

Original Article



Shinjulactone A Blocks Vascular Inflammation and the Endothelial-Mesenchymal Transition

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ABSTRACT

Objective: The endothelial inflammatory response plays an important role in atherogenesis by inducing nuclear factor (NF) κ B-dependent cell adhesion molecule expression and monocyte recruitment. Here, we screened for natural ligands and investigated the ability of shinjulactone A to inhibit interleukin-1 β (IL-1 β)-induced endothelial inflammatory signaling.

Methods: The natural compound library included 880 single compounds isolated from medicinal plants by the Korean Medicinal Material Bank. Primary endothelial cells were pretreated with single compounds before stimulation with IL-1 β to induce endothelial inflammation. Endothelial inflammation was measured by assaying NF κ B activation and monocyte adhesion. The endothelial-mesenchymal transition (EndMT) was evaluated using cell type-specific marker protein expression and morphology.

Results: Shinjulactone A was identified as an efficient blocker of IL-1 β -induced NF κ B activation, with a half-maximal inhibitory concentration of approximately 1 μ M, and monocyte recruitment in endothelial cells. However, it did not affect lipopolysaccharide-induced NF κ B activation in macrophages. Compared to Bay 11-782, a well-known NF κ B inhibitor that shows considerable cytotoxicity during long-term treatment, shinjulactone A did not affect endothelial cell viability. Furthermore, it also significantly inhibited the EndMT, which is known to promote atherosclerosis and plaque instability.

Conclusion: We suggest that shinjulactone A may be an effective and safe drug candidate for atherosclerosis because it targets and inhibits both endothelial inflammation and the EndMT, without impairing NF κ B-dependent innate immunity in macrophages.

Keywords: Inflammation; Atherosclerosis; Endothelial cells

INTRODUCTION

Cardiovascular disease is currently a major cause of death throughout the world. Atherosclerosis is responsible for most mortality from cardiovascular diseases including heart attacks or strokes.

Atherosclerosis is a chronic inflammatory disease, and immune cell recruitment to the endothelium provokes atherosclerotic plaque development under hyperlipidemic conditions.¹

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Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Conceptualization: Yun S; Data curation: Jang Y; Formal analysis: Jang Y, Lee J, Immanuel J; Funding acquisition: Yun S; Investigation: Jang Y, Lee J, Immanuel J; Project administration: Yun S; Resources: Kwon HS, Kwon YJ; Supervision: Yun S, Shin JW; Writing - original draft: Yun S, Jang Y; Writing - review & editing: Yun S, Shin JW.

Endothelial cells respond to inflammatory stimuli, such as disturbed blood flow or multiple inflammatory ligands, by activating the major inflammatory mediator nuclear factor (NF) κ B, which transcriptionally upregulates cell adhesion molecules, including vascular cell adhesion molecule 1, intercellular adhesion molecule 1, E-selectin, and monocyte chemoattractant protein 1 for the endothelial recruitment of monocytes.² Endothelial knockout of NF κ B signaling has been found to protect mice from atherosclerosis.³

Interleukin-1 β (IL-1 β) is an important proinflammatory cytokine produced by macrophages and endothelial cells via the inflammasome pathway and plays an important role in atherogenesis.⁴ The Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial showed that the antibody-mediated blockade of IL-1 β led to reduced atherosclerosis-related cardiovascular events in patients.⁵ The clinical trial also showed a statistically significant increase in infection rates among patients treated with IL-1 β blocking antibodies, which implies that systemic IL-1 β blockade has side effects on immune function.⁶

The endothelial-mesenchymal transition (EndMT) is a de-differentiation process of endothelial cells converting to mesenchymal cell types. During this process, endothelial cells lose multiple endothelial cell-specific marker proteins such as vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule 1 and start to express mesenchymal smooth muscle actin (α -SMA), vimentin, and fibronectin.⁷ Accumulating evidence indicates that the EndMT is involved in multiple vascular diseases, including fibrosis, pulmonary arterial hypertension, and atherosclerosis.⁸ Transforming growth factor- β 1 (TGF- β 1) and inflammatory stimuli, such as disturbed flow or IL-1 β , are required to induce the EndMT *in vitro*.⁹

We screened for natural compounds that can inhibit IL-1 β -induced NF κ B activation in endothelial cells. Among approximately 800 single compounds isolated from various plants that have been medicinally used in Korea for a long time, shinjulactone A (isolated from *Ailanthus altissima* Swingle) was identified as one of the most potent inhibitors of endothelial inflammatory responses in response to IL-1 β treatment.

