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Effects of Osthole on Migration and Invasion in Breast Cancer Cells

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Osthole, a natural coumarin derivative, is extracted from the fruit of *Cnidium monnieri* Cusson. Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death in women. Recent studies have shown that Osthole has anti-tumor activity. However, the effects of Osthole on the migration and invasion of cancer cells have not yet been reported. Here, we found that Osthole is effective in inhibiting the migration and invasion of breast cancer cells by wound healing and transwell assays. Luciferase and zymography assays revealed that Osthole effectively inhibits matrix metalloproteinase-2 promoter and enzyme activity, which might be one of the causes that lead to the inhibition of migration and invasion by Osthole. This is the first report on the inhibitory function of Osthole in migration and invasion in breast cancer cells. Our findings indicate a need for further evaluation of Osthole in breast cancer chemotherapy and chemoprevention.

Key words: Osthole; breast cancer; migration; invasion

Breast cancer is the second most common type of cancer in women, after skin cancer. In spite of advances in treatment strategies, about 25–40% of patients with breast cancer eventually develop metastatic disease that is largely incurable.[1)] Breast cancer principally metastasizes to the regional lymph nodes, bone, liver, lungs, and brain.[2)] In 2007, an estimated 178,480 new cases of invasive breast cancer were diagnosed among women in the USA.[3)] In 2008 it was estimated that in the USA, 40,480 women died from metastatic breast cancer.[4)] Therefore, the development of novel treatments to treat individuals with metastatic tumors is necessary to improve mortality rates.

Osthole (7-methoxy-8-isopentenoxycoumarin) (Fig. 1), a natural coumarin derivative, is extracted from the fruit of *Cnidium monnieri* (L.) Cusson, a Chinese herbal medicine. Osthole has numerious medicinal properties, including anti-inflammation,[5)] anti-oxidation,[6)] anti-osteoporosis,[7)] anti-hepatitis,[8)] and estrogen-like effects.[9)] In addition, accumulating evidence indicates that Osthole possesses an anti-tumor effect by inhibiting tumor cell growth and inducing apoptosis,[10–14)] but the effects of Osthole on the migration and invasion of cancer cells have not yet been reported.

The present study was performed to evaluate the effects of Osthole on migration and invasion in human breast cancer cells as well as to explore the underlying mechanisms. We found that Osthole is effective in inhibiting the migration and invasion of breast cancer cells by wound healing and transwell assays. Luciferase and zymography assays revealed that Osthole effectively inhibits MMP-2 promoter and enzyme activity, which might be one of the causes that lead to migration and invasion inhibition by Osthole. Our findings indicate a need for further evaluation of Osthole in breast cancer chemotherapy and chemoprevention.

Materials and Methods

Chemicals and plasmids. Osthole was purchased from the Jiangsu Institute for Food and Drug Control (Nanjing, China). ECM Gel was from Sigma (St. Louis, MO). ThinCert 24 Well was from Greiner Bio-one (Frickenhausen, Germany). Luciferase reporter plasmid containing a 1,716-bp MMP-2 promoter sequence (MMP-2-Luc) was a generous gift from Dr. Etty (Tika) Benveniste (University of Alabama at Birmingham, AL). Lipofectamine 2000 reagent was from Invitrogen Life Technologies (Grand Island, NY). Luciferase assay system was from Promega (Madison, WI).

Cell culture. Human breast cancer cell lines MCF-7 and MDA-MB 231 were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM medium (Gibco by Invitrogen, Carlsbad, Calif), supplemented with 10% FBS (PAA Laboratories, Etobicoke, Ontario), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

MTT assay. Proliferation of MCF-7 and MDA-MB 231 cells following treatment with Osthole was measured using 3,4-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (Sigma). Briefly, MCF-7 and MDA-MB 231 cells (8×10^3) were seeded in 96-well tissue culture plates and allowed to adhere overnight, and then treated with various concentrations of Osthole (0–100 μM). After treatment for 24 h, 20 μl of MTT dye solution was added to each well, and the plate was incubated for 4 h at 37 °C. Solubilization/stop solution (150 μl) was added for 1 h at 37 °C, and the absorbance at 570 nm was recorded using a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT).

