Sesquiterpene Lactone Suppresses Vascular Smooth Muscle Cell Proliferation and Migration *via* Inhibition of Cell Cycle Progression

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Abnormal vascular smooth muscle cell (VSMC) proliferation and migration are involved in restenosis following percutaneous transluminal angioplasty (PTCA) as well as in the development and progression of atherosclerosis. We investigated the mechanisms underlying the inhibitory effect of the sesquiterpene 3-oxo-5αH,8βH-eudesma-1,4(15),7(11)-trien-8,12-olide (1) on rat VSMC proliferation and migration. VSMCs were isolated from rat aorta, and then the effect of 1 on cell proliferation and migration was examined using methylthiazolyldiphenyl-tetrazolium bromide (MTT) and chemotaxis assays, respectively. Compound 1 had a potent inhibitory effect on fetal calf serum-induced VSMC proliferation. This effect correlated with reduced expression of cyclin D_1. In addition, 1 also inhibited platelet derived growth factor (PDGF)-induced migration of VSMCs. These results indicate that 1 is a promising candidate for additional biological evaluation to further define its potential as an inhibitory modulator of VSMC responses that contribute to restenosis following PTCA and to the development and progression of atherosclerosis.

Key words atherosclerosis; restenosis; migration; vascular smooth muscle cell; cell cycle

Vascular smooth muscle cell (VSMC) proliferation and migration are key features of restenosis following percutaneous transluminal angioplasty (PTCA),[1] whereas VSMC proliferation stabilize atherosclerotic vulnerable plaque which is involved in an acute occlusion of the coronary artery in myocardial infarction.[2] This response is widely believed to be mediated by platelet derived growth factor (PDGF),[3,4] which induces activation of extracellular signal regulated kinase 1/2 (ERK1/2), a key transducer of extracellular signaling that promotes the cell proliferation and migration that is critical for the initiation and progression of vascular lesions.[5,6] Within the arterial media, VSMCs are normally arrested at G_0/G_1 phase of the cell cycle and are thus quiescent (proliferation index, $<0.05\%$).[7] After vessel injury, however, VSMCs migrate into the intima, where they reenter the cell cycle.[8] For many cells, transition through G_1 phase and entry into S phase require activation of cyclin-dependent kinases (CDKs) such as cdk2 and cdk4 through the formation of cyclin/CDK complexes, a process in which cyclin D_1 and cyclin E play major roles.[9,10] The kinase activities of the cyclin/CDK complexes are negatively regulated by CDK inhibitors (CKIs) such as p21 and p27,[11,12] though p21 also plays an essential positive role in the assembly of certain cyclin/CDK complexes.[13—17]

We previously showed that treatment with the water extract of the root of Linderae Radix (*Lindera strychnifolia*) induced significant apoptosis among lung cancer cells and prolonged the survival of tumor-bearing mice.[18] In a search for the mediator(s) of those effects, we isolated two novel sesquiterpene lactones from the ethyl acetate soluble fraction of the water extract through bioassay-guided fractionation and isolation methods.[19] One of two isolates, 3-oxo-5αH,8βH-eudesma-1,4(15),7(11)-trien-8,12-olide (**1**) (Fig. 1) showed a significant inhibitory effect on the proliferation of SBC-3 human small cell lung cancer cells and a lesser inhibitory effect on the proliferation of mouse fibroblasts.[19] The purpose of the present study was to determine whether **1** would also inhibit the proliferation and migration of VSMCs and, if so, to shed light on the mechanism underlying that effect.

MATERIALS AND METHODS

Reagents Compound **1** was kindly provided by the Medicinal Chemistry Research Institute, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Rapamycin and recombinant human (rh)PDGF-BB were purchased from Cal Biochem (San Diego, CA, U.S.A.).

