Magnolol Suppresses Metastasis via Inhibition of Invasion, Migration, and Matrix Metalloproteinase-2/-9 Activities in PC-3 Human Prostate Carcinoma Cells

Eun-Sun HWANG¹,² and Kwang-Kyun PARK²

¹Department of Home Education, Jeonju University, 1200 3Ga, Hyoja-dong, Wansan-gu, Jeonju, Jeonbuk 560-759, Korea
²Department of Oral Biology and Oral Cancer Research Center, College of Dentistry, Yonsei University, Seoul 120-752, Korea

Received October 23, 2009; Accepted February 2, 2010; Online Publication, May 7, 2010
[doi:10.1271/bbb.90785]

Magnolol, a hydroxylated biphenyl compound isolated from the root and stem bark of Magnolia officinalis, has been reported to have anticancer activity, but little is known about its molecular mechanisms of action. Increased expression of cyclooxygenase-2 (COX-2), a key enzyme in arachidonic acid metabolism, has been identified in many cancer types. Matrix metalloproteinases (MMPs) are enzymes involved in various steps of metastasis development. The objective of this study was to study the effects of magnolol on cancer invasion and metastasis using PC-3 human prostate carcinoma cells. Cellular proliferation was determined by MTT colorimetric assay. Magnolol inhibited cell growth in a dose-dependent manner. In an invasion assay conducted in Transwell chambers, magnolol showed 33 and 98% inhibition of cancer cell at 10 μM and 20 μM concentrations, respectively, compared to the control. The expression of MMP-2/-9 and COX-1/-2 was assessed by gelatin zymography and Western blot respectively. The protein and mRNA levels of both MMP-2 and MMP-9 were down-regulated by magnolol treatment in a dose-dependent manner. These results demonstrate the antimetastatic properties of magnolol in inhibiting the adhesion, invasion, and migration of PC-3 human prostate cancer cells.

Key words: magnolol; prostate cancer; invasion; metastasis; matrix metalloproteinase

Magnolol (5,5'-diallyl-2,2'-dihydroxybiphenyl), a hydroxylated biphenyl compound isolated from the stem bark of the Chinese herb Magnolia officinalis (Magnoliaceae), is commonly used to treat acute pain, cough, anxiety, and gastrointestinal disorders in Eastern Asia. Various pharmacological actions have been reported for magnolol, including anti-inflammatory and antimicrobial effects, protection of cortical neuronal cells from chemical hypoxia, and scavenging of hydroxyl free radicals. Magnolol also has been reported to exhibit antiinflammatory activity, including induction of apoptosis in cultured HepG2 human hepatoma and Colo205 colon cancer cells.

Prostate cancer is one of the most common cancers and is the second leading cause of cancer death in American men. Moreover, the number of patients with prostate cancer is increasing in Asia as well as in the United States. The most common site of prostate cancer metastasis is bone, more than 80% of advanced prostate cancer patients being diagnosed with skeletal metastasis.

Cyclooxygenase (COX) is the key enzyme catalyzing the rate-limiting step in prostaglandin biosynthesis. Two forms of COX have been described, a constitutively expressed form (COX-1) and an inducible isoenzyme (COX-2), which is expressed in response to cytokines, growth factors, and other stimuli. COX-1 is present in most tissues and is involved in maintaining normal homeostasis. Expression of COX-1 has been found to be high in all tissue samples, including prostate tissues. However, COX-2 levels were reported to increase in more than 80% of human colon cancers compared to levels in adjacent normal tissues. Increased COX-2 expression has also been confirmed in carcinomas of other organs, including the prostate, lung, stomach, esophagus, and pancreas. Expression of COX-2 was found to increase in prostate cancer cell lines LNCaP (androgen-sensitive) and PC-3 (androgen-insensitive). Tumor metastasis is a major cause of morbidity in cancer patients. Cancer metastasis consists of multiple sequential steps; invasion is one of the most characteristic steps during the cascade of metastasis. Invasion consists of three major processes: adhesion to the extracellular matrix (ECM), degradation of the ECM by several kinds of proteolytic enzymes, and migration. Many studies have demonstrated that inhibition of these steps results in the prevention of metastasis. We began by considering that malignant tumor cells usually produce several matrix-degrading activities with different substrate specificities that are thought to cooperate in the degradation of the ECM. A family of metalloproteinases (MMPs) plays an essential role in matrix degradation, and MMP production has been positively correlated with invasive and metastatic behavior in tumor cell lines. In particular, MMP-2 and -9 degrade components of the basement membrane and are strongly implicated in invasion, by metastasis of malignant tumors. Therefore, inhib-
of MMP activity is important in the prevention of early stage carcinogenesis, particularly the tumor pro-
motion process.

