Inhibitory Effects of Pisiferic Acid on Angiogenesis and Lymphangiogenesis

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Fig. 1   Chemical structures of pisiferic acid (**A**) and carnosic acid (**B**).

Abstract
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Pisiferic acid, a diterpene isolated from *Chamaecyparis pisifera*, has a chemical structure similar to carnosic acid, which has antioxidant activity and exerts various biological effects such as antibiotic and antiangiogenic activity. However, the effect of pisiferic acid on angiogenesis, which is involved in various diseases including cancer and diabetic retinopathy, has not been elucidated. We found that pisiferic acid inhibited angiogenesis and suppressed human umbilical vein endothelial cell tube formation and proliferation but had no effect on human umbilical vein endothelial cell chemotaxis. Furthermore, in lymphangiogenesis assays, pisiferic acid inhibited lymphangiogenesis and lymphatic endothelial cell tube formation and proliferation. Together, these results suggest pisiferic acid to be a useful natural compound, such as carnosic acid, with beneficial effects on angiogenesis- and lymphangiogenesis-related diseases.

Key words
Chamaecyparis pisifera · Cupressaceae · pisiferic acid · angiogenesis · lymphangiogenesis · endothelial cells

Abbreviations
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bFGF:	basic fibroblast growth factor
CA:	carnosic acid
FGF-2:	fibroblast growth factor-2
HUVEC:	human umbilical vein endothelial cell
LEC:	lymphatic endothelial cell
PA:	pisiferic acid
VEGF:	vascular endothelial growth factor

Supporting information available online at http://www.thieme-connect.de/products

Fig. 2   Inhibitory effect of PA in the *ex vivo* angiogenesis model. **A** Representative result of PA (100 µM). **B** Microvessel length was measured on day 7. Values are the means ± SEM (n = 3). Significant difference from the control: ＊＊p < 0.01. CA (50 µM) was used as a positive control.

PA is an abundant natural product found in the leaves of the Japanese tree Sawara [*Chamaecyparis pisifera* (Siebold & Zucc.) Endl.; Cupressaceae] (○**Fig. 1A**) [1], and has been reported to have various biological activities including DNA polymerase inhibition [2], chitin synthase inhibition [3], and antibiotic activity [4]. It also affects cell signaling pathways in HL60 cells and yeast [5,6]. PA has been used for the synthesis of its derivative CA (○**Fig. 1B**) [7]. CA has been extensively studied and reported to have various biological activities such as antioxidant activity, promotion of neurite outgrowth, and a protective effect on the nerv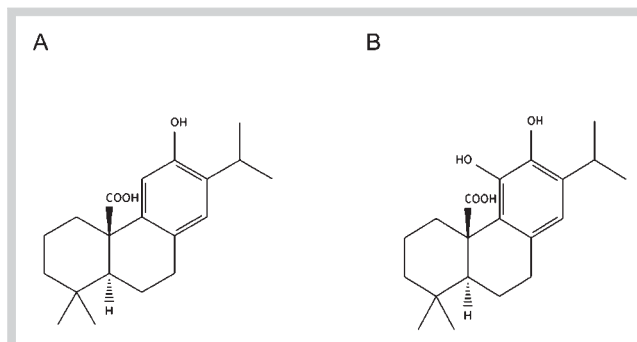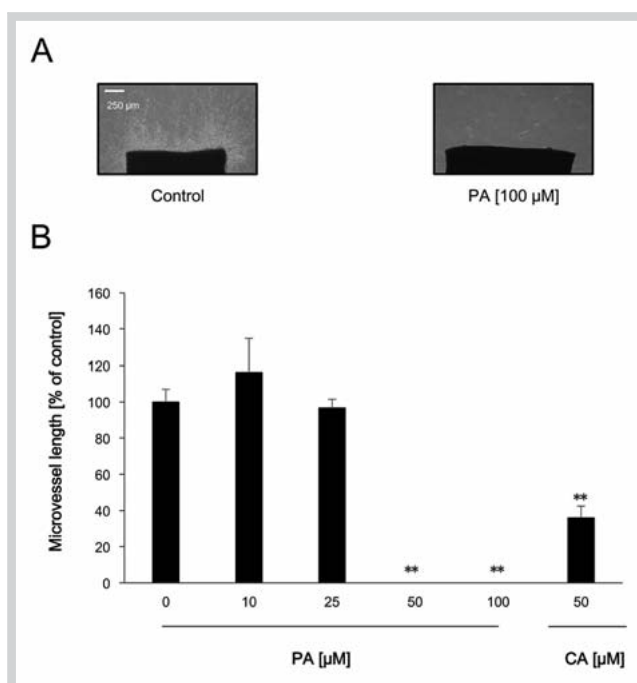e system [8–10]. Recently, we have reported that CA is an inhibitor of angiogenesis [11]. Angiogenesis refers to the formation of new blood vessels and is involved in various diseases such as cancer, diabetic retinopathy, and rheumatoid arthritis [12]. Thus, natural products that inhibit angiogenesis have drawn attention for the development of angiogenesis inhibitors. While CA has been known to inhibit angiogenesis, the effect of PA on angiogenesis has not been reported thus far. In this study, we examined the effect of PA on angiogenesis, and, furthermore, on lymphangiogenesis, which is also involved in inflammation and various diseases [13].

