Novel Antitumor Invasive Actions of p-Cymene by Decreasing MMP-9/TIMP-1 Expression Ratio in Human Fibrosarcoma HT-1080 Cells

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p-Cymene (4-isopropyltoluene) has been reported to have beneficial actions such as anti-inflammatory and antinociceptive activities. To evaluate whether p-cymene exhibits antitumor invasive actions, we examined the effects of p-cymene on the production of matrix metalloproteinase 9 (MMP-9)/gelatinase B and tissue inhibitor of metalloproteinases-1 (TIMP-1) in human fibrosarcoma HT-1080 cells. p-Cymene was found to dose-dependently inhibit the 12-O-tetradecanoylphorbol 13-acetate (TPA)-augmented production and gene expression of MMP-9 in HT-1080 cells. In contrast, p-cymene enhanced the TPA-augmented production and gene expression of TIMP-1 in HT-1080 cells. However, there was no change in the constitutive level of MMP-9 and TIMP-1 mRNAs and TIMP-1 protein in p-cymene-treated cells. In addition, we found that the in-vitro TPA-augmented invasiveness of HT-1080 cells was inhibited by p-cymene in a dose-dependent manner. Furthermore, p-cymene was found to suppress the constitutive and/or TPA-augmented phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) in HT-1080 cells. Thus, these results provide novel evidence that p-cymene is an effective candidate for the prevention of tumor invasion and metastasis through mechanisms that include the inhibition of MMP-9 expression and the augmentation of TIMP-1 production along with the suppression of ERK1/2 and p38 MAPK signal pathways in tumor cells.

Key words p-cymene; tumor invasion; matrix metalloproteinase 9; tissue inhibitor of metalloproteinases-1; extracellular signal-regulated kinase 1/2 (ERK1/2); p38 mitogen-activated protein kinase (p38 MAPK)

The proteolytic degradation of extracellular matrix (ECM) components in basement membranes and stroma tissues is requisite for progressing the invasion and metastasis of malignant tumor cells. Matrix metalloproteinases (MMPs) play a crucial role in ECM degradation, and different sets of MMPs concertedly act in ECM breakdown.1,2 Gelatinase A (72-kDa type IV collagenase)/MMP-2 and gelatinase B (92-kDa type IV collagenase)/MMP-9 have been reported to facilitate tumor cell invasion and epithelial-mesenchymal transition as a promoter and/or modulator in normal and tumor cells in vivo and in vitro.3 On the other hand, the enzymic activity of MMPs and proteolytic activation of the MMP precursor (proMMP) have been reported to be controlled by endogenous inhibitors such as tissue inhibitors of metalloproteinases (TIMPs).4 Since four TIMPs have been identified, designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4, they inhibit MMPs by forming a strong noncovalent complex with a 1:1 stoichiometry.5 Regarding the inhibitory properties among the TIMPs, TIMPs can bind to different sets of MMPs: e.g., the preference inhibition of TIMP-1 against MMP-9, MMP-3, and MMP-1, and that of TIMP-2 against MMP-2 rather than MMP-9.4,5 Therefore, the inhibition of expression and/or enzymic activity of MMP is likely to be an effective target(s) for preventing tumor invasion and metastasis.1,2

Herbal medicine has been reported to exhibit various pharmacological effects, including the prevention of tumor progression by inhibiting cell proliferation and tumor promotion.6 Monoterpenes are the predominant chemical components of the essential oil derived from medicinal herbs and possess therapeutic properties: e.g., anti-inflammatory, antioxidative, analgesic, and antitumorogenic activities.7 p-Cymene (4-isopropyltoluene) (Fig. 1) is a natural monoterpenic hydrocarbon occurring in the essential oil from the resin of Protium species8 and the root of Angelica archangelica.9 In addition, it has been reported that the extract of Angelica archangelica inhibits the growth of mouse breast cancer Crl cells in vivo and in vitro.10 On the other hand, herbal medicine-derived terpenoids have been reported to exhibit not only anti-proliferative but also anti-invasive activity against tumor cells in vivo and in vitro.7 Several studies have shown that triptolide, a diterpenoid trioxide, exhibits the inhibitory activity against both the proliferation and invasion of tumor cells in vivo and in vitro.11-13 However, it remains unclear whether p-cymene

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Fig. 1. Chemical Structure of p-Cymene

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has an antitumor invasive activity.