METHODS

1. Reagents

The antibodies used for immunoblotting in this study were phospho-NF κ B p65 (S⁵³⁶; Cell Signaling Technology, Danvers, MA, USA), β -actin (Cell Signaling Technology, Danvers, MA, USA), anti-rabbit immunoglobulin G (IgG) (R&D Systems, Minneapolis, MN, USA) and anti-mouse IgG (RSA1122, BioActsBM&S, Gimhae, Korea). An NF κ B inhibitor, BAY 11-7085 (Sigma, St. Louis, MO, USA), was used at two concentrations (1 μ M and 10 μ M). Lipopolysaccharide (LPS; used at 1 μ g/mL), IL-1 β (used at 10 ng/mL or 20 ng/mL), and TGF- β 1 (used at 10 ng/mL) were purchased from Sigma.

2. Cell culture

Primary bovine aortic endothelial cells (BAECs) were isolated and maintained in Dulbecco Modified Eagle Medium (DMEM; GenDEPOT, Katy, TX, USA) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. All cell types were maintained at 37°C in a 5% CO₂ incubator. THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin, streptomycin, and 0.05 mM β -mercaptoethanol. Macrophage RAW 264.7 cells were cultured in DMEM with 10% FBS, penicillin, and streptomycin.

3. EndMT

One day after re-plating, the cells were pre-treated with shinjulactone A (10 μ M) and dimethyl sulfoxide (DMSO) as control for 1 hour before incubation with TGF- β 1 (10 ng/mL) and IL-1 β (10 ng/mL). The medium was changed once every 2 days to supply fresh ligands. After 2 and 5 days, cell morphology was examined and harvested for immunoblotting with EndMT marker antibodies.

4. Immunoblotting

The protein sample was loaded in the same amount in the well of a 10% sodium dodecyl sulfate (SDS) –polyacrylamide gel, and the protein was denatured with SDS-running buffer. After transferring the protein to a nitrocellulose membrane (Nitrocellulose Filter; GE Healthcare Life Sciences, Marlborough, MA, USA) and blocking with 5% skim milk, the membrane was incubated overnight at 4°C with p-p65 and actin antibodies. After washing three times for 10 minutes with TBST, anti-rabbit IgG and anti-mouse IgG secondary antibodies were incubated for 1 hour at room temperature. After washing three times with TBST for 10 minutes each, the images were obtained using a chemiluminescent substrate (Thermo Fisher, Waltham, MA, USA). The results were quantified using ImageJ and Prism 5 software (GraphPad LLC, San Diego, CA, USA).

5. Monocyte adhesion

One day after cell seeding, shinjulactone A (1 μ M, 10 μ M) or DMSO as control was added to the endothelial cells 1 hour before treatment with IL-1 β (20 ng/mL). After 6 hours, THP-1 cells were centrifuged and resuspended in 5% bovine serum albumin (GeorgiaChem, Suwanee, GA, USA) in Hanks' balanced salt solution (HBSS, Gibco, Thermo Fisher). After washing the cells once with phosphate-buffered saline (PBS), the resuspended THP-1 cells were incubated on endothelial cell monolayers for 30 minutes in a 37°C incubator under 5% CO₂. After 30 minutes, the unbound THP-1 cells were washed 4 times with 0.5% BSA-containing HBSS. After fixing the cells with paraformaldehyde solution (4%) in PBS (USB, Thermo Fisher) for 10 minutes, the bound monocytes were counted under a microscope. The results were quantified using Prism 5 software (GraphPad LLC).

