4-Methoxyphenyl (E)-3-(Furan-3-yl) Acrylate Inhibits Vascular Smooth Muscle Cell Proliferation

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Abstract: The *Cordyceps* extract exhibits antiproliferative potential in vascular smooth muscle cells (SMCs) through the mitogen-activated protein kinase signaling pathway. In this study, we aimed to identify the active compounds in the *Cordyceps* extract and analyze their role in remodeling the arterial wall. On investigation, we discovered the following active compound: 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate and synthesized it. We performed antiproliferation and antimigration assays in addition to an in vivo vessel wall remodeling experiment. Investigation of the mechanism adopted by the active compound to remodel the vessel was performed. The newly synthesized compound inhibited the proliferation and migration of SMCs. Treatment with the synthesized compound reduced neointima formation in the balloon-injured Sprague–Dawley rat model. In addition, this compound inhibited the activation of matrix metalloproteinase-2 and matrix metalloproteinase-9 in type I collagen-activated SMCs. Moreover, this compound suppressed the expression of cycloxygenase-2 (COX-2) in SMCs. Therefore, this compound can exert potential antiarteriosclerotic effects by modulating vessel wall remodeling. In conclusion, the newly synthesized 4-methoxyphenyl (*E*)-3-(furan-3-yl) acrylate might be an alternative therapeutic intervention for the treatment of atherosclerosis.

Key Words: 4-methoxyphenyl (*E*)-3-(furan-3-yl) acrylate, *Cordyceps*, proliferation, vascular smooth muscle cells

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INTRODUCTION

Atherosclerosis is a disease characterized by the narrowing and hardening of arterial intima. Fat, cholesterol, calcium, cellular waste products, and fibrin are the main causes for the plaque buildup inside the arteries. The major sources of plaque are vascular smooth muscle cells (SMCs) and the extracellular matrix (ECM), and SMCs contribute to numerous processes in atherosclerosis.[1] In addition, it has been reported that there is a close relationship between restenosis and SMC proliferation in the intimal layer.[2]

It is well known that *Cordyceps* are ethnopharmacologically valuable mushrooms and widely used as traditional medicine or tonics in Asian countries. *Cordyceps* display potent anticancer, anti-inflammatory, antiviral, antioxidative, antidiabetic, antinociceptive, and antiobesity pharmacological properties.[3,4] Several pharmaceutical effects of the active components of the extract or KTH-13 [4-isopropyl-2,6-bis(1-phenylethyl) phenol] derived from artificially cultivated *Cordyceps* have been reported.[5–7]

In this study, we extracted 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate from the *Cordyceps* extract and synthesized it. In addition, we investigated the activity of this compound in vessel remodeling and its underlying mechanism. Pharmaceutical activities of 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate might highlight a potential therapeutic intervention for atherosclerosis.

MATERIALS AND METHODS

Preparation of Compounds 1, 2, and 3 and Analysis of Structure

To a solution of (*E*)-3-(3-furyl) prop-2-enoic acid (1, 100 mg, 0.724 mmol), 4-methoxyphenol (2, 180 mg, 1.45 mmol) and dimethylamino pyridine (106 mg, 0.869 mmol) in CH_2Cl_2 (0.7 mL) were added to dicyclohexyl carbodiimide (224 mg, 1.09 mmol) at room temperature. The mixture was allowed to reflux and was stirred overnight. The resulting solution was allowed to cool to room temperature and filtered through a short pad of silica gel while washing with Et_2O. The filtrate was concentrated in vacuo, and the resulting crude product was purified using silica gel column chromatography to obtain the desired 4-methoxyphenyl (E)-3-(furan-3-yl)acrylate (3, 140 mg, 0.573 mmol, 79%) as a white solid (Fig. 1). This compound has been previously reported and spectra data match described.[8] ^{1}H NMR spectra were recorded on a JEOL JNM-AL400 (400 MHz) spectrometer. Chemical shifts are reported in parts per million from tetramethylsilane, with solvent resonance as the internal standard ($CDCl_3$: δ 7.27 ppm) (see **Figure S1**, **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A459). Data are reported as follows: chemical shift, multiplicity (s =

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singlet, d = doublet, t = triplet, q = quartet, quint = quintet, and m = multiplet), coupling constants (Hz), and integration. ^{13}C NMR spectra were recorded on a JEOL JNM-AL400 (100 MHz) spectrometer with complete proton decoupling. Chemical shifts are reported in parts per million from tetramethylsilane, with solvent resonance as the internal standard ($CDCl_3$: δ 77.00 ppm) (see Figure S2, **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A459).

