Potent Inhibition of Platelet-Derived Growth Factor-Stimulated Rat Aortic Vascular Smooth Muscle Cell Cycle and Proliferation by (2E)-3-(4-hydroxy-3-methoxyphenyl)phenylpro-2-en-1-one, a Newly Synthesized Benzylideneacetophenone Derivative

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One of the principal regulators of mitogenesis in vascular smooth muscle cells (VSMCs) is platelet-derived growth factor-BB (PDGF-BB). An increase of PDGF-BB expression has been observed in atherosclerotic lesions. The aim of this study was to elucidate the effects and molecular mechanism of (2E)-3-(4-hydroxy-3-methoxyphenyl)phenylpro-2-en-1-one (KTJ2242), a newly synthesized benzylideneacetophenone derivative, on PDGF-BB-stimulated rat aortic VSMCs. KTJ2242 induced accumulation of cells in the G1 phase of the cell cycle of VSMCs. We observed that KTJ2242 inhibited PDGF-BB-stimulated [3H]-thymidine incorporation into the DNA of VSMCs, and the cell number was significantly reduced in a concentration-dependent manner.

INTRODUCTION

Benzylideneacetophenone analogues have several significant biological activities, including antibacterial, antiviral, anti-inflammatory, antitumor, gastric-protective, and anti-free radical. 1–8 Recent, we reported that a benzylideneacetophenone analogue inhibits the PLCγ1 cascade of rat aortic vascular smooth muscle cell (VSMC) proliferation. 9

The proliferation of VSMCs in arterial walls is involved in cardiovascular problems such as atherosclerosis. One of the principal regulators of VSMC proliferation is platelet-derived growth factor (PDGF)-BB. 10–12 PDGF-BB binds to the PDGF-receptor (PDGF-R) which leads to phosphorylation of the tyrosine residues of the PDGF-Rβ-chain (PDGF-Rβ). 11 The receptor tyrosine kinases for PDGF-BB activate P13-kinase and protein (MAP) kinase. The signaling pathways of both kinases are important in early intracellular mitogenic signal transductions for cell growth and survival. 13 Therefore, inhibition of abnormal proliferation of PDGF-BB-induced VSMCs may be an effective cardiovascular therapy. 14

In this study we focus on understanding the antiproliferative effect and molecular mechanism of a newly synthesized benzylideneacetophenone derivative, KTJ2242 [(2E)-3-(4-hydroxy-3-methoxyphenyl)phenylpro-2-en-1-one], in the PDGF-BB-stimulated signaling pathways of rat aortic VSMCs.

Also, we observed that KTJ2242 decreased PDGF-BB-stimulated extracellular-regulated kinase 1 and 2 (ERK1/2) and Akt phosphorylation. These results suggest the possibility that KTJ2242 may be a potential agent with which to control vascular disorders and its antiproliferative mechanism may be mediated through partial Akt and ERK1/2-dependent signaling pathways.

Key words — vascular smooth muscle cell, proliferation, cardiovascular disease
MATERIALS AND METHODS

Chemical Reagents —— Cell culture materials were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Phospho-extracellular-regulated kinase (ERK) 1/2, phospho-Akt, ERK1/2, and Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). PDGF-BB was obtained from Koma Biotechnology (Seoul, Korea). All other chemicals used were of the highest analytical grade commercially available.

Experimental —— All commercial reagents and solvents were used as received without further purification unless specified. Dichloromethane was distilled from calcium hydride, and tetrahydrofuran was distilled from sodium metal and benzophenone. The reactions were monitored and the Rf values were determined using analytical thin layer chromatography (TLC) with Merck silica gel 60 (Merck, Darmstadt, Germany) and F-254 precoated plates (0.25-mm thickness). Spots on the TLC plates were visualized using ultraviolet light (254 nm) and a basic potassium permanganate solution or cerium sulfate/ammonium molybdate/sulfuric acid solution, followed by heating on a hot plate. Flash column chromatography was performed with Merck silica gel 60 (230–400 mesh). 1H NMR spectra were recorded on Bruker DPX-250 (Bruker, Rheinstetten, Germany) or Varian Unity-Inova 500 (Varian, Palo Alto, CA, U.S.A.) spectrometers. Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ 0.00) or with the solvent reference relative to TMS employed as the internal standard [CDCl3, δ 7.26 ppm; d4-C2D5OD, δ 3.31 ppm, d6-dimethyl sulfoxide (DMSO), δ 2.50 ppm]. Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration). 13C NMR spectra were recorded on Bruker DPX-250 (63 MHz) or Varian Unity-Inova 500 (125 MHz) spectrometers with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard (CDCl3, δ 77.0 ppm; d4-C2D5OD, δ 49.0 ppm, d6-DMSO, δ 39.5 ppm). Infrared (IR) spectra were recorded on a Nicolet Model Impact FT-IR 400 spectrometer (PerkinElmer, Waltham, MA, U.S.A.). Data are reported in wave numbers (cm⁻¹). High resolution mass spectrometer (HRMS) analyses were recorded on an Applied Biosystems 4700 proteomics spectrometer (Applied Biosystems, Vernon Hills, IL, U.S.A.).

