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European Journal of Pharmacology 567 (2007) 193–197
www.elsevier.com/locate/ejphar

Short communication

1-furan-2-yl-3-pyridin-2-yl-propenone inhibits the invasion and migration of HT1080 human fibrosarcoma cells through the inhibition of proMMP-2 activation and down regulation of MMP-9 and MT1-MMP

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Received 26 December 2006; received in revised form 28 March 2007; accepted 1 April 2007
Available online 21 April 2007

Abstract

Matrix metalloproteinases (MMPs) play important roles in solid tumor invasion and migration. In this study, we showed that 1-furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) dose-dependently inhibited HT1080 cell invasion and migration, and decreased MMP-2 and MMP-9 activities. Furthermore, FPP-3 reduced MMP-2 expression at protein and mRNA levels, and suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-enhanced expression of MT1-MMP without changing tissue inhibitors of metalloproteinase (TIMP)-2 level. FPP-3 also suppressed TPA-induced increases in MMP-9 protein and mRNA levels, but did not alter TIMP-1 level. Our results suggest that FFP-3 may be a valuable anti-invasive drug candidate for cancer therapy by suppressing MMP-2, MMP-9, and MT1-MMP.

Keywords: 1-furan-2-yl-3-pyridin-2-yl-propenone; MMP-2; MMP-9; MT1-MMP; TIMP

1. Introduction

The principal mechanisms involved in cancer mortality are migration and invasion, where primary tumor cells disseminate and grow at a distant site resulting in a secondary tumor. Most failures in cancer treatment arise as a result of these processes.

Matrix metalloproteinases (MMPs) are principal enzymes in extracellular matrix degradation, which is essential in the invasive growth and metastasis of cancer (McCawley and Matrisian, 2000). Among the MMPs, MMP-2 and MMP-9 are considered to play critical roles in tumor invasion and metastasis (John and Tuszynski, 2001; Mook et al., 2004). However, since they are produced as latent proenzymes, tumor invasiveness is not necessarily linked with their elevated expression, but with their activity increase. Both of these MMPs are gelatinases, but regulation of their gene expressions and activations are quite different. The promoter of the MMP-2 gene does not contain a 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) sequence, whereas MMP-9 does (Templeton and Stetler-Stevenson, 1991). Activation of MMP-2 occurs on the cell membrane via the membrane-type MMP (MT1-MMP) by forming a trimolecular complex with TIMP-2 (Bernardo and Fridman, 2003). However, in activation of MMP-2, tissue inhibitors of metalloproteinase (TIMP)-2 plays a dual role (Zucker et al., 1998). Low levels of TIMP-2 are related to MT1-MMP-mediated activation of MMP-2, but high TIMP-2 levels directly inhibit MT1-MMP-mediated MMP-2 activation. For MMP-9, activity is primarily regulated based on the balance between proenzyme activation and inhibition by TIMP-1.

A synthetic compound, 1-furan-2-yl-3-pyridin-2-yl-propenone (FPP-3), a dual cyclooxygenase/5-lipoxygenase inhibitor, has shown anti-inflammatory activities in cultured murine macrophages (Jahng et al., 2004) and *in vivo* animal models (Lee et al., 2006). It has also been shown to have inhibitory activity on nuclear factor (NF)-κB activation (Lee et al., 2004). NF-κB is a key player in tumorigenesis, and inhibitors of NF-κB activation have been shown to suppress MMP-2 and MMP-9 and further tumor invasion (Alaniz et al., 2004; Lee et al., 2005; Park

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doi:10.1016/j.ejphar.2007.04.014

et al., 2007). Nonetheless, the possible use of FPP-3 as an anti-invasive agent has not been studied. Thus, we investigated the effects of FPP-3 on cancer cell invasion and migration in relation to the activation and expression of MMP-2 and MMP-9 using HT1080 fibrosarcoma cells.

2. Materials and methods

2.1. Cell culture and treatment

Human fibrosarcoma HT1080 cells from ATCC (American Type Culture Collection, Manassas, VA) were grown in a minimum essential medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 200 IU/ml penicillin (Gibco, Grand Island, NY, USA), and 200 μg/ml streptomycin (Gibco). Cells were treated with different concentrations of FPP-3 in the absence or presence of TPA (12 ng/ml) for 24 h. FPP-3 was synthesized according to the previous report (Jahng et al., 2004), and was dissolved in dimethyl sulfoxide.