Cell Culture

Rat aortic SMCs were purchased from Lonza (Walkersville, MD). SMCs were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) in a 37°C incubator at 5% CO_2.

Cell Proliferation Assay

SMCs were plated in 96-well plates at 3×10^3 cells/well and starved with 0.1% FBS for 24 hours and then treated with various concentrations of the compound for 72 hours. After treatment, cell proliferation was examined using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Japan). CCK-8 allows sensitive colorimetric assays for the determination of cell viability in cytotoxicity or cell proliferation assays. The CCK-8 solution (10 μL) was treated to each well and incubated for 2 hours at 37°C. The absorbance was examined at 450 nm using a microplate reader.

Neointima Formation Study

The animal experiment was approved by the Catholic Kwandong University animal care committee. Balloon denudation of the carotid artery endothelium was evoked in 4-week-old male Sprague–Dawley rats (Daehan Biolink, Chungbuk, Korea) as described in previous reports [5, 8]. In brief, rats were anesthetized with ketamine (10 mg/kg) and xylazine (5 mg/kg). A 2F Fogarty balloon catheter (Edwards Lifesciences, Mississauga, ON) was inserted into the external carotid branch to the aortic arch [5, 8]. Then, the catheter was insufflated to produce slight resistance and withdrawn 3 times. Sham control was induced by the same operation without balloon insertion. Vehicle (saline) or 0.05 mL of the compound (20 μM) was injected daily intraperitoneally for 3 weeks after the balloon injury. We tested the toxicity of the compound for concentration and dose in rats. We did not find any signs of toxicity or change in body weight in rats for 4 weeks.

Hematoxylin and Eosin Staining

Three weeks after the balloon injury, carotid arteries of the Sprague–Dawley rats were harvested and fixed with 4% paraformaldehyde. Within 12 hours after fixation, tissues were embedded in the optimal cutting temperature compound (Sakura, Torrance, CA) and snap frozen in liquid nitrogen. Then, tissues were sectioned into 10 μm thickness and stained with hematoxylin and eosin (H&E). The stained tissues were evaluated with light microscopy. Normal and neointimal areas were analyzed using NIH images.

Cell Migration Assay

Scratch wound assays were performed by a previously reported method with modifications.[9] SMCs were seeded to a final density of 1×10^5 cells/well in a 6-well culture plate and cultured to produce confluent monolayers at 37°C in 5% CO_2. Then, confluent monolayers were scratched using a pipette tip and cultured in low-glucose DMEM supplemented with 100-mg/mL streptomycin, 100-U/mL penicillin, and 10% FBS as a control group. In addition, we cultured SMCs in low-glucose DMEM supplemented with 100-mg/mL streptomycin, 100-U/mL penicillin, and 10% FBS with various concentrations of the compound for 24 hours. Migrated cells were counted using NIH images.

Western Blotting

For the western blot analysis, cells were washed once with PBS, and then, the protein was isolated using a lysis buffer containing 150-mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 50-mM Tris/HCl, and 2-mM EDTA, with a protease inhibitor cocktail. Protein concentrations were determined by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Then, equal amounts of protein were electrophoresed with 12% sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, blocked using tris-buffered saline with Tween 20 containing 5% (wt/vol) nonfat dried skim milk powder for 1 hour at room temperature. Then, the membranes were incubated with primary antibodies (1:1000) in a blocking buffer overnight at 4°C. The membranes were washed with tris-buffered saline with Tween 20 for 10 minutes and then treated with horseradish peroxidase–conjugated secondary antibody at room temperature for 1 hour. Then, bands were detected by the enhanced chemiluminescence western blotting detection reagent. The expression levels were measured using the Davinch-K western image system (Lab plus, Korea). EMMPRIN (35 kDa, ab64616) and β-actin (42 kDa, ab20272) antibodies were purchased from Abcam (Cambridge, MA).