Cell Isolation and Culture All procedures were in accordance with our institutional guidelines for animal research. Male Sprague-Dawley rats (9 weeks old) were purchased from SLC (Shizuoka, Japan). For experimentation, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and then killed by exsanguination. The thoracic aorta was then excised and predigested for 20 min at 37 °C with an enzyme mixture containing 125 U/ml collagenase type I and 2 U/ml elastase type III (Sigma, St. Louis, MO, U.S.A.) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, U.S.A.). The vessels were then cut open and pinned luminal side up on a dissect-

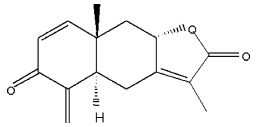

Fig. 1. Structure of 3-Oxo-5αH,8βH-eudesma-1,4(15),7(11)-trien-8,12-olide (**1**)

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ing surface. The residual endothelium was removed with a plastic cell scraper, and the media and adventitia separated by peeling the adventitia from the media. The remaining medial tissue was then minced and digested in the same enzyme mixture for 2 h at 37 °C, after which the cells were harvested and cultured in DMEM supplemented with 20% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C under an atmosphere of 5% CO_2/95% air. Cells from passages 8—11 were used for experimentation; they were identified as smooth muscle based on their typical appearance on light microscopy and immunostaining with anti-smooth muscle α-actin 1A4 (Dako, Carpinteria, CA, U.S.A.).

Cell Proliferation Assays Cells were plated to a density of 1×10^4 cells/well into 96-well plates and cultured for 24 h, after which **1** (1 μg/ml) or rapamycin (0.1 μg/ml),[20] which served as an inhibitory control in these experiments, was added and the cultures were continued for an additional 0, 12, 24 or 48 h. After refreshing the medium at the indicated times, 10 μl of MTT (5 mg/ml in PBS) were added to each well, and the cells were incubated for another 12 h. The accumulated formazan was then solubilized in dimethylsulfoxide (DMSO), and the absorbance at 570 nm was measured.[21]

Cell Cycle Analysis The cells were plated to a density of 6×10^3 cells/cm^2 and cultured in DMEM supplemented with 20% FCS for 24 h, after which they were incubated in serum-free DMEM for 96 h to synchronize VSMCs at G_0/G_1 phase.[22] To examine the effects of **1** and rapamycin on cell cycle progression, cultures were first growth arrested as described above and then incubated in DMEM supplemented with 20% FCS containing **1** (1 μg/ml) or rapamycin (0.1 μg/ml) for the indicated times (0, 15, 18, 21 h). The cells were then harvested by trypsinization and washed with PBS, fixed in ice-cold 70% methanol for 30 min, washed again with PBS, and treated with 1 mg/ml RNase A in buffer containing 1 mM Tris–HCl (pH 7.4) and 1.5 mM NaCl for 30 min at 37 °C. The cells were then collected by centrifugation at 400 ***g*** for 5 min and stained with 250 μl of nuclear staining solution (10 mg propidium iodide, 0.1 mg trisodium citrate, and 0.03 ml Triton X-100 dissolved in 100 ml of H_2O) for 30 min at room temperature in the dark. After adding 750 μl of PBS, flow cytometry (EPICS-XL, BECKMAN COULTER, MF, U.S.A.) was used to determine the DNA content, which was reflected by the propidium iodide signal and served as an index of cell cycle phase.

Western Blot Analysis Cells were lysed in 100 μl of lysis buffer containing 50 mM HEPES (pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 40 mM Na_3VO_4, 0.4 mM NaF, 0.1% NP-40, 10% glycerol and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The lysates were pelleted (1000 ***g***, 10 min) at 4 °C, and the supernatant was assayed for protein using the Bradford method (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Lysate samples containing 10 μg of protein were then separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P Transfer Membrane, Millipore corporation, MA, U.S.A.) in transfer buffer containing 31 mM Tris, 242 mM glycine and 20% methanol. Thereafter, the membrane was first blocked for 1 h in 5% powdered skim milk in Tris-buffered saline (TBS) and then incubated with the appropriate primary antibody for 24 h at 4 °C. The antibodies used were anti-cyclin D_1 (H-295) from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The blot was then washed with four changes of wash buffer (0.05% Tween-20 in TBS) and incubated for 1 h at room temperature in TBS containing anti-rabbit or anti-mouse IgG (Amersham, Arlinton, IL, U.S.A.), 1% powdered skim milk and 0.05% Tween-20. Finally, the blot was again washed with four changes of wash buffer, exposed to ECL Plus WB detection reagents (Amersham, Arlinton, IL, U.S.A.) and developed on Fuji Medical X-ray Film (Fuji Photo Film, Tokyo, Japan).

