Anethole Exerts Antimetastatic Activity via Inhibition of Matrix Metalloproteinase 2/9 and AKT/Mitogen-Activated Kinase/Nuclear Factor Kappa B Signaling Pathways

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Anethole is known to possess anti-inflammatory and anti-tumor activities and to be a main constituent of fennel, anise, and camphor. In the present study, we evaluated anti-metastatic and apoptotic effects of anethole on highly-metastatic HT-1080 human fibrosarcoma tumor cells. Despite weak cytotoxicity against HT-1080 cells, anethole inhibited the adhesion to Matrigel and invasion of HT-1080 cells in a dose-dependent manner. Anethole was also able to down-regulate the expression of matrix metalloproteinase (MMP)-2 and -9 and up-regulate the gene expression of tissue inhibitor of metalloproteinase (TIMP)-1. The similar inhibitory effect of anethole on MMP-2 and -9 activities was confirmed by zymography assay. Furthermore, anethole significantly decreased mRNA expression of urokinase plasminogen activator (uPA), but not uPA receptor (uPAR). In addition, anethole suppressed the phosphorylation of AKT, extracellular signal-regulated kinase (ERK), p38 and nuclear transcription factor kappa B (NF-κB) in HT-1080 cells. Taken together, our findings indicate that anethole is a potent anti-metastatic drug that functions through inhibiting MMP-2/9 and AKT/mitogen-activated protein kinase (MAPK)/NF-κB signal transducers.

Key words anethole; antimetastasis; matrix metalloproteinase; AKT; mitogen-activated kinase; nuclear transcription factor kappa B

Metastasis is a process by which cancer spreads from the place at which it first arose as a primary tumor to distant locations in the body. Thus, metastasis is one of hallmarks of malignant tumor and a major cause of death in cancer.1,2) The matrix metalloproteinase (MMP) family proteins are proteolytic enzymes in extracellular matrix (ECM) that contribute to tumor invasion, angiogenesis, and metastasis.3) Of MMP family, MMP-2 and -9 are mainly involved in the metastasis process.4) The activities of MMPs are inhibited endogenous inhibitors, such like the tissue inhibitor of metalloproteinase (TIMPs)5,6) and also controlled by nuclear factor kappa B (NF-κB) signaling transducers.

Anethole [1-methoxy-4-(1-propenyl)benzene] is a main constituent of fennel, anise, and camphor and has anti-inflammatory and chemopreventive activities.5,11) Chainy and colleagues reported that anethole suppressed tumor necrosis factor (TNF)-induced NF-κB, activator protein 1 (AP-1), c-jun N-terminal kinase (JNK) and mitogen-activated protein kinase kinase (MAPKK), which attenuate the magnitude of apoptosis.12) Reddy and colleagues reported that anethole derivatives anethole trithione and diallyl disulfide inhibited azoxymethane (AOM)-induced colon carcinogenesis with increased activities of phase II enzymes such as glutathione S-transferase (GST), nicotine amide adenine dinucleotide phosphate (NAD(P)H)-dependent quinone reductase, and uridine 5’-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase in the liver and colon.13) However, the anti-metastatic activity of anethole has not been fully investigated. Thus, the present study aims at elucidating the anti-metastatic mechanisms by which anethole attenuates the metastatic process in HT-1080 fibrosarcoma cells.

MATERIALS AND METHODS

Plant Materials The fruits of Foeniculum vulgare were purchased from the oriental drug store, Bohwa Dang (Jeonbuk, Korea). A voucher specimen was deposited at the herbarium of College of Pharmacy, Woosuk University.

General Experimental Procedures 1H- and 13C-NMR spectra were determined on a JEOL JMN-EX 400 MHz spectrometer. TLC work was carried out using plates coated with silica gel 60 F254. Detection was performed by spraying with 10% H2SO4 in ethanol followed by heating at 100—120°C for 30 s. Column chromatography was performed on Merck silica gel 60 (230—400 mesh) and Sephadex LH-20. Preparative HPLC was carried out on a Jaigel GS310 column (Japan).