6. Cell viability

BAECs were treated with shinjulactone A (1–10 μ M) or BAY 11-782 (1–10 μ M) and maintained at 37°C in an incubator under 5% CO₂ incubator. Live cells were counted every 24 hours after trypan blue staining (Thermo Fisher) using a Countess II FL automated cell counter and the results were quantified using Prism 5 software (GraphPad LLC).

RESULTS

1. Shinjulactone A mediated the suppression of endothelial inflammation

We examined NF κ B activation to investigate the effect of shinjulactone A on vascular inflammation. After pretreatment with 0–10 μ M shinjulactone A, endothelial cells were incubated with IL-1 β for 6 hours. NF κ B activation was monitored by immunoblotting for pS³⁶-p65, which decreased gradually with increasing doses of shinjulactone A compared to the DMSO control. The known NF κ B inhibitor Bay 11-782 almost completely blocked NF κ B activation at a 1 μ M concentration, and 5 μ M shinjulactone A led to the same level of NF κ B suppression (**Fig. 1**).

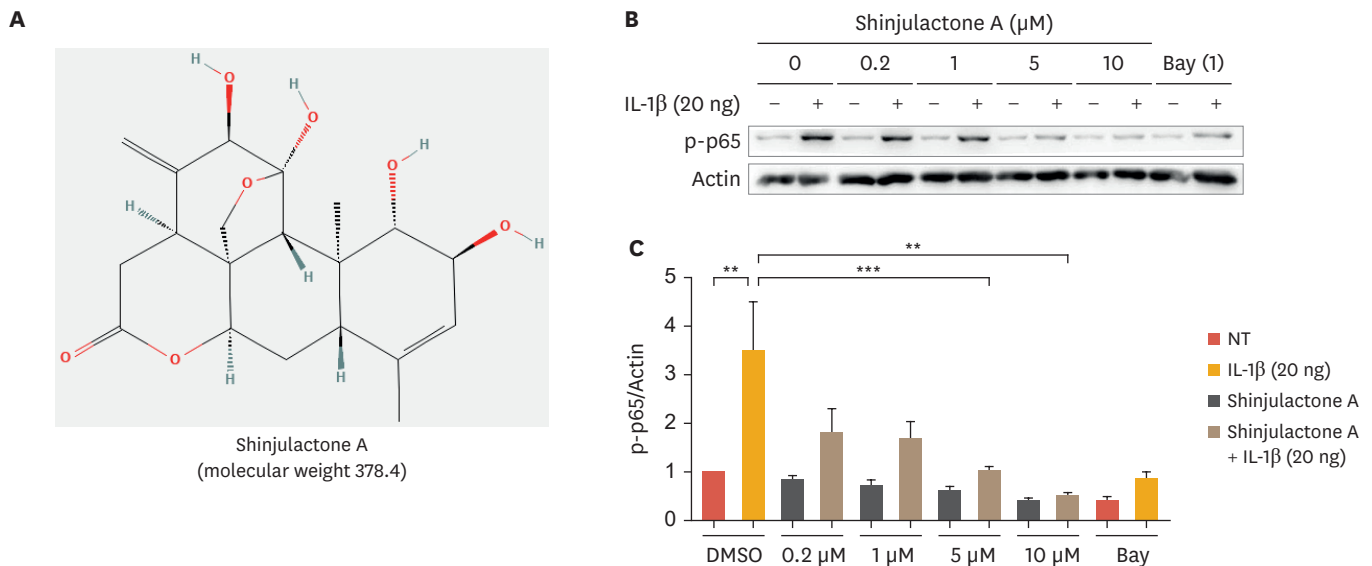


Fig. 1. Shinjulactone A decreased the expression level of p-p65 in a dose-dependent manner. (A) Structural formula and molecular weight of shinjulactone A. (B) Immunoblotting analysis of p-p65 and actin. BAEC cells were pretreated with 0.2 μM, 1.0 μM, 5.0 μM, and 10.0 μM concentrations of shinjulactone A, then treated with Bay and IL and incubated for 6 hours. (C) Quantification of immunoblotting result (n=3). BAEC, bovine aortic endothelial cell; IL, interleukin; NT, no treated cells; DMSO, dimethyl sulfoxide. ***p*<0.005, ****p*<0.0005.