Cell Migration Assay Cell migration was measured using modified Boyden chambers (Neuro Probe, Gaithersburg, MD, U.S.A.) each housing a polycarbonate filter with 8-μm pores (Neuro Probe, Gaithersburg, MD, U.S.A.), which had been incubated with 0.1 mg/ml collagen (Centrix, Santa Clara, CA, U.S.A.) in 0.2 M acetic acid for 24 h prior to each migration assay. For each assay, 27 μl of DMEM to which 20 ng/ml rhPDGF-BB plus 0.2% bovine serum albumin (BSA; Sigma) had been added were loaded in the lower section of the Boyden chambers. DMEM containing the BSA without PDGF served as a negative control. Then after pretreating VSMCs for 48 h with **1** or rapamycin (plates without drug served as controls), the cells were trypsinized, washed three times with PBS, and equal numbers (2×10^5 cells/ml) in 50 μl of medium were placed in the upper section of the Boyden chambers. After incubating 6 h at 37 °C, non-migrating cells were scraped from the upper surface of the filters. Cells on the underside of the filters were deemed to have migrated through the filter and were fixed with methanol and stained with Diff-Quik (Baxter Healthcare Co., Miami, FL, U.S.A.). The numbers of VSMCs on the lower surface of the filter were then determined microscopically by counting five high-power (400×) fields per well.[20]

Statistical Analysis Data are expressed as means±S.D. Statistical comparisons were made using two-way repeated measurements of analysis of variance (ANOVA) with *post hoc* test (Bonferroni/Dunn test). Values of $p<0.05$ were considered significant.

RESULTS

Compound 1 Inhibits Proliferation of Rat VSMCs We first evaluated the effects of **1** on rat VSMC proliferation. VSMCs were incubated with **1** for 48 h, after which cell viability was assessed using the MTT method. Treatment with either **1** (1 μg/ml) or rapamycin (0.1 μg/ml), which served as an inhibitory control, incubation time-dependently and significantly inhibited VSMC proliferation (Fig. 2A), though comparison with untreated cultures showed there was no effect on cell morphology (Fig. 2B). On the other hand, treatment with **1** (<0.5 μg/ml) did not affect the VSMC proliferation (data not shown).

Compound 1 Induces G_1 Cell Cycle Arrest and Modulates G_1 Cell Cycle-Associated Protein To evaluate the cytostatic activity of **1**, we investigated its effect on cell cycle progression using propidium iodide staining. For this experiment, rat VSMCs were first synchronized at G_0/G_1 phase by serum starvation for 96 h, after which the medium was exchanged for DMEM containing 20% FBS. Among control cells, cell cycle progression from G_0/G_1 phase to S and G_2/M phase was observed within 21 h after addition of medium

containing 20% FCS. In the presence of **1** (1 μg/ml) or rapamycin (0.1 μg/ml), however, no cell cycle progression was observed—*i.e.*, the cells remained at G_0/G_1 phase (Fig. 3A).

We next used Western analysis to examine the expression of cell cycle regulatory molecule to determine whether the **1**-mediated inhibition of VSMC proliferation is associated with reduced activation of the cell cycle machinery. We found that FCS-induced expression of the G_1-associated factor cyclin D_1 was diminished in **1** treated-VSMCs (Figs. 3B, C).

Compound 1 Suppresses VSMC Migration Rat VSMCs added to the upper sections of Boyden chambers did not migrate if rhPDGF-BB was omitted from the lower section (Fig. 4, first column). rhPDGF-BB (20 ng/ml) added to the lower section acted as a chemoattractant, stimulating the migration of the VSMC from the upper section to the underside of the filters separating the upper and lower sections (second column). Treating VSMCs with **1** (third—fifth col-