In the present study, we investigated the effect of p-cymene on tumor invasiveness in vitro by monitoring the regulation of MMP and TIMP expression and the phosphorylation of extracellular signal receptor-activated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) in human fibrosarcoma HT-1080 cells. p-Cymene inhibited the production of proMMP-9, but not proMMP-2, and augmented TIMP-1 production in 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated HT-1080 cells. We further demonstrated the possible mechanisms by which the p-cymene-mediated inhibition of proMMP-9 expression was due to the inhibition of constitutive and TPA-augmented phosphorylation of ERK1/2 and p38 MAPK in HT-1080 cells. These results suggest that p-cymene is an attractive candidate with antitumor invasive activity due to the decrease of MMP-9/TIMP-1 expression ratio by inhibiting the ERK1/2 and p38 MAPK signal pathways.

MATERIALS AND METHODS

Cell Culture and Treatment Human fibrosarcoma HT-1080 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Eagle's minimum essential medium (MEM) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (JRH Bioscience, Tokyo, Japan) and nonessential amino acids (Invitrogen). After reaching confluence, the cells were treated with p-cymene (ZeLang Medical Technology Co., NanJing, China) in MEM/0.2% (w/v) lactalbumin hydrocymene (ZeLang Medical Technology Co., NanJing, China) and nonessential amino acids (Invitrogen). The gel was washed with a washing buffer [50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM CaCl2/1% (v/v) Triton X-100] to remove SDS and then incubated at 37°C in an incubation buffer [50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM CaCl2/1% (v/v) ZnCl2/0.1% (v/v) Triton X-100] to remove SDS and then incubated at 37°C in an incubation buffer [50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM CaCl2/1% (v/v) ZnCl2]. Thereafter, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.), and gelatinolytic activity was detected as unstained bands on a blue background.

Western Blot Analysis The harvested culture medium (1 mL) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% (w/v) acrylamide gel containing gelatin (0.6 mg/mL) (Becton Dickinson, Tokyo, Japan). The gel was washed with a washing buffer [50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM CaCl2/1% (v/v) ZnCl2] to remove SDS and then incubated at 37°C in an incubation buffer [50 mM Tris–HCl (pH 7.5)/0.15 M NaCl/10 mM CaCl2/1% (v/v) ZnCl2]. Thereafter, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co.), and gelatinolytic activity was detected as unstained bands on a blue background.

Statistical Analysis All data are expressed as the mean ± standard deviation (S.D.) from at least three independent experiments. Data were analyzed by Student’s t-test; p<0.05 was considered to indicate a statistically significant difference.

RESULTS

Inhibition of TPA-Augmented MMP-9 Expression by p-Cymene in HT1080 Cells Since MMP-9 has been reported to participate in the invasion of malignant tumor cells, which are activated by tumor promotive reagents such as TPA, 14)
we first examined the effects of \( p \)-cymene on the expression of MMP-9 in TPA-stimulated HT-1080 cells. TPA was found to increase the production of proMMP-9 (10.8±0.6 fold in mRNA level vs. untreated cells, \( p<0.001 \)), while the constitutive expression of proMMP-9 was negligible in HT-1080 cells (Figs. 2A, B). In addition, the suppression of TPA-induced proMMP-9 production by \( p \)-cymene was found to be due to the inhibition of MMP-9 mRNA expression (38.4% inhibition at 600 \( \mu \)M vs. TPA-treated cells, \( p<0.05 \)) (Fig. 2B). Furthermore, the constitutive gene expression of MMP-9 was not influenced in \( p \)-cymene-treated HT-1080 cells (Fig. 2B). On the other hand, we have previously reported that HT-1080 cells constitutively express proMMP-2, and the proMMP-2 activation which is facilitated by TPA. \(^{14} \) As shown in Fig. 2A (upper panel), \( p \)-cymene did not alter the expression and activation of proMMP-2 in HT-1080 cells treated with or without TPA. Thus, these results suggest for the first time that \( p \)-cymene transcriptionally suppresses the production of proMMP-9 in TPA-stimulated HT-1080 cells.