Extraction and Isolation The plant materials (500 g) was extracted twice with MeOH under 50°C. The resultant MeOH extract (65 g) was suspended in water, and then fractionated successively with equal volumes of methylene chloride, ethyl acetate and n-ButOH, leaving residual water soluble fraction. Each fraction was evaporated in vacuo to yield the residues of n-hexane (10 g), methylene chloride (8 g), ethyl acetate (6 g) and n-ButOH (15 g) soluble fractions. The n-hexane soluble fraction was chromatographed on silica gel column using n-hexane—ethyl acetate (20:1) as an eluent, to give five fractions (H1—H5). H3 fraction was purified by HPLC with Jaigel GS310 column (MeOH), to confirm compound 1 (2.9 g) shown in Fig. 1A.
NMR Analysis for Compound 1 (trans Anethole) The chemical structure of trans anethole was confirmed by NMR analysis as follows: A colorless oil, \(^1\)H-NMR (400 MHz, CDCl\(_3\)) \(\delta: 7.23\) (2H, d, \(J=8.8\) Hz, H-2, 6), 6.82 (2H, d, \(J=8.8\) Hz, H-3, 5), 6.34 (1H, d, \(J=14.5\) Hz, H-1’), 6.09 (1H, d, m, H-2’), 3.78 (3H, s, OCH\(_3\)), 1.85 (3H, d, \(J=6.8\) Hz, H-3’), \(^13\)C-NMR (100 MHz, CDCl\(_3\)) \(\delta: 158.5\) (C-4), 131.0 (C-1), 130.4 (C-1’), 126.8 (C-2,6), 123.5 (C-2’), 114.0 (C-3), 113.0 (C-5), 55.4 (OCH\(_3\)), 18.5 (C-3’).

Cell Culture Human sarcoma cell line HT-1080 was obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 g/l sodium bicarbonate, 1% penicillin–streptomycin.

Cytotoxicity Assay To evaluate cytotoxicity of anethole in HT-1080 cells, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed. Briefly, the cells were seeded at a density of 5000 cells/well on a 96-well plate and treated with various concentrations of anethole (0, 50, 100 or 200 \(\mu\)M) for 24, 48 or 72 h. After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min, rinsed three times for 30 min with washing buffer (50 mM Tris–HCl buffer (pH 7.6) containing 5 mM CaCl\(_2\), 0.02% Brij-35, and 0.2% sodium azide) and incubated at 37°C overnight. The gels were stained with 0.5% Coomassie brilliant blue R-250 solution containing 10% acetic acid and 20% methanol for 30 min, and then destained with 7.5% acetic acid solution containing 10% methanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Invasion Assay The Boyden chamber (Neuro Probe Inc., Gaithersburg, MD, U.S.A.) was used to evaluate the spontaneous invasion of HT-1080 cells as described.\(^{10}\) The method was based on the passage of cells across porous filters separating the upper and lower wells of the migration chamber. Polyvinyl-pyrrolidone-free polycarbonate filters (8 \(\mu\)m pore size) were used in this experiment. The filters were coated with the reconstituted basement membrane Matrigel (50 \(\mu\)g/filter). HT-1080 cells were pre-cultured in serum-free medium for 24 h and applied onto the upper chamber at a density of \(1\times10^5\) cells/well/50 \(\mu\)l of serum-free medium with 0, 50 or 100 \(\mu\)M anethole. The chamber was incubated at 37°C for 8 h and the filters were removed and fixed in methanol. Non-migrated cells on the upper surface of the filter were removed with a cotton swab, while migrated cells, adherent on the lower filter surface, were stained with Diff-Quick (Mertz-Dade AG, Dade International, Milan, Italy) and counted under a light microscope (\(\times400\) in 10 random fields) per each well. Each experiment was performed in triplicates. Migration values were expressed as means±S.D. of (the number of migrated cells/total cells)\(\times100\)% counted on the lower surface of filter.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis Total RNA was prepared from HT-1080 cells by using the Trizol reagent according to the manufacturer’s instructions and reverse transcribed to cDNA using oligo-dT and random primers. The cDNA was amplified by PCR using the specific primers (Table 1).
and the amplified products were separated on 1% agarose gels.

**Western Blotting** Whole cell extracts were prepared by using protein lysis buffer (50 mm Tris–HCl, pH 7.4, 300 mm NaCl, 0.5% Triton X-100, 5 mm ethylenediaminetetraacetic acid (EDTA), 1 mm Na3VO4, 1 mm NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mm iodoacetamide, and 2 mm phenylmethylsulfonyl fluoride (PMSF)). The extracts were incubated for 30 min, at 14000 g for 20 min at 4 °C and the supernatants were collected. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, U.S.A.), and 50—100 μg of protein was separated on 1% agarose gels.