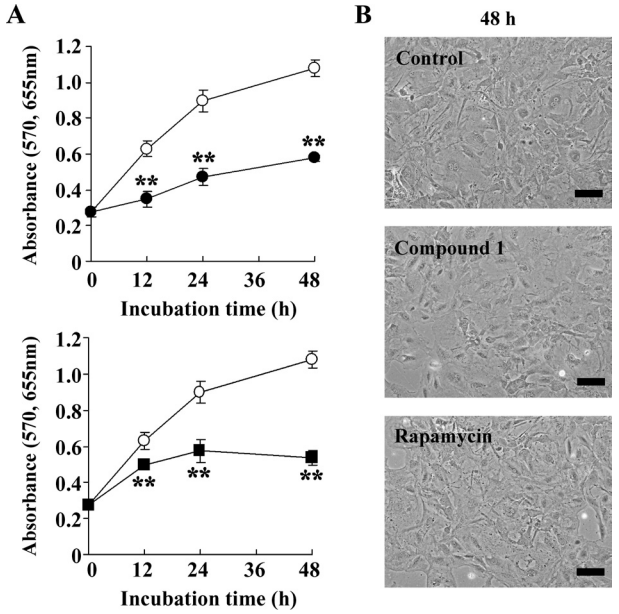

Fig. 2. Effects of **1** and Rapamycin on VSMC Proliferation

(A) Rat VSMCs were incubated for 48 h with the indicated concentrations of **1** or rapamycin. The degree of proliferation was determined using the MTT method as described in Materials and Methods. Values represent means±S.D. of 4 wells: open circles, control; closed circles, **1** (1 μg/ml); closed squares, rapamycin (0.1 μg/ml). $^{**}p<0.01$ *vs.* control. (B) Culture in the presence of **1** (1 μg/ml) or rapamycin (0.1 μg/ml) had no effect on rat VSMC morphology; compare control cells to cells cultured for 48 h with **1** or rapamycin. Scale bars represent 100 μm.

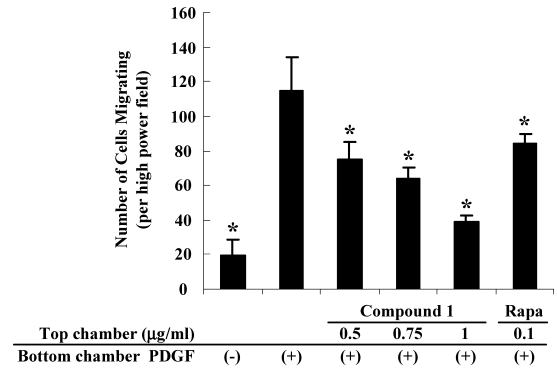

Fig. 4. Effects of **1** and Rapamycin on VSMC Migration

Cells were seeded into the upper sections of Boyden chambers, and chemotaxis was induced by adding rhPDGF-BB (20 ng/ml) to the lower section; as a negative control, PDGF was omitted from some chambers. The assay was then allowed to run for 6 h under the indicated conditions. The y-axis shows the numbers of cells per high power field that migrated to the underside of the filter. Each column represents means±S.D. of 6 wells. $^{*}p<0.05$ *vs.* control (PDGF(+)).