**\( p \)-Cymene Increases the Production and Gene Expression of TIMP-1 in TPA-Stimulated HT1080 Cells** Since the enzymic activity of MMP has been reported to be controlled by not only their expression level but also TIMPs \(^{4,16,17} \) we next examined the effects of \( p \)-cymene on the expression of TIMP-1 in TPA-stimulated HT-1080 cells. Figure 3A shows that \( p \)-cymene dose-dependently enhanced the TPA-augmented production of TIMP-1 but did not influence the constitutive level of TIMP-1 in

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**Fig. 2.** Suppression of MMP-9 Expression by \( p \)-Cymene in TPA-Stimulated HT1080 Cells

HT-1080 cells were treated for 24h with or without \( p \)-cymene (200–600 \( \mu \)M) in the presence or absence of TPA (10 nM). A: The harvested culture medium was subjected to gelatin zymography for proMMP-2 (upper panel) and proMMP-9 (lower panel) as described in Materials and Methods. iMMP-2, an intermediate form of MMP-2, aMMP-2, an active form of MMP-2. B: Isolated RNA (an equivalent of 2.5 ng of total RNA) from the cells was subjected to the analysis of MMP-9 mRNA expression as described in Materials and Methods. Data are shown as the mean±S.D. of three different experiments. *** Significantly different from untreated cells (Cont) (\( p<0.001 \)). * Significantly different from TPA (10 nM)-treated cells (\( p<0.05 \)).

**Fig. 3.** Enhancement of TIMP-1 Expression by \( p \)-Cymene in TPA-Stimulated HT1080 Cells

The harvested culture medium (A) and RNA (B) from the cells treated with or without \( p \)-cymene (200–600 \( \mu \)M) and TPA (10 nM) were subjected to Western blotting and quantitative RT-PCR, respectively, for TIMP-1 as shown in Fig. 2. Data are shown as the mean±S.D. of three different experiments. ** Significantly different from untreated cells (Cont) (\( p<0.01 \)). * Significantly different from TPA (10 nM)-treated cells (\( p<0.05 \)).
 HT-1080 cells. In addition, the gene expression of TIMP-1 was further increased by co-treatment of TPA and p-cymene (1.3-fold increase at 600 µM vs. TPA-treated cells, \( p < 0.05 \)), while p-cymene alone did not influence TIMP-1 mRNA expression (Fig. 3B). Furthermore, we confirmed that there was no change in cell viability in HT-1080 cells treated with up to 800 µM of p-cymene, while an antitumor agent, doxorubicin, dose-dependently decreased the cell viability (Fig. 4). Therefore, these results suggest that, unlike in the case of MMP-9, p-cymene transcriptionally facilitates the production of TIMP-1 in TPA-stimulated HT-1080 cells.

**p-Cymene Inhibits an in-Vitro Invasive Activity of HT-1080 Cells** Since it has been reported that HT-1080 cells exhibit invasive activity due to the deregulation of the MMP/TIMP equilibrium in vitro and in vivo,\(^ {14,18} \) we next examined the effect of p-cymene on the invasiveness of HT-1080 cells using Matrigel-coated insert chambers as previously reported.\(^ {14} \) As shown in Fig. 5, TPA was found to increase the invasive activity of HT-1080 cells (2-fold vs. untreated cells). The TPA-augmented invasiveness was dose-dependently inhibited by p-cymene (87% inhibition at 600 µM). In addition, p-cymene was found to decrease the invasive activity of HT-1080 at basal level (87% inhibition at 600 µM). Taken together with our previous report that not only exogenous administration of TIMPs but also the relative augmented protein level of TIMP-1 against MMP-9 suppresses the invasiveness of HT-1080 cells,\(^ {18} \) it is suggested that p-cymene exerts an anti-invasive action by a mechanism in which at least the proteolytic activity of MMP may be inhibited in HT-1080 cells.