Wound healing assay. MCF-7 cells were seeded in 6-well tissue culture plates. When cell confluence reached about 90%, wounds were created in confluent cells using a 200-μl pipette tip. The cells were then rinsed with medium to remove any free-floating cells and debris. Medium containing various concentrations of Osthole without serum

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Abbreviations: MTT, 3,4-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; MMP, matrix metalloproteinase

was then added, and culture plates were incubated at 37 °C. Wound healing was observed at different time points within the scrape line, and representative scrape lines were photographed. The experiment was repeated 3 times.

Cell invasion assay. MDA-MB 231 cells were seeded at a density of 3×10^5 cells onto ECM gel-coated inserts (8 μm pore size) in 24-well tissue culture plates. The cells were incubated in DMEM medium supplemented with 2% serum and various concentrations of Osthole. After 24 h of incubation, the cells on the upper side of the inserts were removed with a cotton swab. The cells that migrated to the lower side of the inserts were stained with 1% crystal violet and counted under a microscope.

Cell transient transfection and luciferase assay. MCF-7 cells were plated on 12-well tissue culture plates to achieve 90–95% confluence for transfection on the following day. Transfection was performed with MMP-2-Luc reporter plasmids using Lipofectamine 2000 reagent. β-Galactosidase vector was used as an internal control for transfection efficiency. Three h after transfection, the cells were treated with various concentrations of Osthole for 24 h. Then they were washed with PBS and lysed using 1× lysis buffer, and 20 μl of cell extract was assayed for luciferase activity using the Luciferase assay kit following the manufacturer's instructions. β-Galactosidase activity was measured to normalize the luciferase activity. This experiment was repeated at least 3 times.

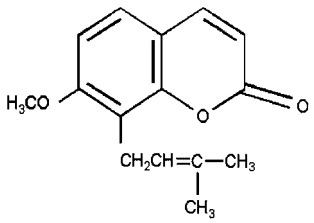

Fig. 1. Chemical Structure of Osthole.

Zymography. MDA-MB 231 cells were seeded in 6-well tissue culture plates and allowed to adhere in the presence of serum. Media subsequently were replaced with 0.5 ml of media containing various concentrations of Osthole without serum for 24 h. The medium was centrifuged to remove cellular debris. Concentrated samples with equal amounts of proteins were mixed with SDS sample buffer without reducing agent and subjected to 8% SDS–PAGE containing 0.1% gelatin A (Sigma). After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 h at room temperature to remove the SDS, and then incubated for 24–48 h at 37 °C in buffer containing 5 mM $CaCl_2$ and 1 mM $ZnCl_2$. Thereafter, the gels were fixed and stained with 0.25% Coomassie Blue R-250 for 4 h, and then destained in 45% methanol and 10% acetic acid. The molecular weights were estimated by reference to prestained SDS–PAGE markers.

Statistical analysis. Results are expressed as mean ± SD. Data were analyzed by one-way analysis of variance between groups (ANOVA) with least significant difference. Statistical analysis was performed with statistical analysis software SPSS 10.0.

Results

Osthole inhibited migration of MCF-7 cells

The effects of Osthole on MCF-7 cell migration were assessed by wound healing assay. As shown in Fig. 2A, photomicrographs taken 24 h after wounding showed delayed wound closure by MCF-7 cells treated with Osthole at concentrations ranging from 20 to 100 μM as compared with untreated control cultures. Quantitation of the wound closure over time revealed a significant inhibitory effect of Osthole on MCF-7 motility (Fig. 2B).