The extracts were incubated on ice for 30 min, at 14000 g for 20 min at 4 °C and the supernatants were collected. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, U.S.A.), and 50—100 μg of protein was separated on 1% agarose gels.

**Electrophoretic Mobility Shift Assay (EMSA)** The NF-κB–DNA binding was analyzed by electrophoretic mobility shift assay (EMSA) by using Gelshift Chemiluminescence (ECL) transfer membrane and analyzed with anti-phospho-AKT, AKT, phospho-ERK, ERK, phospho-p38 MAPK and p38 MAPK antibodies (Cell Signaling Technology, Beverly, MA, U.S.A.). Cytosolic and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extract reagents and subjected to Western blotting with anti-IκBα and NF-κB p65 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), respectively.

**Statistical Analysis** All data were expressed as means±S.D. The statistically significant differences compared with untreated group were calculated by Student’s t test.

**RESULTS**

**Cytotoxic Effect of Anethole on HT-1080 Cells** To evaluate cytotoxic effect of anethole, MTT assay was performed. HT-1080 cells were plated onto 96-well plate and treated with anethole (0, 50 or 100 μM) for 24, 48 or 72 h. As shown in Fig. 1b, anethole exerted no cytotoxicity at the concentration of up to 100 μM following 72 h culture.

**Inhibitory Effect of Anethole on the Adhesion and Invasion of HT-1080 Cells** Tumor metastasis process is initiated by disaggregation of invasive cells from the primary tumor.\(^{(17)}\) Invasion is dependent upon adhesive interactions of tumor cells with extracellular matrix (ECM) components.\(^{(17,18)}\) To evaluate the effect of anethole on ECM of tumor cells, adhesion assay was performed. HT-1080 cells with or without anethole treatment were added to Matrigel-coated plates. Anethole significantly inhibited 14, 18 and 24% of the adhesion of HT-1080 cells at the concentrations of 25, 50 and 100 μM anethole, respectively, compared with untreated control (Fig. 2A).

The effect on invasion of HT-1080 cells was then evaluated by in vitro tumor cell invasion assay using Boyden-chamber. As shown in Fig. 2B, anethole reduced 40 and 85% of HT-1080 cells to invade into Matrigel at the concentrations of 50 and 100 μM anethole, respectively, in comparison with untreated control.

**Effect of Anethole on the Expression of MMPs, TIMPs and uPA in HT-1080 Cells** MMPs are important proteins in tumor metastasis.\(^{(19,20)}\) To test whether anethole affects the activity of MPs, gelatin zymography and RT-PCR analyses were employed to evaluate the effect of anethole on MMP-2 and -9 activities. The gelatinolytic activities of MMP-9 and -2 were constitutively activated in HT-1080 cells, whereas anethole suppressed both MMP-2 and -9 enzymatic activities in a concentration-dependent manner (Fig. 3A). RT-PCR analysis also showed that mRNA transcripts of MMP-2 and -9 were significantly decreased by anethole (Fig. 3B). It is also known that TIMPs are secretory glycoproteins that can inhibit MMP activity, thereby attenuating ECM degradation.\(^{(21)}\) and uPA receptor (uPAR) facilitates tumor cell invasion.\(^{(22)}\) Thus, mRNA expression of TIMP-1/2 (Fig. 3C) and uPA/uPAR (Fig. 3D) were analyzed by RT-PCR. Anethole significantly increased TIMP-1, but not TIMP-2. The gene expression of uPA, but not uPAR, was suppressed by anethole, which was in a concentration-dependent manner (Fig. 3D).

**Table 1. The Specific Primer Sequences Used in RT-PCR**

<table>
<thead>
<tr>
<th>Gene (a)</th>
<th>Primer sequences</th>
<th>Product size</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>S: 5’-TATGGGCTTCGCCGAGGAC-3’</td>
<td>970 bp</td>
<td>65 °C</td>
</tr>
<tr>
<td>MMP-9</td>
<td>S: 5’-GCTGTCAGCCGCTTCCA-3’</td>
<td>540 bp</td>
<td>60 °C</td>
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<tr>
<td>TIMP-1</td>
<td>S: 5’-TGGCATCTGGACCACTGGAAGTACG-3’</td>
<td>550 bp</td>
<td>59 °C</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>S: 5’-TTATGGCTCTGAGTCCGACG-3’</td>
<td>581 bp</td>
<td>59 °C</td>
</tr>
<tr>
<td>uPA</td>
<td>S: 5’-TGACAGCTGGCTCCGGGAGC-3’</td>
<td>447 bp</td>
<td>59 °C</td>
</tr>
<tr>
<td>uPAR</td>
<td>S: 5’-GATGCTAGTCAAGACCAAGGCG-3’</td>
<td>253 bp</td>
<td>65 °C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>S: 5’-GACACTTTCGAGGGG-3’</td>
<td>700 bp</td>
<td>58 °C</td>
</tr>
</tbody>
</table>