**Inhibition of ERK1/2 and p38 MAPK Phosphorylation by p-Cymene in HT1080 Cells** As the intracellular signal pathways of ERK1/2 and p38 MAPK have been reported to participate in the regulation of MMP-9 and TIMP-1 expression in various cell species,\(^ {19,20} \) we examined whether p-cymene may modulate the phosphorylation of ERK1/2 and p38 MAPK in TPA-stimulated HT-1080 cells. Figures 6A and F show that the TPA-augmented ERK1/2 phosphorylation (2.3±0.1 fold vs. untreated cells, \( p < 0.001 \)) was inhibited by p-cymene (33.6 and 49.7% inhibition at 400 and 600 µM, respectively, vs. TPA-treated cells, \( p < 0.001 \)). In addition, the TPA-augmented p38 MAPK phosphorylation (1.6±0.1 fold vs. untreated cells, \( p < 0.001 \)) was suppressed by p-cymene in a dose-dependent manner (29.6 and 60.1% inhibition at 400 and 600 µM, respectively, vs. TPA-treated cells, \( p < 0.001 \)) (Figs. 6C, G). Furthermore, the basal levels of phosphorylated ERK1/2 and p38 were significantly decreased in p-cymene at 600 µM (74.1 and 80.9% inhibition, respectively, vs. untreated cells, \( p < 0.001 \)). However, there was no change in protein levels of ERK1/2, p38, and β-actin in p-cymene and/or TPA treated HT-1080 cells (Figs. 6B, D, E). Thus, these results suggest that p-cymene possesses dual inhibitory actions to ERK1/2 and p38 MAPK phosphorylation in HT-1080 cells.

**DISCUSSION**

Monoterpenes have been identified as the major constituents of the essential oil from medicinal herbs, which are widely used in traditional Chinese medicine.\(^ {7} \) For instance, aucubin, a monoterpenoid based compound, has been reported to inhibit the proliferation of human non-small cell lung cancer A549 cells through the augmentation of p53 expression and the activation of the Fas ligand-mediated apoptosis pathway.\(^ {21} \) In addition, it has been reported that \( d \)-limonene has antiangiogenic and proapoptotic effects on human gastric cancer BGC-823 cells implanted into nude mice, which thereby inhibits tumor metastasis in vivo.\(^ {22} \) Since p-cymene has been reported to be a natural monoterpen in the essential oil of the resin of \textit{Protium} species\(^ {9} \) and the root of \textit{Angelica archangelica},\(^ {20} \) it remains unclear whether p-cymene exhibits anti-tumor invasive activity or not. In the present study, we found that p-cymene transcriptionally suppresses the production of proMMP-9 in TPA-stimulated HT-1080 cells. Furthermore, p-cymene is found to inhibit the TPA-enhanced invasiveness of HT-1080 cells. Taken together with previous reports that the augmented expression of MMP-9 is implicated in the

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**Fig. 4. No Effect of p-Cymene on Cell Viability in HT-1080 Cells**

HT1080 cells (1×10⁴ cells/well) in a 96-well multplate were treated for 24 h with p-cymene (0–800 µM) or doxorubicin (1–4 µM), and then cell viability was measured using a CellTiter-Blue\(^ {23} \) reagent as described in Materials and Methods. Data are shown as the mean±S.D. of six individual wells. ***Significantly different from untreated cells (Cont) \( p < 0.001 \).

**Fig. 5. p-Cymene Suppresses the Invasiveness of HT-1080 Cells in Vitro**

HT-1080 cells were treated with p-cymene (200–600 µM) in the presence or absence of TPA (10 nM) in the Matrigel invasion models and the invasive activity is analyzed as described in Materials and Methods. Data are shown as the mean±S.D. of three individual chambers by taking untreated cells (Cont) as 1. ** and ***Significantly different from untreated cells (Cont) \( p < 0.01 \) and 0.001, respectively). ###Significantly different from TPA (10 nM)-treated cells \( p < 0.001 \).
metastatic phenotype of many cancers in vivo and in vitro,\textsuperscript{23,24} $p$-cymene is likely to be useful for the prevention of tumor invasion and metastasis.