Osthole inhibited invasion of MDA-MB-231 cells

To determine the role of Osthole in cell invasion, MDA-MB-231 cells, a highly metastatic breast cancer

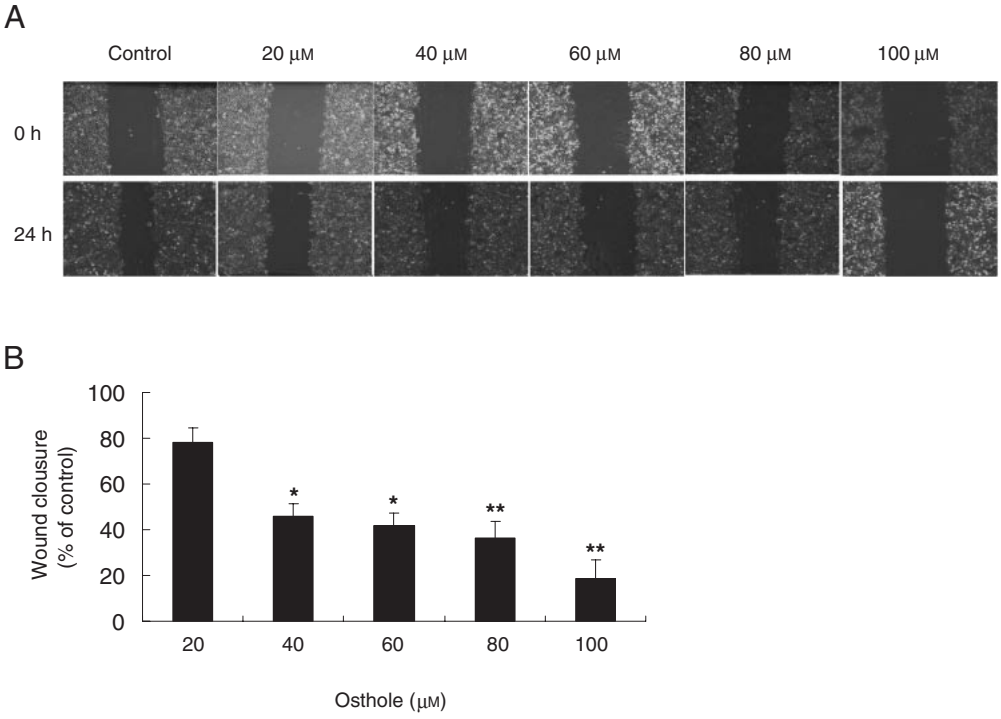

Fig. 2. Effects of Osthole on MCF-7 Cell Migration.
A monolayer of confluent MCF-7 was scraped with a sterile pipette tip and treated with increasing concentrations of Osthole (0, 20, 40, 60, 80, and 100 μM) for 24 h. Wound closure was observed by phase-contrast microscopy and photographed at 0 and 24 h. A, Digital pictures of non-treated and treated cells were taken at 0 h as well as at 24 h; B, Wound closure was quantified as percentage as the mean ± SD of three independent experiments. Control wound closure was set at 100%, and the Osthole treatments were represented as percent of control. $^{*}p < 0.05$; $^{**}p < 0.01$.