The adhesion of monocytes to endothelial cells leads to EC transmigration and further inflammation.¹⁰ To investigate the effects of shinjulactone A on monocyte adhesion, confluent BAEC monolayers pretreated with shinjulactone A (1–10 μM) for 1 hour were stimulated by IL-1β (20 ng/mL) for 6 hours. Then, the THP-1 monocyte cells were added and incubated for 30 minutes. After washing the non-bound monocytes, the adherent monocytes were counted and quantified. IL-1β induced monocyte adhesion to BAEC was significantly reduced in the shinjulactone A-treated cells, supporting the anti-inflammatory activity of shinjulactone A (Fig. 2).

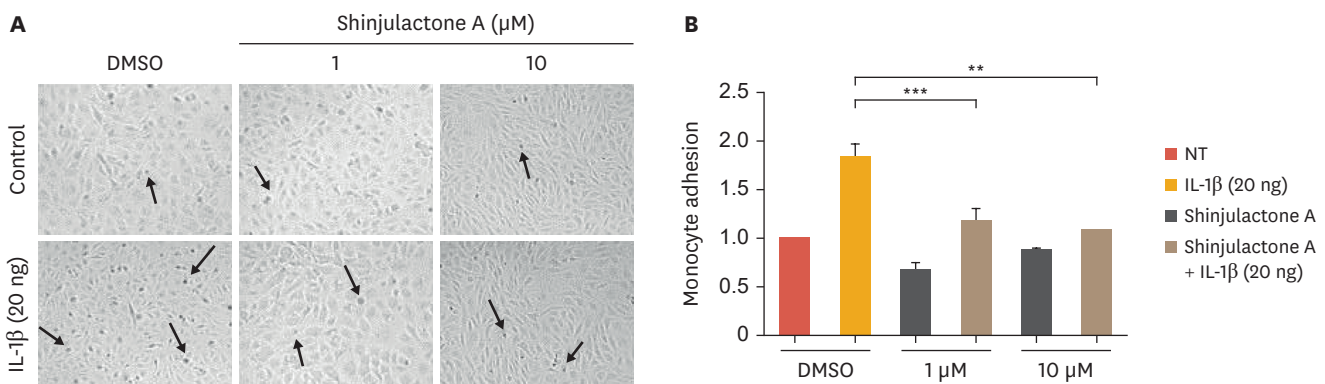


Fig. 2. Shinjulactone A reduces monocyte adhesion by IL-1β. (A) Representative micrographs at 10× original magnification of monocyte adhesion to the BAEC cells. In BAEC cells with shinjulactone A 1 μM and 10 μM, the small round cells are adhered THP-1 monocytes (arrowheads). Total numbers of adherent cells in a total of three randomly selected microscopic fields. (B) Quantification of monocyte adhesion (n=3). IL, interleukin; BAEC, bovine aortic endothelial cell; NT, no treated cells; DMSO, dimethyl sulfoxide. ***p*<0.005, ****p*<0.0005.