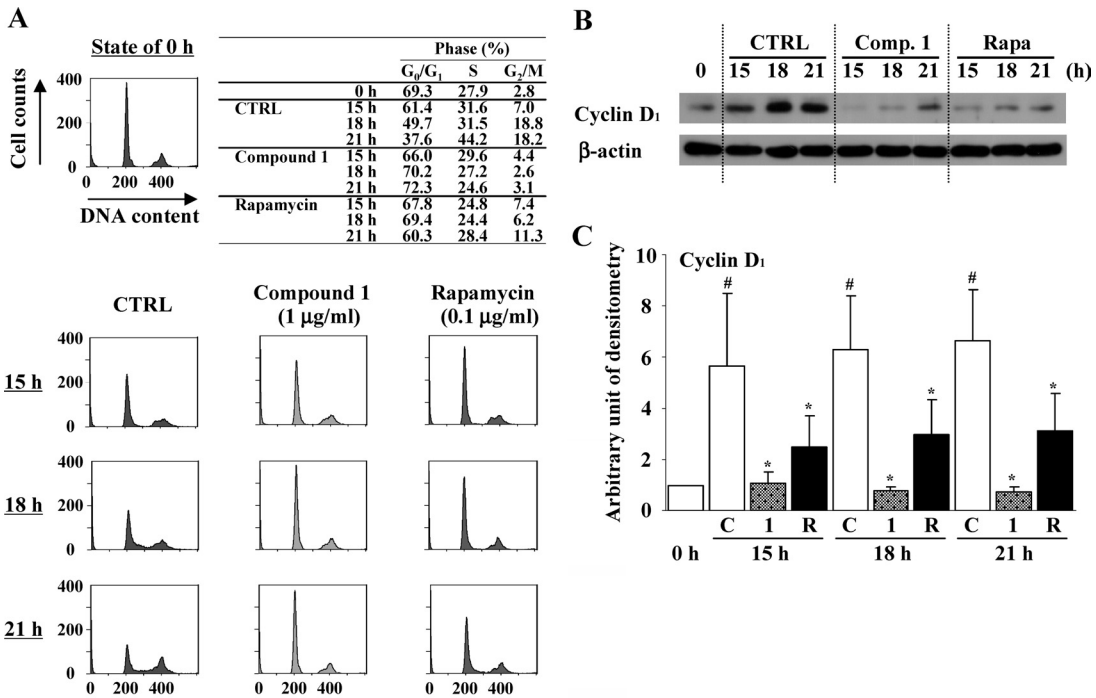

Fig. 3. Effects of **1** and Rapamycin on Cell Cycle Progression in Rat VSMCs Previously Synchronized at G_0/G_1 Phase by Serum Starvation

The cells were initially maintained in serum-free DMEM for 96 h to arrest cell cycle progression at G_0/G_1 phase. The cells were then stimulated for the indicated times with 20% FCS in the presence of **1** (1 μg/ml) or rapamycin (0.1 μg/ml). (A) The cells were then harvested and stained with propidium iodide, after which cell cycle progression was assessed by flow cytometric analysis of DNA content. Similar results were observed in three independent experiments. (B) The cells were then lysed, and lysate samples containing 10 μg of protein were subjected to SDS-PAGE and Western analysis. (C) Y-axis indicates arbitrary unit of cyclin D_1 densities. Each column represents means±S.D. of three independent experiments. $^{\#}p<0.05$ *vs.* 0 h group, $^{*}p<0.05$ *vs.* control group.

umn) or rapamycin (sixth column) for 48 h prior to the assay significantly inhibited migration.

DISCUSSION

VSMC proliferation and migration play key roles in the restenosis following PTCA and in the development and progression of atherosclerosis. In arterial media, VSMC are normally quiescent and remain in the G_0/G_1 phase of cell cycle. In response to atherogenic stimuli or vessel injury, VSMC migrate into the intimal layer of the arterial wall, where they leave their quiescent state and reenter the cell cycle.[8] Cell cycle progression from G_1 to S phase is controlled by several CDK complexes, including cyclin D_1/cdk4 and cyclin E/cdk2,[9,10] the activities of which are dependent on the balance of cyclins and CKIs, such as p27 and p21.[11,12] Cyclin D_1 is expressed in low abundance in quiescent cells, but quickly accumulates upon the stimulation with serum or mitogen.[23]

The aim of the present study was in part to determine whether compound **1**, which we isolated from the root of Linderae Radix, might also exert an anti-atherogenic effect on VSMCs, as it was previously shown to significantly inhibit proliferation of human small cell lung cancer cells.[19] Rapamycin, which served as an inhibitory control in the present study, was initially developed as an antibiotic and then used as an immunosuppressant, while paclitaxel was initially developed as anticancer drug. However, both of those drugs exert an inhibitory effect of VSMC proliferation and have recently been used to prevent restenosis after placement of coronary arterial stents.[24,25] Consistent with that idea, treating VSMCs with **1** time-dependently inhibited their proliferation (Fig. 2A), and dose-dependently inhibited their migration (Fig. 4). In addition, compared to rapamycin, **1** had greater inhibitory effect on migration rather than proliferation, despite their suppressive effect on cyclin D_1 expression level were comparable (Figs. 3B, C). Cell morphology was unaffected by **1** and rapamycin (Fig. 2B), suggesting **1** and rapamycin do not exert an effect on the skeletal components of the cells, which are associated with migration. Therefore, the possibility exists that **1** affect the Rho/Rho kinase pathway, which plays an important role in VSMC contraction, proliferation, gene expression and migration.[26] In fact, Rho kinase inhibitor suppressed the migration of VSMC.[27] However, further detailed study is required to clarify this possibility.

In conclusion, our finding show that **1** had a potent inhibitory effect on rat VSMC proliferation and migration by inhibiting cell cycle progression through coordinate effects on positive regulator, cyclin D_1. Sesquiterpene **1** is a promising candidate for additional biological evaluation to further define its potential as an inhibitory modulator of VSMC responses that contribute to restenosis following PTCA and to the development and progression of atherosclerosis.

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