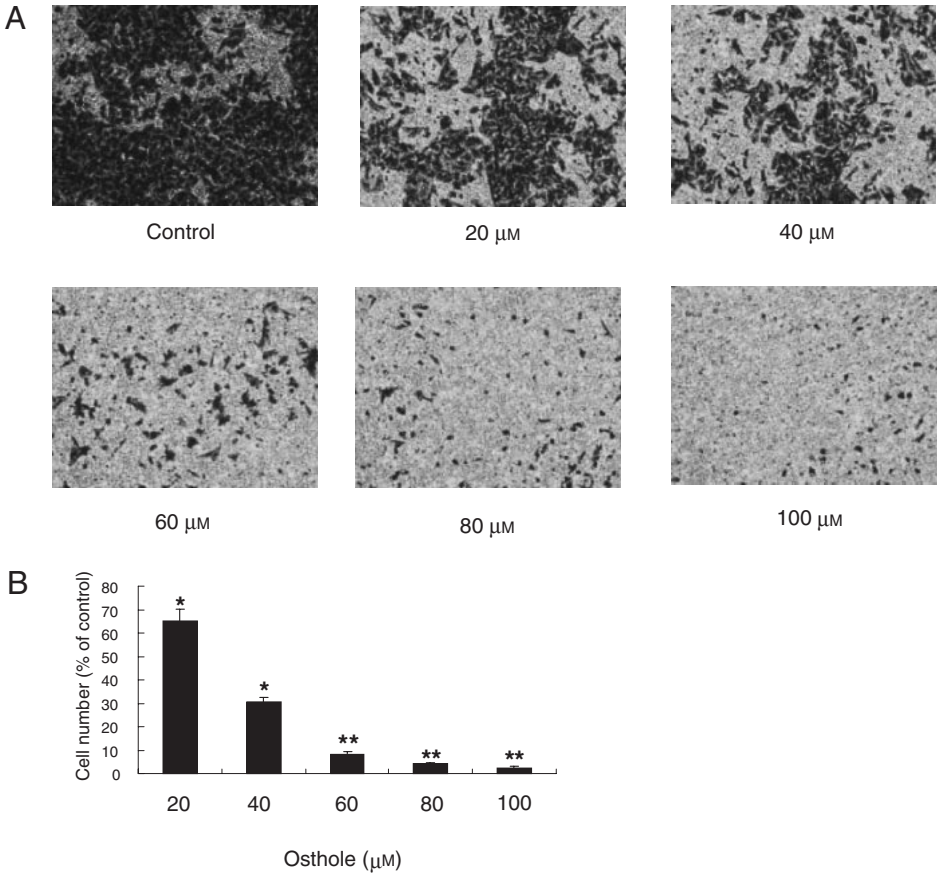

Fig. 3. Effects of Osthole on MDA-MB 231 Cell Invasion.

MDA-MB 231 cells were seeded at a density of 3×10^5 cells onto ECM gel-coated inserts in 24-well plates, and treated with the indicated concentrations of Osthole. After 24 h of incubation, the cells on the upper side of the inserts were removed with a cotton swab. Cells that migrated to the lower side of the filters were stained with 1% crystal violet and counted under a microscope. A, Digital pictures of invasive cells. Stained areas represented numbers of invasive cells. Images are representative of three independent experiments. B, Invasive cell number compared to control (expressed as percentage of control). $^*p < 0.05$; $^{**}p < 0.01$.

cell line, were treated with the indicated concentrations of Osthole for 24 h. Osthole significantly inhibited MDA-MB-231 cell invasion as compared with the untreated control culture (Fig. 3A and B). The invasion of MDA-MB-231 cells was inhibited by 97.6% by 100 μM Osthole treatment for 24 h as compared with the control.

To exclude the possibility that decreased numbers of migrating and invading cells were a consequence of reduced proliferation, we performed proliferation assays using MCF-7 and MDA-MB 231 cells treated at the same concentration of Osthole as that used in migration and invasion assays for 24 h. No significant difference was detected between the treated cells and the controls (Fig. 4A and B).

Osthole inhibited MMP-2 promoter and enzyme activity

To explore the underlying mechanisms by which Osthole inhibits the invasion of MDA-MB 231 cells, we performed luciferase reporter assay and zymography analysis. MCF-7 cells were transfected with MMP-2-Luc reporter construct and then treated with the indicated concentrations of Osthole for 24 h. As shown in Fig. 5, Osthole inhibited MMP-2 promoter activity in a dose-dependent manner. It inhibited MMP-2 promoter activity by 68% at a concentration of 80 μM.

Since MMP-2 and MMP-9 play critical roles in tumor cell invasiveness, we further examined the effects of Osthole on the enzyme activities of MMP-2 and MMP-9. Gelatin zymography was conducted using conditioned media harvested from Osthole-treated MDA-MB 231 cells. The gelatinolytic activity of MMP-2 was reduced in MDA-MB 231 cells after treatment with increasing concentrations of Osthole (Fig. 6A and B). MMP-2 enzyme activity decreased by 47% under 100 μM Osthole treatment for 24 h (Fig. 6B). These data suggest that the inhibition in cell invasion by Osthole is at least partially due to suppression of MMP-2 enzyme activity. MMP-9 enzyme activity was not obviously affected by Osthole treatment (Fig. 6C and D).

Discussion

In the present study, we evaluated the effects of Osthole on migration and invasion in breast cancer cells by wound healing and transwell analysis. We found that Osthole inhibited the migration and invasion of breast cancer cells. Moreover, luciferase reporter assay and zymography analysis revealed that Osthole effectively suppressed MMP-2 promoter and enzyme activity.