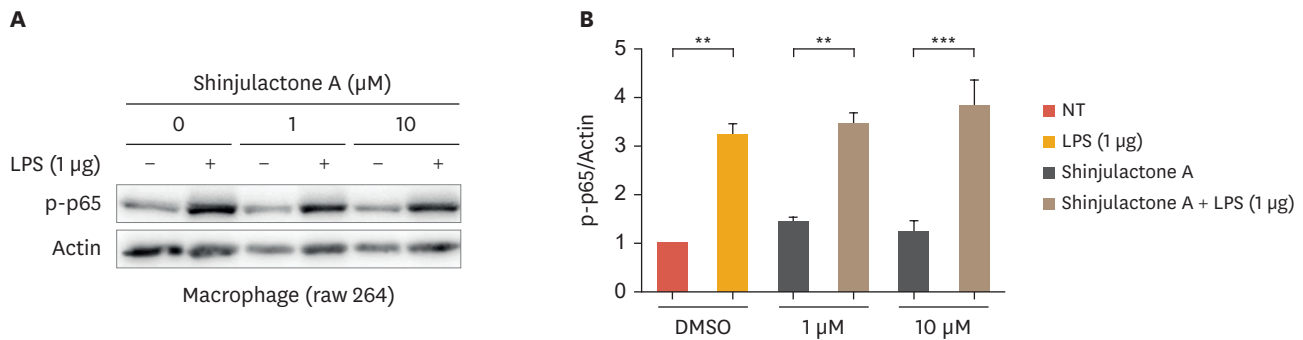


Fig. 3. LPS-induced p65 phosphorylation was not blocked by shinjulactone A in macrophage. (A) Immunoblotting analysis of p-p65 and actin. In macrophage, the phosphorylation level of p65 was confirmed by pre-treatment with shinjulactone A 1 μM and 10 μM, followed by treatment with LPS for 6 hours. (B) Quantification of immunoblotting result (n=3). LPS, lipopolysaccharide; NT, no treated cells; DMSO, dimethyl sulfoxide. **p<0.005, ***p<0.0005.

2. Shinjulactone A did not block LPS-induced NFκB activation in macrophages

We next tested whether shinjulactone A-dependent NFκB inhibition is EC-specific. LPS is well known to elicit the inflammasome pathway by NFκB activation in macrophages.¹¹ LPS efficiently induced NFκB activation in macrophage cells; however, shinjulactone A could not block NFκB activation in this cell type (**Fig. 3**). This result suggests that shinjulactone A does not inhibit inflammation in EC by directly controlling the NFκB activation machinery, as is the case for Bay 11-782; instead, it controls cell type-specific or ligand-specific signaling.

3. Shinjulactone A did not have cytotoxicity

To assay cytotoxicity, the cultured BAECs treated with shinjulactone A (1–10 μM) were compared with the control and BAY 11-782 (1–10 μM)-treated cells. The cells were observed for 5 days after treatment. Viable cells were counted every 24 hours using an automated cell counter after trypan blue staining. This assay revealed that the viability of the cells treated with shinjulactone A (1–10 μM) was similar to the non-treated cells, which indicates that shinjulactone A (1–10 μM) is not toxic to the cells (**Fig. 4**).

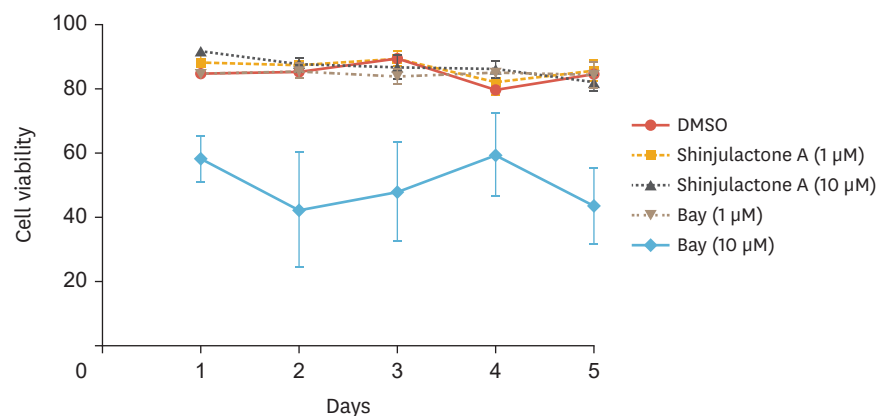


Fig. 4. Shinjulactone A did not have cytotoxicity. Viability of BAEC cells treated with shinjulactone A 1 μM, 10 μM and Bay 1 μM, 10 μM. After treatment, cells were incubated for 1 to 5 days and cell viability was detected by cell counting. BAEC, bovine aortic endothelial cell; DMSO, dimethyl sulfoxide.