The processes regulating tumor cell invasion and metastasis depend in part on the activation of at least
two major MMPs, MMP-2 (72 kDa) and MMP-9 (92 kDa). MMP-9 digests denatured collagens, such as
the major component of the basement membrane type IV collagen; its protein structure has three repeats of a
type II fibronectin domain at the catalytic site, which
binds to substrates such as collagens, gelatin, and
laminin.36) MMP-2 is structurally related to MMP-9 and
cleaves ECM proteins such as collagen types I and IV.36) MMP activity has been reported to be associated
with invasive and metastatic behavior in malignant
tumors based on elevated expression of them in relation
to invasion and metastasis. Inhibition of MMPs may be
useful for inhibiting cancer cell invasiveness and could
consequently decrease the incidence of metastasis.
MMP production has been positively correlated with
the invasive and metastatic behaviors of tumor cell
lines.31)

In the present study, we determined the anti-meta-
static effect of magnolol in PC-3 cells using migration
and invasion assays. We also investigated the effects of
magnolol on MMP-2/9 and COX-1/2 expression at both
the protein and the gene level in PC-3 human prostate
carcinoma cells.

Materials and Methods

Cell culture. PC-3 human prostate carcinoma cells were obtained
originally from the American Type Culture Collection (Rockville,
MD), and cell culture supplies were purchased from Sigma-Aldrich
(St. Louis, MO). PC-3 cells were maintained at 37 °C with 5% CO2 in
RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10%
FBS, 0.37% NaHCO3, 100 U/ml of penicillin, 100 µg/ml of strepto-
mycin. Magnolol (99.9%) was obtained from LKT Laboratories (St.
Paul, MN). Magnolol was dissolved in dimethyl sulfoxide (DMSO)
and added directly to cell culture medium at a final concentration of 0.01% DMSO. This concentration had no effect on
cell proliferation or other assays.

Cell proliferation assay. Proliferation was determined by MTT
 assay. To evaluate the effect of magnolol on cell viability, cells were seeded at a density of 5 × 103 cells per well containing 200 µl of
culture medium in 96-well plates. After cultivation for 24 h, the
medium was changed to 200 µl of fresh medium supplemented with
magnolol. After cultivation for 24–72 h, all the medium was removed
and cell viability was evaluated as follows: MTT (Sigma M5665,
Sigma) was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml
and filtered to sterilize and remove small amounts of insoluble residue.
A mixture of 10 µl of the MTT stock solution and 90 µl of phenol red
and serum free medium was added to all the wells of the 96-well plate
culture. After the plates were incubated for 4 h at 37 °C, DMSO was
added to the wells and mixed by pipetting to dissolve the dark-blue
formazan product. The absorbance of the dissolved formazan in each
well was measured with a microplate spectrophotometer at 570 nm.
Cell viability was calculated by the formula (magnolol-treated
Abs570/1) (control Abs570) × 100 (%).

Cell adhesion assay. The 24-well tissue culture plates were coated
with 25 µg/well of Matrigel and left to air-dry for 40 min. Cells
(5 × 104) suspended in RPMI-1640 that contained 0.5% BSA, in the
presence and the absence of different concentrations of magnolol,
were dispensed into each well of the 24-well culture plate, incubated in 5%
CO2 at 37 °C for 1 h, and gently washed 3 times with PBS to remove
the unattached cells. The cells that remained attached to the bottom of
the plate were stained with hematoxylin and eosin and counted under a
microscope (Olympus IX70; Olympus, Okaya, Japan). The experiments
were performed at least 4 times.