2.2. In vitro invasion assay

An *in vitro* invasion assay was performed using a 24-well transwell unit (8 μm pore size) with polycarbonate filters (Corning Costar, Cambridge, MA) by methods previously described (Kim et al., 2003). The upper and lower sides of the transwell filter were coated with 20 μL of Matrigel (1.5 mg/ml) (BD Biosciences, Bedford, MA) and type I collagen (0.5 mg/ml), respectively. The lower compartment was filled with medium containing 10% FBS. Cells were placed in the upper part of the transwell plate and incubated with FPP-3 for 18 h at 37 °C. The cells that invaded to the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. We determined invasive phenotypes by counting the cells that migrated to the lower side of the filter using microscopy at ×200. Three fields were counted for each filter.

2.3. Wound migration assay

A cell migration assay was performed using a 6-well plate as previously described (Kim et al., 2003). Cells were pretreated with mitomycin C (25 μg/ml) for 30 min before an injury line was made with a 1 mm width tip on cells that were plated in culture dishes at 90% confluency. After being rinsed with HBSS the cells were allowed to migrate in complete medium in the presence of FPP-3, and photographs were taken (×100) in series at different time points.

2.4. Zymography

The enzymatic activities of MMP-2 and MMP-9 were assayed by gelatin zymography (Herron et al., 1986) in the absence of serum. Supernatants from FPP-3-treated cultures were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin. The gel was washed twice with washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100, and then incubated with incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3) at 37 °C. After incubation, the gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO), and then destained. MMP activity was represented by a clear zone of gelatin digestion.

2.5. Western blot analysis

HT1080 cells treated with TPA (12 ng/ml) and different concentrations of FPP-3 for 24 h were lysed in freshly prepared extraction buffer [10 mM Tris-HCl (pH 7.6), 0.1% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin (Sigma-Aldrich, MO, USA)] for 30 min at 4 °C. The lysates were centrifuged at 20,000 g for 10 min at 4 °C. The supernatant samples were then separated by 10% SDS-PAGE and transferred to Hybond ECL (Amersham Life Science, Buckinghamshire, England). The membranes were blocked with 5% skimmed milk in Tween-20 containing Tris buffered saline (TTBS) [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], and then incubated with primary anti-human MMP-2 (Cell Signaling Technology, Beverly, MA) and horseradish peroxidase-conjugated anti-IgG antibody (Santa Cruz Biotechnology, CA). Peroxidase was detected by an enhanced chemiluminescence assay kit (Amersham Biosciences, Buckinghamshire, England). The protein band densities were measured using an Image Analyzing System (UVP, Upland, USA).

2.6. MMP-9 ELISA assay

The extracellular MMP-9 was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Oncogene Research Products) according to the manufacturer's protocol (specificity: human pro-MMP-9 protein and MMP-9/TIMP-1 complex). Three independent experiments with duplicate were performed.

2.7. RT-PCR (mRNA analysis)

The total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using a Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, USA). PCR was performed in the presence of 0.5 U Taq DNA polymerase (Takara, Japan) using primer sets that were specific to the following: human MMP-2 (5′-GTG CTG AAG GAC ACA CTA AAG AAG A-3′, 3′-GGA TGT TGA AAC TCT TCC TAC CGT T-5′); MMP-9 (5′-CAC TGT CCA CCC CTC AGA GC-3′, 3′-GGA ATA GCG GCT GTT CAC CG-5′); TIMP-1 (5′-TGC ACC TGT GTC CCA CCC CAC CCA CAG ACG-3′, 3′-TGG ACC GTC AGG GAC GCC AGG GTC TAT CGG-5′); TIMP-2 (5′-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3′, 3′-GAG CTG TAG CTC CTG GGT ATT TTC GAA GG-5′); MT1-MMP (5′-CGC TAC GCC ATC CAG GGT CTC AAA-3′, 3′-AAA ACA CGA CGG GCT ACT

ACT GGC-5′); and GAPDH (5′-GGT GAA GGT CGG AGT CAA CG-3′, 3′-CCA GTA GGT ACT GTT GAA AC-5′). The PCR products were separated on a 2% agarose gel containing ethidium bromide (0.5 μg/ml), visualized, and photographed using a gel documentation system (UVP, Cambridge, UK).