4. Shinjulactone A decreases the EndMT

In order to examine the effect of shinjulactone A on the EndMT, we treated endothelial cells with IL-1 β and TGF- β 1 and examined the levels of endothelial and mesenchymal markers. In DMSO-treated control cells, the level of the endothelial marker protein VE-cadherin decreased and that of the mesenchymal marker α -SMA remarkably increased after TGF- β 1/IL-1 β treatment. However, shinjulactone A treatment partially blocked the downregulation of VE-cadherin and upregulation of α -SMA after 5 days of treatment (Fig. 5A and B). In the absence of IL-1 β and TGF- β 1, endothelial cells had a cobblestone-like phenotype and remained closely attached to each other. Upon co-treatment for 2 days, the endothelial cells showed a spindle-shaped morphology and loss of cell-cell adhesion, but when shinjulactone A was administered, the cell morphology did not change significantly compared to the no-treatment control cells. With co-treatment for 5 days, shinjulactone A still maintained the cell-to-cell adhesion of endothelial cells (Fig. 5C).

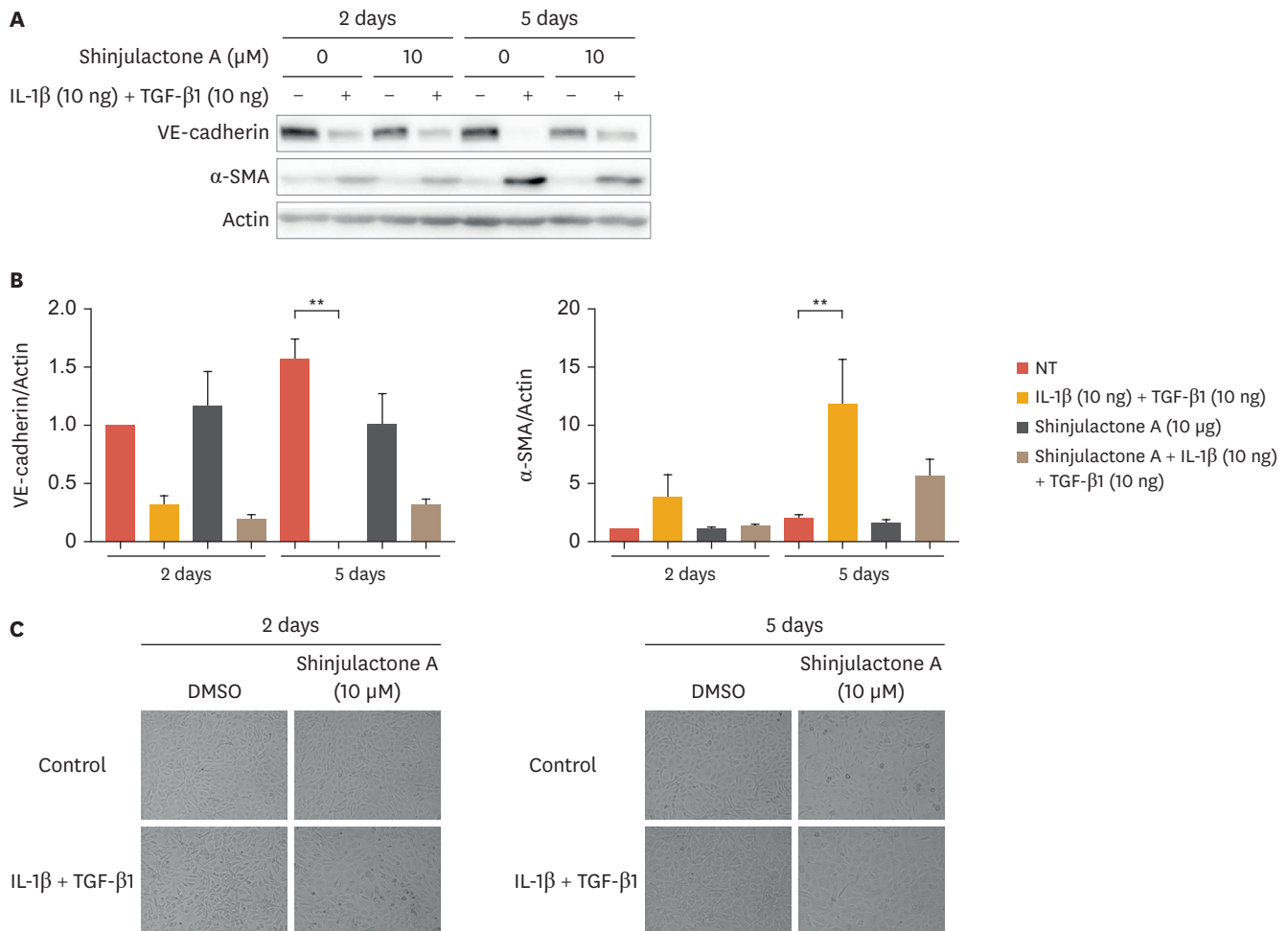


Fig. 5. Shinjulactone A suppressed TGF- β 1/IL-1 β -induced EndMT. (A) Immunoblotting analysis of endothelial marker (VE-cadherin) and mesenchymal marker (α -SMA) expression levels for 2 days and 5 days. (B) Quantification of Immunoblotting result. (C) Morphology of BAEC cells cultured for 2 days and 5 days upon treatment with IL-1 β or TGF- β 1 (n=3). TGF, transforming growth factor; IL, interleukin; VE, vascular endothelial; α -SMA, α -smooth muscle actin; BAEC, bovine aortic endothelial cell. **p<0.005.

DISCUSSION

Shinjulactone A is a quassinoid from *Ailanthus altissima* Swingle that has been used for the treatment of colds and gastric diseases in Asian countries.¹² Unlike the very similar quassinoid ailanthone (which has a carbonyl group at C₂ instead of a hydroxyl group), which has been widely studied for its anti-cancer effect, little is known about the function of shinjulactone A except that it exerts mild anti-tumor activity.¹³ Our analysis revealed a novel function of shinjulactone A in suppressing vascular inflammation and showed potential as a therapeutic for vascular inflammatory diseases.