Cell invasion assay. A Transwell system that incorporated a
polycarbonate filter membrane with a diameter of 6.5 mm and pore size
of 3µm (Corning, Corning, NY) was used to assess the rate of cell
invasion. Matrigel (12.5 µg in 50 µl of PBS) was added to the filter
to form a thin gel layer, dried in a laminar hood overnight, and
reconstituted in 100 µl of PBS at 37 °C for 2 h. Cells at 90% confluency
were harvested using a cell dissociation solution. The cells (1 × 105)
were suspended in 100 µl of serum-free RPMI-1640 medium in the
presence and the absence of different concentrations of magnolol, and
added to the upper chamber of the Transwell insert. The lower chamber
was filled with 500 µl of the same medium as the upper chamber. After
18h of incubation at 37 °C, the cells on the upper surface of the filter
were removed using a cotton swab. Cells that penetrated to the lower
surface of the filter were stained with hematoxylin and counted under an
Olympus IX70 microscope in 13 randomized fields at 400× magnification.
The assay was performed on at least four separate occasions.

Wound migration assay. Cell motility was examined by wound
migration assay. Cells were cultured to 100% confluency in a 6-well
plate in the presence or absence of different concentrations of magnolol
for 18 h. The cells were pretreated with mitomycin C (25 µg/ml) for
30 min before the injury line was made by applying a plastic pipette tip
to the cells across the center of the well, producing a clean 1-mm-wide
wound area. After rinsing with PBS, the cells were allowed to migrate
in the medium. A computer-based microscopy imaging system
(Olympus) was used to determine wound healing at 0 h with a
microscope at 200× magnification. At the indicated time-points,
migration was assessed by counting the number of cells across the
wound. This number was then compared to the same frame at 0 h.
Several wound areas (at least five per plate) were quantified for
cell migration. The migration distances between the leading edge of the
migrating cells and the edge of the wound were compared. Migration
rate = (migration distances of magnolol-treated cells/migration dis-
tances of untreated cells) × 100%. The experiments were performed
at least 4 times.

Gelatin zymography. Cells were incubated with 0–20 µM magnolol
to sub-confluence, and were rinsed with PBS and then incubated in
serum-free RPMI-1640 medium for 48 h. Conditioned media were
collected, and the cell numbers were determined. Cell number
was standardized conditioned media were resolved in 10% SDS–PAGE
containing 1 mg/ml of gelatin. The gel was washed twice with 2.5%
Trion X-100 for 30 min to remove SDS, and was subsequently
incubated in buffer containing 50 mM Tris–HCl (pH 7.6), 200 mM
NaCl, and 5 mM CaCl2; for 18 h at 37 °C. The gel was stained with 0.5%
Coomassie Blue for 1 h and then destained in water containing 10%
glacial acetic acid and 30% methanol. Proteolysis was detected as a
white zone on a dark field. The intensity of the bands was quantitated
using ImageJ software (version 1.34) (NIH, Bethesda, MD).

Isolation of total RNA and semiquantitative RT-PCR. Total RNA
was isolated from 105 cells using TRIzol reagent (Life Technologies,
Rockville, MD). The homogenized samples were incubated for 5 min
at room temperature so that the nucleoprotein complexes completely
dissociated. After the addition of 0.2 volumes of chloroform, the
samples were shaken vigorously for 15 s, incubated for 2–3 min, and
centrifuged at 12,000 g for 15 min at 4 °C. The total RNA remaining in
the upper aqueous phase was precipitated by mixing it with an equal
volume of isopropanol. The mixtures were then incubated for 15 min at
4 °C for 15 min and centrifuged at 12,000 g for 15 min at 4 °C. The resulting
total RNA pellet was washed with 70% ethanol, dried, and dissolved in
RNase-free water. The concentration and purity of total RNA were
calculated on the basis of the absorbance at 260 and 280 nm.