2.8. Statistical analysis

The data are expressed as means ± S.E.M. and were analyzed using one-way analysis of variance (ANOVA) and the Student–Newman–Keul's test for individual comparisons. P values of <0.05 were considered statistically significant.

3. Results

3.1. Effects of FPP-3 on the invasion and migration of HT1080 cells in vitro

To investigate whether FPP-3 inhibits tumor invasion and migration, Matrigel invasion and wound migration assays were

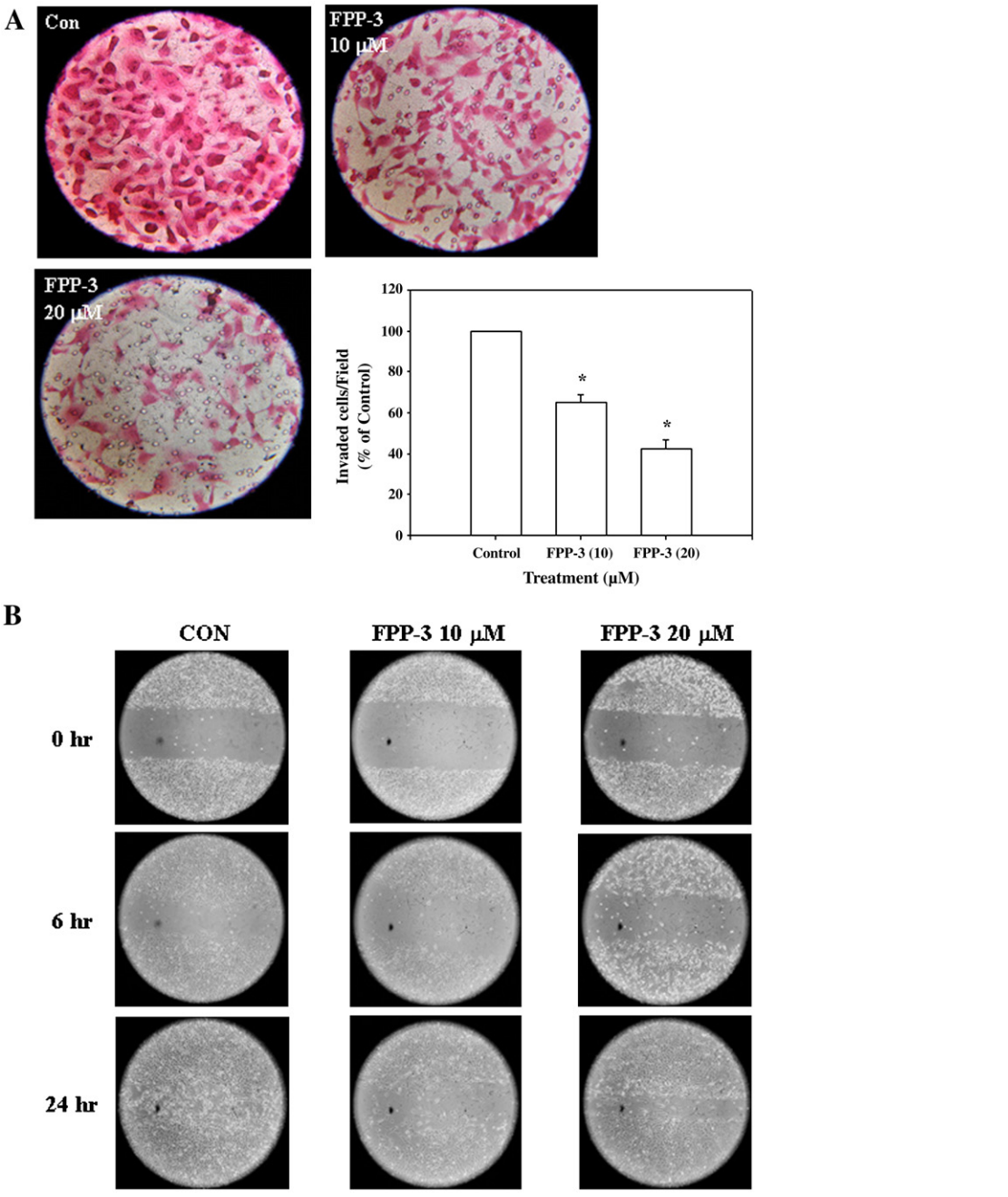

Fig. 1. Effects of FPP-3 on invasion and migration of HT1080 cells. (A) HT1080 cells were treated with FPP-3 for 24 h in a Matrigel-coated transwell. After incubation, the cells were fixed, stained, and photographed using a digital camera attached to a phase contrast microscope at ×200 magnification. The bar graphs represent the relative number of invaded cells. *$P<0.05$ compared to the vehicle-treated control group. (B) Confluent cultures of HT1080 cells were wounded with a tip, and incubated with FPP-3 in the presence of mitomycin C (25 μg/ml).