Reverse Transcription Polymerase Chain Reaction and Analysis

Reverse transcription polymerase chain reaction (RT-PCR) was conducted as reported previously.[10] In brief, total RNA was isolated using RNA-stat (Ambion, Austin, TX), and extracted RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Perkin-Elmer Applied

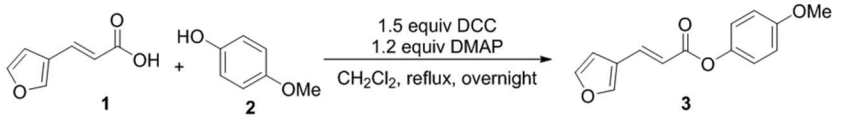

FIGURE 1. Procedure for the synthesis of 4-methoxyphenyl (*E*)-3-(furan-3-yl) acrylate.

Biosystems, Foster City, CA). The synthesized cDNA was subjected to RT-PCR using primers and probes. RNA transcript levels were quantitatively measured by an ABI Prism 7000 (Applied Biosystems, Foster City, CA). Relative mRNA expression was normalized to GAPDH expression, and those were calculated as described previously.[11]

RT-PCR Primers

The primers used for RT-PCR were as follows: matrix metalloproteinase (MMP)-2, fwd 5′-GCGGATCCAGC-GCCCAGAGAGACAC-3′, rev 5′-TTAAGCTTCCAC TC-CGGGCAGGATT-3′, MMP-9, fwd 5′-TGGACGATGC-CTGCAA CGTG-3′, rev 5′- GTCGTGCGTGTCCAA-AGGCA–3′, COX-2, fwd 5′-TCCAATCGCTGTACAA-GCAG-3′, rev 5′ TCCCCAAAGATAGCAT CTGG-3′; GAPDH, fwd 5′-CTCCCAACGTGTCTGT TGTG-3′, and rev 5′-TGAGCTTGAC AAAGTGGTCG-3′. GAPDH was used for the internal standard.

Statistical Analysis

Statistical significance was determined by Student's *t* test and analysis of variance with Bonferroni's multiple comparison tests using SPSS v12.0 software. All data were shown as mean ± SD from at least 3 independent experiments. Data with $P < 0.05$ were considered statistically significant.

RESULTS

In Vitro Antiproliferative Effect on SMCs

Through fractionation process of *Cordyceps* extracts by preparative high-pressure liquid chromatography, we identified some compounds from the peaks in the chromatogram. Because mass producing these compounds was difficult, we designed and synthesized their structural derivatives. Next, we tested these compounds for antiatherosclerotic potential.

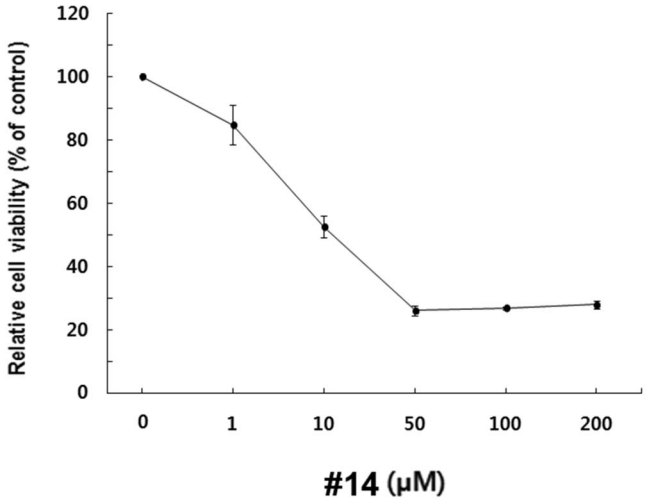

FIGURE 2. Antiproliferative effects of compound #14 on SMCs. SMCs (1×10^6 cells/mL) were treated with compound #14 as evaluated by the CCK assay (**P < 0.01, n = 6 per group).