KTJ2242 was prepared according to a conventional method. Briefly, 4-hydroxy-3-methoxy cinnamaldehyde was used in a four step strategy involving a protecting reaction, a Grignard reaction, an oxidation reaction, and a deprotection reaction of the tert-butylidemethylsilyl (TBS) group at the hydroxyl group of a protected enone using tetrabutylammonium fluoride (TBAF) in tetrahydrofuran RF = 0.4 (20% ethyl acetate/hexanes); mp 86–87°C (85–90°C); IR (neat, NaCl) 3321, 3001, 2960, 1739, 1656, 1420, 1285, 1038 cm⁻¹; 1H NMR (400 MHz, CDCl3) δ 8.02 (d, J = 5.4 Hz, 2 H), 7.76 (d, J = 11.7 Hz, 1 H), 7.58 (t, J = 5.1 Hz, 1 H), 7.50 (t, J = 5.4 Hz, 2 H), 7.38 (d, J = 11.7 Hz, 1 H), 7.21 (d, J = 6.0 Hz, 1 H), 7.14 (s, 1 H), 6.96 (d, J = 6.0 Hz, 1 H), 5.66 (brs, 1 H), 3.95 (s, 3 H); 13C NMR (100 MHz, CDCl3) δ 191.1, 149.0, 147.4, 145.7, 138.9, 132.9, 128.9, 128.8, 127.8, 123.8, 120.1, 115.4, 110.6, 56.4; HRMS calcd. for C16H15O3: 255.1051 [M+H]+, found: 255.1038.

Cell Culture —— Rat aortic VSMCs were obtained from BioBud Co. Ltd. (Seoul, Korea). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 8 mM HEPES, and 2 mM L-glutamine at 37°C in a humidified 5% CO2 incubator.

Cell Cycle Analysis —— Cell cycle was determined by flow cytometry as previously described. Briefly, cells were trypsinized and centrifuged at 1500 × g for 7 min. Pellets were suspended in 1 ml of 1 × phosphate buffered saline (PBS), washed twice, and re-centrifuged. Pellets were then suspended in 70% ethanol and fixed overnight at 4°C. The fixed VSMCs were briefly vortexed and centrifuged at 15000 × g for 5 min. Ethanol was discarded and pellets were stained with 0.4 ml of propidium iodide (PI) solution [50 µg/ml PI in buffer containing 100 µg/ml of ribonuclease (RNase) A]. Samples were incubated for 1 hr at room temperature prior to analysis by flow cytometry. The PI-DNA complex within each cell nucleus was measured using a FACS Calibur (BD Biosciences, San Jose, CA, U.S.A.). The rates of G0/G1, S and G2/M phases were determined using the computer program ModFit LT (Verity Software House, Topsham, ME, U.S.A.).

Cell Count Assays —— Rat aortic VSMCs were seeded into 12-well culture plates at 1 × 10⁵ cells/ml and cultured in DMEM containing 10% FBS at
37°C until 70% confluent. Media was then replaced with serum-free medium containing KTJ2242. Cells were stimulated for 24 hr with 25 ng/ml PDGF-BB, trypsinized with trypsin-EDTA, and counted using a hemocytometer.