performed in FPP-3-treated HT1080 cells. As shown in Fig. 1A, serum-induced invasion of the cells was reduced by the FPP-3 treatment in a concentration-dependent manner. In addition, FPP-3 suppressed the migration of HT1080 cells across the wounded space (Fig. 1B).

3.2. Inhibitory effects of FPP-3 on the activities and expressions of MMP-2, MMP-9, and MT1-MMP

As shown in Fig. 2A, the gelatinolytic activities of both MMP-2 and MMP-9 were decreased by FPP-3. Enhanced activation of MMP-2 and MMP-9 by the treatment with TPA, which is known to enhance MMP production through the activation of transcription factors such as NF-κB (Lee et al., 2005; Park et al., 2007), was significantly suppressed by pretreatment with FPP-3 (Fig. 2B). Next, we measured the expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and MT1-MMP to examine whether reduced MMP activities were due to changes in their protein levels or in the levels of other regulatory molecules involved in the activation process. The expressions of MMP-2 at protein (Fig. 2D) and mRNA (Fig. 2E) levels were not changed much by TPA, but both expressions were significantly decreased by FPP-3. Moreover, FPP-3 significantly reduced TPA-enhanced MT1-MMP expression, an important regulator of MMP-2 activation, while the expression of TIMP-2, another regulator of MMP-2 activation, was not changed by either TPA or FPP-3 (Fig. 2E). In addition, FPP-3 suppressed TPA-induced increases in MMP-9 protein (Fig. 2C) and mRNA (Fig. 2E) levels, but did not alter TIMP-1 level, which forms a specific complex with pro-MMP-9 and thus inhibits activation of pro-MMP-9.

4. Discussion

In this study we demonstrated that FPP-3 significantly suppressed cancer cell invasion, migration, and MMP-2 and MMP-9 activities, critical factors in tumor invasion and metastasis. FPP-3 at the tested concentrations did not significantly affect the cell viability except only 20 μM concentration showing 75% cell viability (Data not shown).

Unlike secretory MMP, MT1-MMP is expressed on the cell surface in an active form (Sato et al., 1994). It has been reported that TPA is a potent inducer of MT1-MMP (Lohi et al., 1996), and that enhanced MT1-MMP production correlates with pro-MMP-2 activation (Sato et al., 1994). For pro-MMP-2 activation to occur, the balance between MT1-MMP and TIMP-2 is important, meaning low levels of TIMP-2 are related to MT1-MMP-mediated activation of MMP-2, but high TIMP-2 levels directly inhibit MT1-MMP-mediated MMP-2 activation. Here, FPP-3 significantly reduced TPA-stimulated MT1-MMP transcription (Fig. 2E). However, neither TPA nor FPP-3 exerted its action on the transcriptional regulation of TIMP-2 (Fig. 2E). Thus, suppression of TPA-stimulated MT1-MMP expression by FPP-3 seems to be a major factor leading to MMP-2 activity reduction.

MT1-MMP, in addition to its role in activating pro-MMP-2, acts as an extracellular matrix degrading enzyme (Imai et al., 1996). It has been reported that MT1-MMP expression is elevated in various types of human cancers, and that MMP-2 activation and the co-expression of MT1-MMP and MMP-2, increases tumor aggressiveness (Gilles et al., 1996; Tokuraku et al., 1995). The inhibitory effect of FPP-3 on MT1-MMP expression further emphasizes FPP-3's potential as an inhibitor of cancer invasion and metastasis.