Some preclinical studies and clinical trials have suggested that anti-inflammatory drugs can be an alternative strategy for atherosclerosis treatment.¹⁴ However, the sustained suppression of systemic inflammation could also lead to a higher risk of infection. Therefore, targeting tissue- or target-specific inflammation would be important. Our study suggested the possibility that shinjulactone A might inhibit IL-1 β -induced inflammatory signaling in an endothelial cell-specific manner without affecting immune cells such as macrophages. In particular, it is expected to be effective in inhibiting atherosclerosis regulated by NF κ B. Inhibition of NF κ B in endothelial cells is well known to inhibit the development of atherosclerosis by preventing the expression of proinflammatory mediators and recruitment of immune cells to the arterial wall.¹⁵ Another study demonstrated that the inhibition of endothelial NF κ B in ApoE mice fed a cholesterol-rich diet impaired the recruitment of macrophages from endothelial cells to atherosclerotic plaques and reduced the expression of cytokines and chemokines in the aorta.¹⁶ However, NF κ B is also involved in the resolution of inflammation,¹⁷ which makes it infeasible to use systemic inhibition of NF κ B for atherosclerosis treatment or prevention. We found that shinjulactone A inhibited NF κ B in a cell type-dependent manner, suggesting that we can target NF κ B activity more specifically in endothelial cells by perturbing cell type-specific NF κ B activation pathways.

The EndMT is known to play an important role in several adult cardiovascular diseases.¹⁸ In particular, mesenchymal cells induce the secretion of proinflammatory molecules in atherosclerosis, influence matrix and collagen production, and regulate plaque integrity.¹⁹ We found that a mesenchymal marker (α -SMA) significantly increased and an endothelial marker (VE-cadherin) completely disappeared in response to TGF- β and IL-1 β treatment for 5 days. Shinjulactone A significantly reduced the increased α -SMA expression level and partially restored the VE-cadherin expression level. These results suggest that shinjulactone A treatment can be very useful since it targets multiple pro-atherosclerotic disease mechanisms, including inflammation and the EndMT.

In summary, our study identified a novel therapeutic agent, shinjulactone A from a medicinal plant, that can control endothelial inflammation and the EndMT. The application of this compound in a hyperlipidemic animal model would reveal its *in vivo* efficacy and detailed atherosclerotic phenotypes.

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