SMCs were treated for 72 hours with several of the synthesized compounds to examine their antiproliferative effect. On analyzing the compounds, we observed that an increased concentration of the 3-furanylacrylate derivative [4-methoxyphenyl (*E*)-3-(furan-3-yl) acrylate: #14] significantly decreased the extent of proliferation in SMCs, and over 50 μM of compound #14 markedly inhibited the proliferation of SMCs by approximately 80% (Fig. 2).

In Vivo Antiatherosclerotic Effects in the Balloon Injury Rat Model

To investigate the in vivo antiatherosclerotic effects, we induced a balloon injury in the carotid artery of the rat model. The effect of compound #14 on neointimal hyperplasia was examined by histomorphometric analysis. The vehicle (PBS)-treated group showed abundant neointimal hyperplasia due to vascular injury at 3 weeks after the balloon injury. However, 20 μM of compound #14 injected intraperitoneally resulted in a significant reduction of neointimal hyperplasia (Fig. 3).

Effects of In Vitro SMC Migration

To examine the SMC migration effect of compound #14, we performed a scratch wound assay. Results showed that the increased concentration of compound #14 significantly reduced SMC migration compared with untreated control SMCs (Figs. 4A, B).

Effects of the ECM Metalloproteinase Inducer (EMMPRIN) and MMP Activation

To investigate the effect of compound #14 on the extracellular MMP inducer, EMMPRIN, we performed western blot analysis. The activation level of EMMPRIN was higher in the collagen type I–treated group than in the control group. It has been known that collagen type I acts as a signaling molecule, which affects vascular SMC growth and migration.[12] The activation level of EMMPRIN significantly decreased in response to treatment with increased concentrations of compound #14 (Figs. 5A, B).

To examine the activity of MMPs, we performed RT-PCR. Collagen type I treatment in SMCs increased the activities of MMP-2 and MMP-9 (Figs. 5C, D). However, treatment with compound #14 reduced the activities of MMP-2 and MMP-9 in a dose-dependent manner (Figs. 5C, D).

Effects of Cyclooxygenase (COX)-2 Activation

Generally, it has been reported that COX-2 expression is related to the mitogen-activated protein kinase pathway.[13,14] Accordingly, we examined the mRNA expression levels of COX-2 in SMCs treated with collagen type I. Interestingly, treatment with more than 20 μM of compound #14 inhibited the collagen type I-induced COX-2 expression in a dose-dependent manner (Figs. 6A, B).

DISCUSSION

In this study, we first demonstrated the newly synthesized 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate to be a potential chemical compound that exhibits

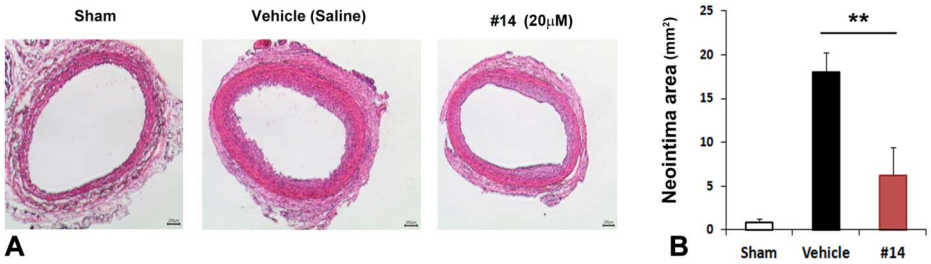

FIGURE 3. Effect of compound #14 on neointima formation in balloon-injured rats. Femoral arteries were excised at 3 weeks after balloon injury. A total of 20 μM compound # 14 of the *Cordyceps* extract was administered for 3 weeks after the balloon injury. The sections were stained with H&E, and neointima formation was evaluated. A, Representative photographs of H&E staining. B, Quantification of neointimal areas performed by ImagePro software (**P < 0.01, n = 3 per group).