Metastasis is the overwhelming cause of mortality in patients with breast cancer.[15] Although the exact mechanism by which metastasis develops is not yet fully understood, it is generally agreed that cells must possess both invasive and migratory activities,[16] and that type IV collagenases or gelatinases (MMP-2 and -9)

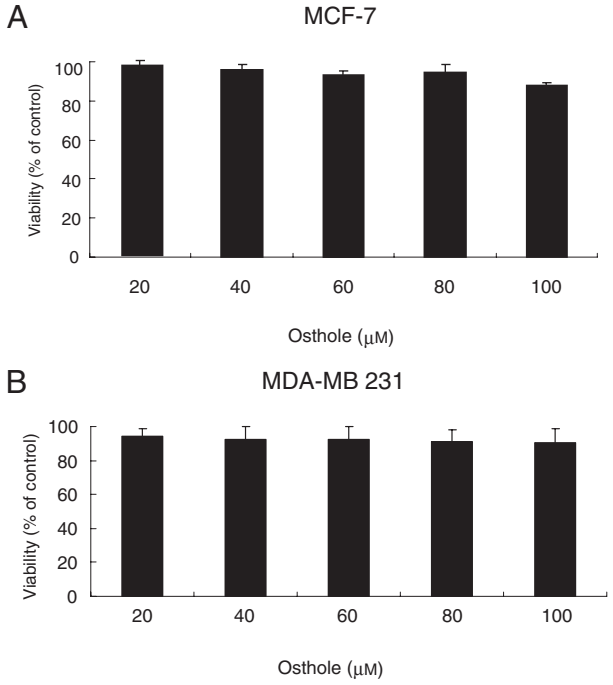

Fig. 4. Effects of Osthole on the Proliferation of MCF-7 and MDA-MB 231 Cells.

MCF-7 and MDA-MB 231cells were treated with 0, 20, 40, 60, 80, and 100 μM Osthole for 24 h, and cell proliferation was examined by MTT assay. A, MCF-7 cells; B, MDA-MB 231 cells. Data are represented as mean ± SD for three independent experiments. $^{*}p < 0.05$.

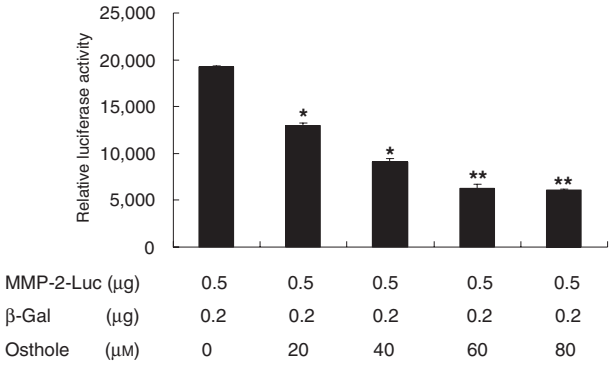

Fig. 5. Effects of Osthole on MMP-2 Promoter Activity.

MCF-7 cells were transfected with MMP-2-Luc reporter plasmids and β-Galactosidase vector and treated with the indicated concentrations of Osthole for 24 h. Cells were lysed and assayed for luciferase activity using the Luciferase assay kit. β-Galactosidase activity was measured to normalize luciferase activity. Transfection was performed at least 3 times. $^{*}p < 0.05$; $^{**}p < 0.01$.

are critical for cell migration, leading to invasion and metastasis of cancer.[17,18] It has been reported that lymph node-positive breast cancer patients have higher MMP-2 and proMMP-9 activity than those with node-negative cancer.[19] MMP-2 and MMP-9 are usually used to evaluate the effects of certain natural compounds on anti-metastasis of breast cancer.[20,21] The results of the transwell assay showed that Osthole significantly inhibited the invasion of MDA-MB 231 cells. To explore the mechanisms by which Osthole inhibits cell invasion, we performed zymography and luciferase assay. Osthole effectively reduced MMP-2 promoter activity (Fig. 5) and MMP-2 enzyme activity (Fig. 6A and B), but there