The primer sets used in this study are listed in Table 1. First-strand
cDNA was synthesized with 1 µg of total RNAs and 1 µl oligo(dT15)
primer using Omniscript Reverse Transcriptase (Quagen, Valencia,
CA). Using the Taq PCR Master Mix kit (Quagen), subsequent PCR as
performed with 0.5 µl of first-strand cDNA and 20 pmol of primers.
PCR consisted of an initial denaturation at 94 °C for 3 min; 30 3-step
Magnolol Suppresses Metastasis in PC-3 Human Prostate Carcinoma Cells

Table 1. Sequences of Primers Used in This Study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Sense</td>
<td>GGC CCT GTC ACT CCT GAG AT</td>
<td>474</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGC ATC CAG GTT ATC GGG GA</td>
<td>573</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Sense</td>
<td>CGG AGC ACG GAG ACG GGT AT</td>
<td>494</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGA AGG GGA AGA CCG ACA GC</td>
<td>774</td>
</tr>
<tr>
<td>COX-1</td>
<td>Sense</td>
<td>GTT CAA CAC CTC CAT GTT GGT GGA C</td>
<td>501</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGG TGT TGA GGC AGA CCA GTC TC</td>
<td>774</td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense</td>
<td>GGT CTG GTG CCT GTG CTG ATG ATG</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GTC CTT TCA AGG AGA ATG GTG C</td>
<td>983</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>TGA AGG TCG GAG TCA AGT TTG GT</td>
<td>983</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CAT GTG GGC CAT GAG GTC CAC CAC</td>
<td>983</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of Magnolol on PC-3 Human Prostate Carcinoma Cell Proliferation as Measured by MTT Assay.

The optical density was determined at 570 nm, and is expressed as cell survival relative to control. Data are mean ± SD values for 4 independent experiments.

Results

Cell proliferation

Figure 1 illustrates the dose-dependent effect of 0–40 μM magnolol on the growth of PC-3 human prostate carcinoma cells after various incubation times. After 24 h of incubation, magnolol inhibited the proliferation of PC-3 cells in a dose-dependent fashion. The numbers of cells were significantly reduced by 5.2–88% at magnolol concentrations of 0–40 μM as compared to the control. Similarly, after 48 and 72 h of incubation, 40 μM magnolol reduced the cell numbers by 61 and 71% respectively, and magnolol dose-dependently inhibited PC-3 cell proliferation.

Cell adhesion

To determine the effects of magnolol on the adhesion of PC-3 cells, adhesive cells were quantitated using an ELISA reader. Incubation of PC-3 cells with 1–20 μM magnolol for 60 min significantly inhibited cell adhesion to the Matrigel-coated substrate in a concentration-dependent manner (Fig. 2). Approximately 31 and 65% reduction was seen under the 10 and 20 μM magnolol treatments respectively.

Cell invasion

We further evaluated the anti-metastatic activity of magnolol using the Transwell assay. We tested the ability of PC-3 cells to invade through a reconstituted basement membrane barrier (Matrigel) with and without

Statistical analysis

All experiments were performed in triplicate and are presented as mean ± SD. Appropriate comparisons were made by the Student-Newman-Keuls method for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.
magnolol. Similarly to its effects in the adhesion assay, magnolol in the range of 0–20 μM inhibited the invasion of PC-3 cells in a dose-dependent manner. When PC-3 cells were grown on Matrigel, treatment with 5–20 μM magnolol for 18 h produced a significant reduction in the number of invasive cells compared to the control (fresh medium alone). The level of invasion was reduced to 32.9% of the control level at 10 μM magnolol (Fig. 3). Magnolol showed significant inhibition at 5 μM (31.2% reduction) and almost complete inhibition at 20 μM (98% reduction).

**Cell migration**

To evaluate the anti-metastatic activity of magnolol, we assessed the effect of magnolol on the migration of PC-3 cells on wound migration assay. The migration distance between the leading edge and the wound line was compared between magnolol-treated cells and untreated cells. As shown in Fig. 4, cellular motility was controlled by 10 μM magnolol in a time-dependent manner, with up to 94 and 82% motility at 24 and 48 h of incubation respectively ($p < 0.001$).