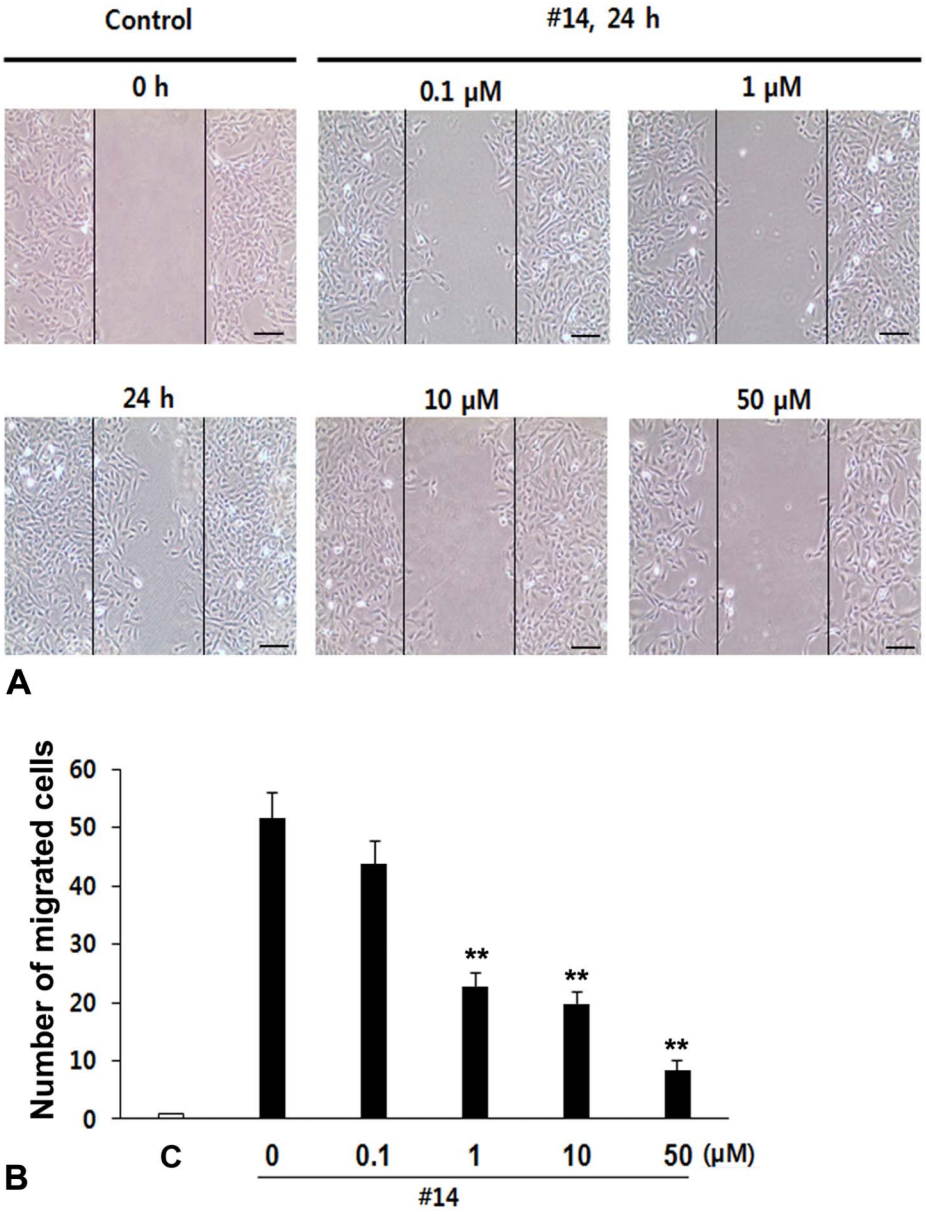

FIGURE 4. Effect of compound #14 on SMC migration. Migration assays were performed using scratch wound closure analysis. A, Representative photograph of SMC wound closure after incubation with compound #14. Bars = 200 μM. B, Quantification of cell migration using migrated cell numbers. Control, (C). **P < 0.01 versus untreated SMCs, n = 3 per group.