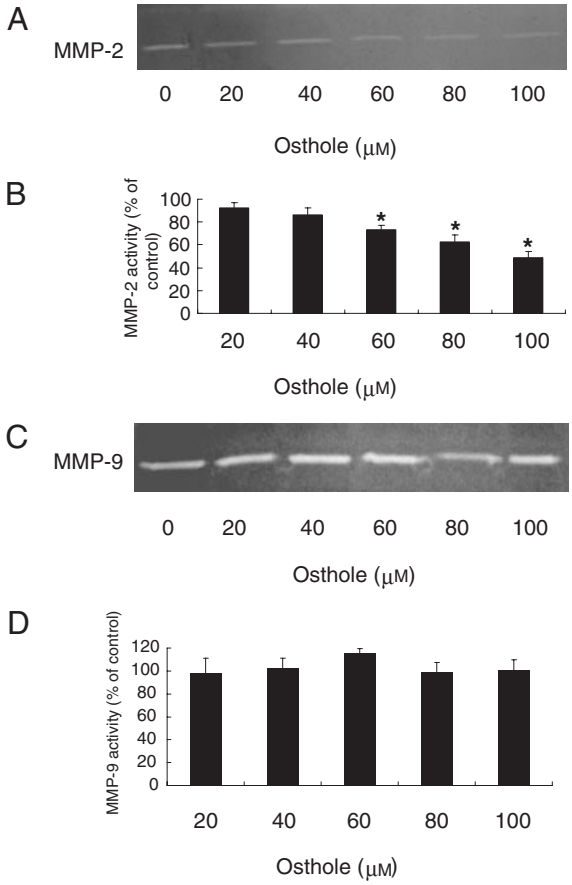

Fig. 6. Effects of Osthole on MMP-2 and MMP-9 Enzyme Activity.

MDA-MB 231 cells were incubated with serum-free medium and Osthole (0, 20, 40, 60, 80, and 100 μM) for 24 h. Supernatants were harvested and subjected to zymography, as described in "Materials and Methods." A and C, Representive images of three independent experiments. B and D, Quantitation analysis of the above experiment and two others. Control MMP-2 and MMP-9 enzyme activities were set at 100%, and the Osthole treatments were represented as percent of control (mean ± SD). $^{*}p < 0.05$.

was no obvious effect of Osthole on MMP-9 enzyme activity (Fig. 6C and D). These data indicate that the invasion inhibition observed in MDA-MB-231 cells following Osthole treatment is at least partially due to suppression of MMP-2 enzyme activity.

The results of the wound healing assay indicate that Osthole effectively inhibits the migration of MCF-7 cells (Fig. 2A and B). Several factors contribute to cancer cell motility, including hepatocyte growth factor/scatter factor (HGF/SF), insulin-like growth factor II (IGF-II), and autotaxin (ATX), *etc*.[22–24] Whether Osthole inhibits the migration of MCF-7 cells by affecting the activity of these molecules should be investigated further.

To exclude the possibilities that decreased numbers of migrating and invasion cells were a consequence of reduced proliferation, we performed an MTT assay on MCF-7 and MDA-MB 231 cells treated with Osthole using the same concentrations and times as used in the wound-healing and transwell assays. There were no significant differences between the treated cells and the controls (Fig. 4A and B). This suggests that the decreased numbers of migrating and invasion cells after Osthole treatment were not due to proliferation inhibition. Osthole significantly inhibited cell growth

and induced apoptosis when MCF-7 and MDA-MB 231 cells were treated with Osthole for longer times, such as 36 h and 48 h (data not shown). This is in accord with reports that Osthole has anti-tumor effects by inhibiting tumor cell growth and inducing apoptosis.[10–14)]

In conclusion, Osthole, as an active compound of *Cnidium monnieri* (L.) Cusson, has been reported to possess several biological functions. We are the first to report that Osthole inhibited the migration and invasion of breast cancer cells, and that the mechanism might be associated with suppression of MMP-2 enzyme activity by Osthole. Because of Osthole's diverse pharmacological activities, the possibility of its development as a promising lead compound for drug discovery has been proposed.[25)] Our findings indicate a need for further evaluation of Osthole in breast cancer chemotherapy and chemoprevention.

Acknowledgments

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