**DNA Synthesis Assays** —— DNA synthesis was determined by [3H]-thymidine incorporation as previously described. Briefly, VSMCs were seeded in 24-well culture plates under the above conditions, and 2 µCi/ml of [3H]-thymidine was added to the medium for 4 hr. Reactions were terminated by aspirating the medium and washing the cultures with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). The acid-insoluble [3H]-thymidine was extracted using 250 µl/well of 0.5 M NaOH, this solution was then mixed with 3 ml of scintillation cocktail (Ultimagold, Packard Bioscience, Meriden, CT, U.S.A.) and analyzed using a liquid scintillation counter (LS3801, Beckman, Düsseldorf, Germany).

**Western Blot Analysis** —— Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on cell lysates using 7.5–10% acrylamide gels according to the method described by Ahn et al. Briefly, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, U.S.A.), which were then blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% skim milk powder. Membranes were then incubated with a 1:2000 dilution of phospho-ERK1/2 and phospho-Akt antibodies. Blots were washed with TBS/T and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, U.S.A.). Proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection reagent (GE Healthcare, Buckinghamshire, U.K.).

**Statistical Analysis** —— Experimental results are expressed as mean ± S.E. One-way analysis of variance (ANOVA) followed by Dunnett’s test was used for multiple comparisons. p values of <0.05 and <0.01 represent statistically significant differences.

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**RESULTS AND DISCUSSION**

**Effects of KTJ2242 on PDGF-BB-Stimulated Cell Cycle Progression, DNA Synthesis and Proliferation of Rat Aortic VSMCs**

![Chemical Structure of KTJ2242](image1)

![Effect of KTJ2242 on PDGF-BB-stimulated Cell Cycle Progression in VSMCs](image2)

The proliferation of VSMCs is involved in a broad spectrum of cardiovascular disorders. These can be triggered by various extracellular mitogenic stimuli, such as PDGF-BB, that activate a variety of signaling events involved in the regulation of cell growth and division. In addition, an association between PDGF and VSMC proliferation has been demonstrated in animal experiments, which showed increases in PDGF-BB after arterial injury were correlated with neointimal cellular proliferation.

To determine whether KTJ2242 (Fig. 1) inhibits PDGF-BB-stimulated cell cycle progression of rat aortic VSMCs, flow cytometry was performed with propidium iodide. Rat aortic VSMCs were precultured in serum-depleted medium for 24 hr, followed by stimulation with PDGF-BB 25 ng/ml for 24 hr. As shown in Fig. 2, KTJ2242 induced accumulation of cells in the G1 phase of the cell cycle in a concentration-dependent manner. The effect of KTJ2242 on DNA synthesis and cell number was assessed. The antiproliferative activity of KTJ2242
Fig. 3. Effect of KTJ2242 on PDGF-BB-stimulated Cell Number and DNA Synthesis of VSMCs
(A) Cells were precultured in serum-free medium in the presence or absence of KTJ2242 (1–20 µM) for 24 hr, then stimulated with 25 ng/ml PDGF-BB for 24 hr. Cells were trypsinized and counted using a hemocytometer. (B) Cells were precultured in serum-free medium in the presence or absence of KTJ2242 (1–20 µM) for 24 hr, then stimulated with 25 ng/ml PDGF-BB for 20 hr. [3H]-thymidine (2 µCi/ml) was added to the medium, and cells were incubated for 4 hr. Radioactivity was measured using a liquid scintillation counter. Data are expressed as the mean ± S.E. from triplicate experiments. *p < 0.05, **p < 0.01 vs. positive control value stimulated by PDGF-BB alone.

Fig. 4. Effect of KTJ2242 on ERK1/2 and Akt Activation in PDGF-BB-stimulated VSMCs
Cells were precultured in serum-free medium in the presence or absence of KTJ2242 (1–20 µM) for 24 hr, then stimulated with 25 ng/ml PDGF-BB for 10 min. The cells were then lysed, and proteins from these lysates were analyzed with SDS-PAGE. The data from triplicate experiments were quantified by densitometry. **p < 0.01 vs. positive control value stimulated by PDGF-BB alone.