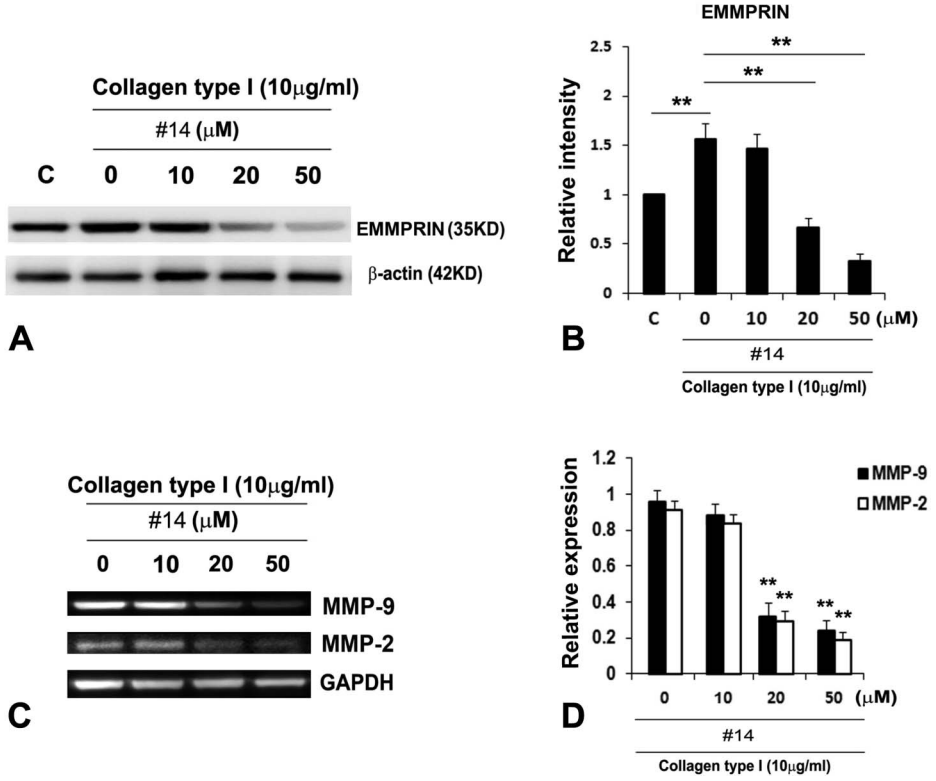

FIGURE 5. The inhibitory effects of compound #14 on the expression of EMMPRIN and MMPs in SMCs. SMCs were serum starved and treated with various concentrations of compound #14 for 2 hours before the exposure with 10-μg/mL collagen type I for 3 hours. A, The expression level of EMMPRIN protein was determined by western blot. Control, C. B, Quantification of protein expression by western blot analysis. **P < 0.01, n = 3 per group. C, Representative photograph of the RT-PCR result. Agarose gel was electrophoresed and visualized by ethidium bromide staining. D, Quantification of mRNA expression by RT-PCR analysis. **P < 0.01, n = 3 per group.

antiatherosclerotic activity. The important findings were as follows: First, the synthesized compound inhibited the proliferation and migration of aortic SMCs. Second, this compound significantly reduced in vivo neointimal hyperplasia. Third, this compound markedly inhibited the activation of EMMPRIN, MMP-2, and MMP-9. These results suggest that the newly synthesized compound can reduce restenosis by regulating vessel wall remodeling in the intimal layer.

Generally, it has been known that atherosclerosis caused by a neointimal lesion in the arterial lumen leads to various vascular diseases, such as peripheral vascular disease, myocardial infarction, and stroke. One of the important factors responsible for the pathogenesis of atherosclerosis is SMC. The abnormal proliferation of SMCs can contribute to restenosis, which is characterized by a narrowed vessel, due to neointimal thickening. In addition, thrombosis is a main cause of early restenosis, whereas fibrocellular proliferation, including SMCs and inflammatory cells, is the major cause of late restenosis. Thus, we explored natural compounds as antiatherosclerotic drugs to treat vascular disease.