TPA also stimulated MMP-9 mRNA expression and secretion, which was significantly suppressed by FPP-3. However, the mRNA level of TIMP-1, which binds and inhibits pro-MMP-9 activation, was not greatly altered by TPA or FPP-3. Although we

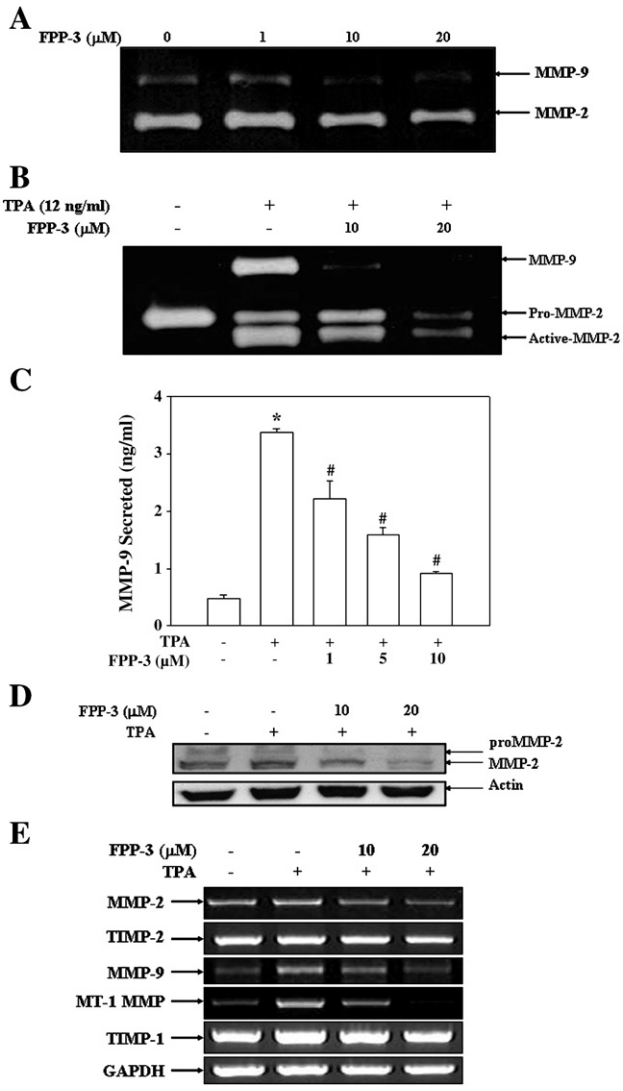

Fig. 2. Inhibitory effects of FPP-3 on activities and expressions of MMP-2, MMP-9, and MT1-MMP. HT1080 cells were treated with FPP-3 for 24 h in the absence and presence of TPA. Conditioned media were collected and analyzed for the gelatinolytic activities (A and B) by zymography, and for the secreted amount of MMP-9 (C) by ELISA method. Total amount of MMP-9 was normalized by total number of viable cells, determined using a MTT method. *$P<0.01$ compared to the vehicle-treated control group. #$P<0.01$ compared to the TPA-treated group. (D) Total cellular proteins were immunoblotted with MMP-2 antibody. (E) Analysis of MMP-2, TIMP-2, MMP-9, MT1-MMP, and TIMP-1 mRNA expression in HT1080 cells treated with FPP-3 using RT-PCR. The sizes of RT-PCR products for MMP-2, TIMP-2, MMP-9, MT1-MMP, TIMP-1, and GAPDH are 605 bp, 590 bp, 243 bp, 497 bp, 551 bp, and 496 bp, respectively. The data presented in (A) through (E) were the representative of three independent experiments.

did not measure TIMP-1 protein level, the results indicate that decreased MMP-9 activity by FPP-3 may be due to the suppression of MMP-9 expression without any influence by TIMP-1. It has been reported that TPA-induced activation and upregulation of MMP-2 and MMP-9 is mediated through NF-kB activation (Lee et al., 2005; Park et al., 2007), and the promoter region of the MT1-MMP contains NF-kB binding sites (Han et al., 2001). Since FPP-3 has an inhibitory activity on NF-kB activation (Lee et al., 2004), the suppressive action of FPP-3 on the TPA-induced expressions of MMP-2, MMP-9, and MT1-MMP is believed to be mediated through suppressions on NF-kB activation.

In conclusion, our data strongly imply that FPP-3 inhibits tumor invasion and migration, which may be mediated by suppressing MMP-2, MMP-9, and MT1-MMP expression.

Acknowledgments

This work was supported by a grant No. RTI04-01-04 from the Regional Technology Innovation Program of the Ministry of Commerce, Industry, and Energy (MOCIE).

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