(a) Human.
Effect of Anethole on AKT, MAPK and NF-κB Signaling in HT-1080 Cells

Metastasis process is known to induce through the activation of MAPK, AKT and NF-κB pathways, in which MMP-2 and MMP-9 are often involved. Western blotting revealed that anethole down-regulated the phosphorylation of AKT, ERK and p38 MAPK in a concentration-dependent manner (Fig. 4A). In addition, anethole blocked degradation of IκBα phosphorylation of IκBα in the cellular extracts and inhibited the expression of p65 of NF-κB in the nuclear extracts (Fig. 4B), possibly via interfering with IκBα degradation and phosphorylation in HT-1080 cells. Consistently, anethole reduced the NF-κB binding on its target DNA sequence in gel shift mobility assay (Fig. 4C), suggesting that anethole blocks NF-κB activation.

DISCUSSION

The metastasis is a series of sequential steps, including tumor-induced angiogenesis, tumor invasion, and establishment of metastatic foci at the secondary site involving various molecules. Western blotting revealed that anethole derived from Foeniculum vulgare Mill., attenuated the metastatic process in highly metastatic human HT-1080 fibrosarcoma cells. We found that anethole effectively inhibited the proliferation, adhesion and invasion of HT-1080 cells to extracellular matrix (ECM) at nontoxic concentrations, implying the potent antimeetastic activity of anethole.

Matrix metalloproteinases (MMPs) and zinc-dependent proteolytic enzymes play important roles in matrix degradation during the tumor growth, invasion, and tumor-induced angiogenesis. MMPs mediate invasion and metastasis through the degradation of the extracellular matrix and the basement membrane, and this process facilitates the tumor cells to invade surrounding tissues and enter the bloodstream to travel to distant sites. Several MMP inhibitors have been identified from natural sources such as resveratrol, theaflavins, catechins, curcumin and genistein. In this study, we showed that anethole inhibited the activity of MMP-2 and -9 and simultaneously increased the activity of TIMP-1 in HT-1080 cells. The data suggest that anethole possesses the antimeetastic activity and is a potent drug for anti-cancer treatment.

The serine protease uPA converts inactive plasminogen into plasmin and thereby plays an essential role to initiate proteolytic steps in the degradation of the ECM. uPA is found in cellular structure at the leading edge of migrating cells that are involved in adhesion, migration, invasion, in-
travasation and metastasis. Although our study demonstrated that uPAR gene expression was not affected by anethole, the amount of uPA transcripts was dramatically suppressed upon the same treatment, which may account, in part, for the anti-metastatic effect of this nature compound. Studies indicated that AKT, JNK1/2, ERK1/2, p38 MAPK and NF-kB pathways are involved in the regulation of the activities of MMP-2 and MMP-9 in various types of cells. Silibinin, isolated from Silybum marianum, inhibits cell invasion and MMP-2 expression through inactivation of phosphatidylinositol 3-kinase (PI3K)-AKT and MAPK signaling pathways. Evodiamine, an alkaloidal component extracted from the fruit of Evodiae fructus, abolished constitutive and inducible NF-kB activation, thereby suppressing AKT and MMP-9. Also, glucosamine inhibited the expression and synthesis of MMP-3 induced by IL-1β, NF-kB, AP-1, AKT, and p38 MAPK pathways. Our study revealed a similar result, in which anethole down-regulated the phosphorylation of AKT, ERK, p38 MAPK and suppressed the activation of NF-kB by blocking IκBα degradation and NF-kB activation. Cytosolic and nuclear extracts were fractionated and Western blotting was performed for IκBα and NF-kB p65 in cytosolic and nuclear extracts, respectively. (C) Effect of anethole on NF-kB-DNA binding activity. Nuclear extracts were prepared and analyzed for nuclear NF-kB levels by EMSA.

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**REFERENCES**