was quantitated using a [3H]-thymidine incorporation assay. As shown in Fig. 3A, KTJ2242 reduced PDGF-BB-induced [3H]-thymidine incorporation in a concentration-dependent manner. Cytotoxic effects of KTJ2242 were measured on rat aortic VSMCs and no evidence was found of cytotoxicity at 20 µM. Significant inhibition of [3H]-thymidine incorporation after PDGF-BB stimulation was observed at KTJ2242 1 µM (26.19 ± 4.09%), 5 µM (47.34 ± 3.45%), 10 µM (55.93 ± 2.42%), and 20 µM (89.34 ± 6.28%). We also assessed the inhibitory effects of KTJ2242 by direct cell counting. Pretreatment with KTJ2242 decreased the total number of cells following PDGF-BB stimulation in a concentration-dependent manner, with inhibition of 12.57 ± 1.81, 56.81 ± 18.18, 81.66 ± 17.12, and 92.12 ± 8.48% at concentrations of 1, 5, 10, and 20 µM, respectively (Fig. 3B). Therefore, our results indicate that KTJ2242 inhibits PDGF-BB-induced VSMC proliferation without cytotoxicity.

Effects of KTJ2242 on ERK1/2 and Akt Phosphorylation in PDGF-BB-Stimulated Rat Aortic VSMCs
The binding of PDGF-BB to PDGF-R activates several intracellular signaling cascades, with inhibition of 12.57 ± 1.81, 56.81 ± 18.18, 81.66 ± 17.12, and 92.12 ± 8.48% at concentrations of 1, 5, 10, and 20 µM, respectively (Fig. 3B). Therefore, our results indicate that KTJ2242 inhibits PDGF-BB-induced VSMC proliferation without cytotoxicity.

ERK1/2 and Akt are major signal-transducing molecules involved in the regulation of cellular proliferation, differentiation, and apoptosis.
Their activation is associated with the development and progression of proliferative cardiovascular diseases, such as hypertension and atherosclerosis.\textsuperscript{20–28} Therefore, understanding of the inhibitory role of ERK1/2 and Akt in the prevention of PDGF-BB-stimulated VSMC proliferation is important for developing new methods of treating cardiovascular diseases.

We hypothesized that the antiproliferative properties of KTJ2242 were caused by modulation of the signaling cascade involved in VSMC proliferation. The inhibitory effects of KTJ2242 on ERK1/2 and Akt phosphorylation levels were examined using Western blotting. Rat aortic VSMCs were precultured in the presence or absence of KTJ2242 (1–20\(\mu\)M) in serum-free medium for 24 hr and then stimulated with 25 ng/ml PDGF-BB. As shown in Fig. 4, ERK1/2 phosphorylation was significantly inhibited by KTJ2242 (1–20\(\mu\)M) in a concentration-dependent manner. Significant inhibition of ERK1/2 phosphorylation after PDGF-BB stimulation was observed with KTJ2242 at concentrations of 1\(\mu\)M (4.76 ± 18.17\%), 5\(\mu\)M (12.70 ± 6.91\%), 10\(\mu\)M (43.65 ± 3.96\%), and 20\(\mu\)M (52.78 ± 10.48\%). Pretreatment with KTJ2242 decreased the Akt phosphorylation after PDGF-BB stimulation was observed with KTJ2242 at concentrations of 1, 5, 10, and 20\(\mu\)M, respectively (Fig. 4B). Several groups have reported that the MAP kinase (MAPK) signaling pathway is critical in abnormal proliferation of VSMCs, which have been shown to be activated in fibroblasts and smooth muscle cells.\textsuperscript{29)–31) Among the signaling pathways studied, our data suggests that inhibition of the ERK1/2 and Akt cascade may be involved in the KTJ2242-induced inhibition of rat aortic VSMC proliferation and cell cycle progression. Recently, our group reported that BST-406, a newly synthesized benzylideneacetophenone derivative inhibits rat aortic VSMC proliferation via the inhibition of PLC\(\gamma\)1 pathway.\textsuperscript{9) In this study, we report that KTJ2242 inhibits cell cycle and growth through the inhibition of partial Akt and ERK1/2 activation as rat aortic VSMC proliferation signaling. However, these compounds are the differences structurally. Therefore, we believe that these compounds can account for the different mechanisms of action. Although, at present, the molecular mechanism by which KTJ2242 exerts its effects on rat aortic VSMC proliferation has not been fully explained, a possibility of KTJ2242 can be a potential agent to control vascular disorders.

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