**MMP expression**

We determined the inhibitory effects of magnolol on MMP production by zymography in PC-3 cells. The PC-3 cells constitutively secreted high levels of MMP-9 and low levels of MMP-2. Gelatin zymography of conditioned medium demonstrated that the PC-3 cells secreted a 72-kD protein with gelatinolytic activity, corresponding to pro-MMP-2. Two gelatinolytic bands, corresponding to the latent (72 kD) and activated (62 kD) forms of MMP-2, were detected in the PC-3 cells. As shown in Fig. 5, gelatinolytic activity in the zymogram was inhibited by magnolol in a dose-dependent manner. Magnolol at concentrations of 10 μM and higher clearly suppressed MMP-2 activity, and MMP-9 activity was inhibited in a dose-dependent manner by 0–20 μM magnolol. Thus, in the PC-3 cell system, both MMP-2 and MMP-9 were downregulated by magnolol treatment.

We also quantified MMP-2/9 mRNA expression in PC-3 cells by RT-PCR with an internal standard (GAPDH) (Fig. 6). In the presence of magnolol, MMP-9 and MMP-2 activities were reduced in a dose-dependent manner. MMP-9 expression was inhibited by 30 and 86% at 5 μM and 20 μM magnolol respectively as compared to the control. MMP-2 mRNA expression was

![Fig. 3. Effects of Magnolol on PC-3 Cell Invasion.](image)

**Fig. 3.** Effects of Magnolol on PC-3 Cell Invasion.

Invasiveness of PC-3 cells towards endothelial cell-conditioned medium was measured using Transwell chambers with tissue culture-treated filters with 8 μm pores. Each bar represents the mean ± SD for three separate experiments. Values marked * and ** indicate significant differences from 0 magnolol treatments ($p < 0.05$).

![Fig. 4. Effects of Magnolol on Wound Healing Migration of PC-3 Cells.](image)

**Fig. 4.** Effects of Magnolol on Wound Healing Migration of PC-3 Cells.

Wounds were introduced by scraping confluent cell layers with a pipette tip. Representative photographs of invading cells magnolol-treated and untreated cells.

![Fig. 5. Effects of Magnolol on MMP Expression.](image)

**Fig. 5.** Effects of Magnolol on MMP Expression.

MMP-2/9 protein expression was analyzed by gelatin zymography, which was performed on media conditioned by $2 \times 10^5$ PC-3 cells treated with magnolol for 48 h (A). Data shown are representative of three different experiments yielding essentially the same results. The enzyme activities of MMP-2 and MMP-9 in the treated cells were expressed as percentages of their activities in untreated cells (B). Values marked * and ** indicate significant differences from 0 magnolol treatments ($p < 0.05$).
also inhibited at all doses of magnolol used in this study, with 36 and 99% inhibition after a 24-h exposure to 5 \( \mu \)M and 20 \( \mu \)M magnolol respectively.

**COX-1 and COX-2 expression**

As COX-2 is also a potential target molecule for the anti-metastatic activity as well as anti-inflammatory activity of chemopreventive agents, we investigated the effects of magnolol on COX-2 protein expression in PC-3 human prostate cancer cells. Figure 7 shows the Western immunoblots for COX-1 and COX-2 proteins after treatment of PC-3 cell with different concentrations of magnolol. Magnolol markedly reduced the expression of COX-2 protein in a dose-dependent manner, except at concentrations as low as 2.5 \( \mu \)M. Treatment with 5 \( \mu \)M and 20 \( \mu \)M magnolol suppressed COX-2 activity by 24 and 77% respectively. Magnolol inhibited the level of COX-2 mRNA in a dose-dependent manner except at concentrations as low as 5 \( \mu \)M (Fig. 8). COX-1 protein expression was not reduced by magnolol treatment. Magnolol inhibited COX-2 mRNA expression at concentrations as low as 2.5 \( \mu \)M after 24 h of treatment, as well as at higher concentrations. To determine if magnolol simultaneously inhibited the expression of COX-1 mRNA, we studied its effect on COX-1 mRNA expression in PC-3 cells after the same treatment. COX-1 mRNA expression remained unaltered under magnolol treatment. Inhibition of COX-2 protein expression correlated with the COX-2 mRNA expression data, suggesting that no post-translational modification of the mRNA transcript was necessary to account for the effect of magnolol.
Discussion