We demonstrated that natural products derived from *Cordyceps* showed antiproliferation activity of SMCs.[7] We identified and synthesized its specific chemical compounds. The newly synthesized compound was shown to exhibit antimigrative and antiproliferative activity for SMCs. We speculated that the compound damaged SMCs and affected the cell death and migration function.

This compound also suppressed the enzyme activity of MMPs and its inducer EMMPRIN. EMMPRIN, also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells.[15] In fact, neointimal SMCs within atherosclerotic lesions synthesize novel ECM components and induce the activation of matrix-degrading proteases that remodel the neointimal environment. During atherosclerosis and restenosis, matrix-degrading metalloproteinase (MMP) has been reported to be associated with the induction of SMC migration and hyperplasia.[16,17] MMPs, which degrade most of the ECM and basement membranes, are the proteinases that affect the proliferation and migration of SMCs.[18,19] In a previous study, the correlation between MMP activation and SMC migration was demonstrated using a balloon-injured carotid artery rat model.[17] The expression level of each MMP (MMP-2, -3, -7, -9, -12, and -13) in SMCs differs depending on the cellular state and environment. MMP-2 is expressed in quiescent SMCs, whereas the others are expressed in injured or atherosclerotic arteries.[20,21] It has been reported that MMP-2 and MMP-9 are involved in SMC migration, which contributes to the intimal thickening of vascular lesions in vivo. In addition, overexpression of MMP-9 in SMCs has been shown to induce expansive remodeling in the carotid artery of an injured rat because of the increase in SMC migration and the decrease in ECM. EMMPRIN has also been known to play an important role as the inducer or activator in MMP expression and cell migration.[22]

COX comprises 2 isoforms, COX-1 and COX-2. The induction of COX-2 is mediated by inflammation, and this

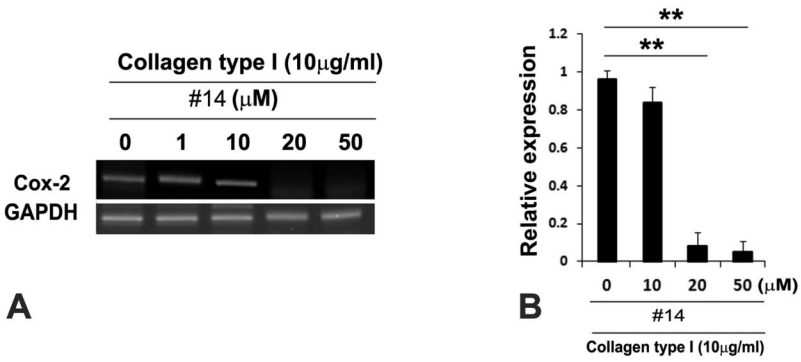

FIGURE 6. The inhibitory effects of compound #14 on the expression of COX-2 in SMCs. SMCs were serum starved, and the SMCs were treated with various concentrations of compound #14 for 2 hours before the exposure of 10 μg/mL of collagen type I for 3 hours. A, Representative photograph of the RT-PCR result. The agarose gel was electrophoresed and visualized using ethidium bromide staining. B, Quantification of mRNA expression performed by RT-PCR analysis. **P < 0.01, n = 3 per group.

regulates cellular processes involved in vessel remodeling, including MMPs and chemokines.[23] COX-2 expression is associated with SMC proliferation and mitogen-activated protein kinase and mediates proliferation of SMCs.[24,25] Consistent with these findings, we observed that treatment with collagen type I enhanced the expression levels of COX-2, MMP-2, and MMP-9 in SMCs. However, the compound treatment reduced the collagen type I-induced expression of COX-2. Thus, these results confirm that the newly synthesized compound possesses anti-inflammatory or antiatherosclerotic potential.

Therefore, in this study, we demonstrated that 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate regulates molecules of neointimal SMCs involved in the remodeling of the vessel wall and ECM. In conclusion, the newly synthesized 4-methoxyphenyl (E)-3-(furan-3-yl) acrylate might be an alternative therapeutic option for treating atherosclerosis.

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