in the metastatic breast cancer cells, when compared with the normal breast epithelial cell line or nonmetastatic breast cancer cells. In addition, the overexpression of recombinant PI3Kγ was reported to be able to increase the metastatic ability of nonmetastatic breast cancer cells [46]. Moreover, PI3K inhibitor could lead to a reduction in MMP-2 activation, cell migration and cell invasion [47]. Some traditional Chinese medicine monomers and compounds have been reported to have inhibitory effects on the migration and invasion of cancer cells via reducing the expression of MMPs via the PI3K/Akt signaling pathway [48–56]. It was also reported that formononetin inhibited the breast cancer cell proliferation by decreasing the
expression of cyclin D1 via the IGF1/PI3K/Akt pathways [17]. In our study, we have demonstrated that treatment with formononetin significantly reduced PI3K and Akt phosphorylation (Fig. 3c, d), which indicated an inhibitory effect to the PI3K/Akt signaling pathway by formononetin. Therefore, we suggest that formononetin inhibits the invasion of breast cancer cells by reducing the expression of MMP-2 and MMP-9 through PI3K/Akt signaling pathways.

In conclusion, the present study has demonstrated that formononetin decreased the invasive ability of MDA-MB-231 and 4T1 breast cancer cells, including migration and invasion, by inhibiting MMP-2 and MMP-9 expressions. This effect might be attributed to the inactivation of PI3K/Akt pathway. These results provide new insights into molecular mechanisms involved in the anti-invasive activity of formononetin in breast cancer cells. This finding strongly suggests formononetin to be a potentially useful anti-invasive agent for breast cancers.

Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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