Our study suggests that magnolol at a more than 5 μM concentration inhibits tumor invasion and metastasis through decreased COX-2 expression in PC-3 human prostate carcinoma cancer cells. Several in vitro studies have shown that magnolol inhibits the cancer cell growth of B16-BL6, THP-1, BAEC, and HT1080 cells. Furthermore, magnolol inhibited cell proliferation at 10–40 μM and induced apoptotic cell death at 80–100 μM within 24 h in human lung squamous carcinoma CH27 cells.

Our results indicate that magnolol decreased the metastasis of PC-3 cells, as determined by adhesion, invasion, and migration assays. To define the inhibitory effects of magnolol on metastasis, we focused on MMP expression, because degradation of the ECM is required for tumor metastasis. We evaluated MMP-2/-9 expression because the main component of the basement membrane, type IV collagen, is degraded by collagenase, and several reports have indicated a correlation between decreased MMP-2/-9 expression and metastatic potential in various cancer cells. Furthermore, Attiga et al. reported inhibition of MMP-2 and MMP-9 by COX-2 inhibitors in prostate cancer. As found in the current study, magnolol suppressed the secretion of MMP-2/-9 into the culture medium as well as the expression of MMP-2/-9 mRNA and protein.

Magnolol inhibited HT1080 fibrosarcoma cell invasion of the reconstituted basement membrane in vitro and markedly inhibited MMP-9 degradation of type IV collagen. In an animal model, Ikeda et al. evaluated the anti-metastatic effects of magnolol in an experimental liver and spleen metastasis model using L5178Y-ML25 lymphoma, and in an experimental and spontaneous lung metastasis model using B16-BL6 melanoma. Magnolol significantly inhibited B16-BL6 cell invasion of the reconstituted basement membrane (Matrigel) without affecting cell growth. The anti-metastatic action of magnolol is thought to result from its ability to inhibit tumor cell invasion.

Several studies have reported that COX-2 stimulates endothelial cell migration and affects MMP-2 and activated collagenase levels. Expression of COX-2 is also significantly correlated with MMP-2 in colorectal cancer. Xi et al. reported that COX-2 affects tumor progression by modulating cancer cell motility and invasive potential in colorectal cancer, and that COX-2 is a possible biomarker for colorectal cancer. They found that 67.9% of 128 cases of colorectal cancer were positive for COX-2. Expression of COX-2 was significantly correlated with the depth of invasion, stage of disease, and metastasis (lymph node and liver). Among 45 cases of colorectal cancer with lymph node metastasis, 86.7% of primary lesions were positive for COX-2, and diffuse cytoplasmic staining for COX-2 protein was detected in cancer cells from 100% of the metastatic lesions of the lymph nodes. MMP-2 expression was detected in 88 of 128 colorectal tumors (68.8%) and was closely correlated with COX-2 expression. The rate of MMP-2 expression in the COX-2-positive group (79.6%) was higher than in the COX-2-negative group (20.4%).

Sun et al. used immunohistochemical staining to investigate the expression of COX-2 and MMP-9 in 96 resected tumor specimens from gastric carcinoma patients. They showed that overexpression of COX-2 and MMP-9 is related to tumor invasion and lymph node metastasis in gastric carcinoma. These results provide evidence that COX-2 contributes to gastric cancer development by promoting MMP-9 expression.

Nevertheless, the underlying molecular mechanisms of action of COX-2 and MMP-2/-9 remain unknown, and further research is required.

References

Magnolol Suppresses Metastasis in PC-3 Human Prostate Carcinoma Cells