The enzymic activity of MMP is controlled by TIMPs, but the affinities of TIMPs vary for different MMP-TIMP pairs.\textsuperscript{4} Since TIMP-1 has been reported to prefer to inhibit MMP-9 rather than MMP-2,\textsuperscript{4,5} the augmentation of TIMP-1 expression has resulted in the inhibition of tumor metastasis by decreasing the overall MMP-9 activity in patients with prostate cancer.\textsuperscript{17} In contrast, the deregulation of the MMP-9/TIMP-1 equilibrium has facilitated the invasion of human MDA-MB-231 breast cancer cells.\textsuperscript{25} In the present study, we demonstrated that $p$-cymene transcriptionally augments the production of TIMP-1 in TPA-stimulated HT-1080 cells, under which the invasiveness of HT-1080 cells was attenuated. Thus, these results suggest a possible mechanism in that the antitumor invasive activity of $p$-cymene includes not only the down-regulation of MMP-9 production but also the up-regulation of TIMP-1 production.

It has been reported that different transcriptional factors and signal mediators divergently and complicatedly participate in the regulation of MMP-9 and TIMP-1 gene expression.\textsuperscript{26} As the regulation of MMP-9 expression has been dependent on activator protein-1 (AP-1) and/or nuclear factor-kappa B (NF-$\kappa$B) signal,\textsuperscript{27,28} we demonstrated that MMP-9 expression was augmented by TPA, which activates both signaling pathways.\textsuperscript{29} In addition, it has been reported that the activation of the ERK1/2 and/or p38 MAPK pathways is involved in the up-regulation of MMP-9 expression in some tumor cell lines such as human fibrosarcoma, oral squamous cell carcinoma, and cholangiocarcinoma.\textsuperscript{30,31} We demonstrated that $p$-cymene suppressed the phosphorylation of ERK1/2 and p38 MAPK in TPA-treated HT-1080 cells, suggesting that the concomitant suppression of the ERK1/2 and p38 MAPK signal pathways by $p$-cymene at least partially results in the down-regulation of MMP-9 production. Moreover, as monoterpenoids including $p$-cymene have been reported to potentially inhibit the NF-$\kappa$B signal pathway,\textsuperscript{7,32} the inhibition of NF-$\kappa$B may be associated with the $p$-cymene-decreased expression of MMP-9 in TPA-stimulated HT-1080 cells. Further experiments are needed to clarify this hypothesis.

Regarding the regulation of TIMP-1 expression, Zhang et al.
al. 31) have reported the augmentation of TIMP-1 production by inhibiting p38 MAPK phosphorylation by baicalein, a flavonoid from Scutellaria baicalensis, in human glioma U87MG cells, under which the production of proMMP-9 is suppressed. In contrast, Fields et al. 32) have reported that a p38 selective small molecular inhibitor, SB203580, decreases the gene expression of TIMP-1 in IL-1β-treated human astrocytes. On the other hand, we previously reported that the TPA-augmented TIMP-1 production is not suppressed by another selective p38 MAPK inhibitor, SB202190, but enhanced that of TIMP-1 by a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY-294002, in HT-1080 cells. 33) Thus, it is suggested that the involvement of p38 MAPK signaling in the regulation of TIMP-1 expression is dependent on cell species and/or experimental conditions. Moreover, a PI3K signaling pathway may be associated with the p-cymene-enhancement of TIMP-1 expression in TPA-stimulated HT-1080 cells.

In conclusion, we demonstrated for the first time that p-cymene transcriptionally suppressed the expression of MMP-9 but enhanced that of TIMP-1 in TPA-stimulated HT-1080 cells, which causes the inhibition of in-vitro invasiveness of HT-1080 cells. Furthermore, p-cymene interfered the TPA-augmented phosphorylation of ERK1/2 and p38 MAPK. Thus, these results provide novel evidence that p-cymene is an attractive candidate that exerts an antitumor invasive action by decreasing the MMP-9/TIMP-1 expression ratio due to the inhibition of the ERK1/2 and p38 MAPK signal pathways.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

23) Liu Z, Li L, Yang Z, Luo W, Li X, Yang H, Yao K, Wu B, Fang W. Increased expression of MMP9 is correlated with poor prognosis of...