In an *ex vivo* angiogenesis assay using the rat aortic ring, PA suppressed the outgrowth of microvessels that grew in a week, as shown in ○**Fig. 2**. At 50 µM, PA completely inhibited angiogenesis, whereas CA suppressed angiogenesis by about 40 % at the same concentration. However, PA did not show a suppressive effect at 25 µM, but CA significantly suppressed angiogenesis at this concentration [11].
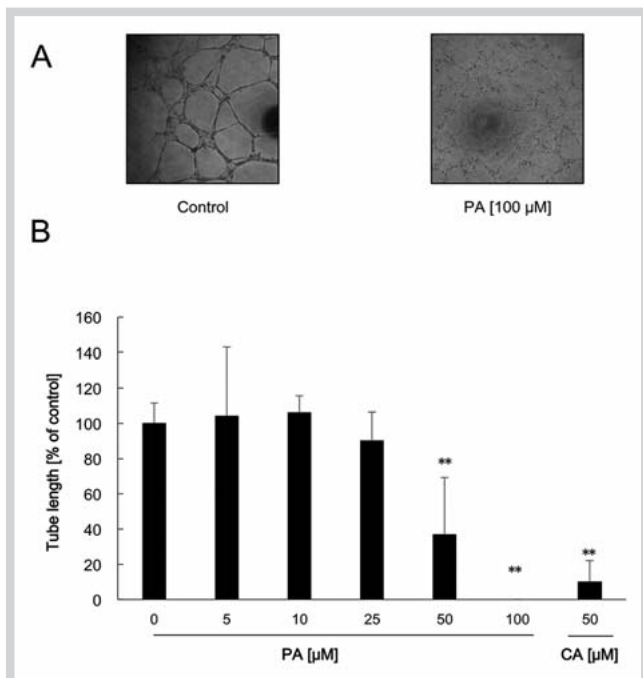
Fig. 3 Effect of PA on HUVEC tube formation on a reconstituted basement membrane. (**A**) HUVEC tube formation on reconstituted gel was observed at 12 h, and the capillary length (**B**) was measured. Values are the means ± SD (n = 7–12). Significant difference from the control: **p < 0.01. CA (50 μM) was used as a positive control.

Fig. 4 Effect of PA on HUVEC proliferation. Values are the means ± SD (n = 10). Significant difference from the control: *p < 0.05 and **p < 0.01. CA (50 μM) was used as a positive control.

Fig. 5 Effect of PA on HUVEC chemotaxis. Migrated cells after 6 h incubation were counted. Values are the means ± SEM (n = 3). Significant difference from the control: **p < 0.01. CA (50 μM) was used as a positive control.

To determine how PA inhibited angiogenesis, we examined its effect in some models using HUVECs. PA significantly suppressed HUVEC tube formation and proliferation at 50 μM and 25 μM, respectively (○ **Figs. 3** and **4**), but had no effect on HUVEC migration induced by VEGF (○ **Fig. 5**). Thus, PA exerts antiangiogenic activity by suppressing endothelial cell capillary formation and proliferation. CA exerted suppressive effects in all three HUVEC assays as previously reported [11]. These results suggest that the inhibitory mechanisms by which both compounds exert antiangiogenic activity are different, and that the difference could be attributed to their structural difference. In the HUVEC migration assay, VEGF was used to induce HUVEC migration, but in HUVEC tube formation and proliferation assays, the used medium contained bFGF, and not VEGF, suggesting that PA may not affect the VEGF receptor-mediated cell signaling pathway. Recently, the antiangiogenic activity of fucoxanthin and siphonaxanthin, both natural compounds, has been reported [14, 15]. Both carotenoids did not affect VEGF-induced HUVEC migration, and we demonstrated that both carotenoids downregulated the FGF-2 (bFGF) signaling pathway [16]. Thus, PA might exert its inhibitory effect on angiogenesis in a similar manner. Other derivatives of PA might have unique effects on endothelial cells and angiogenesis.

Lymphangiogenesis is also an important target for preventing various diseases such as cancer and inflammatory conditions. To clarify whether PA affects lymphangiogenesis, we examined its effect in an *ex vivo* lymphangiogenesis model [17]. Since the method was slightly modified, immunohistochemical analysis was performed to confirm the induction of lymphangiogenesis in the model. The morphology of capillaries was the same as that reported previously (○ **Fig. 6**), and the anti-LYVE-1 antibody reacted with the capillarie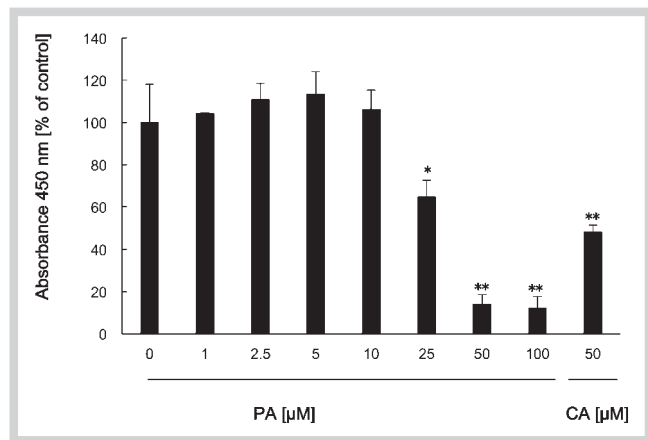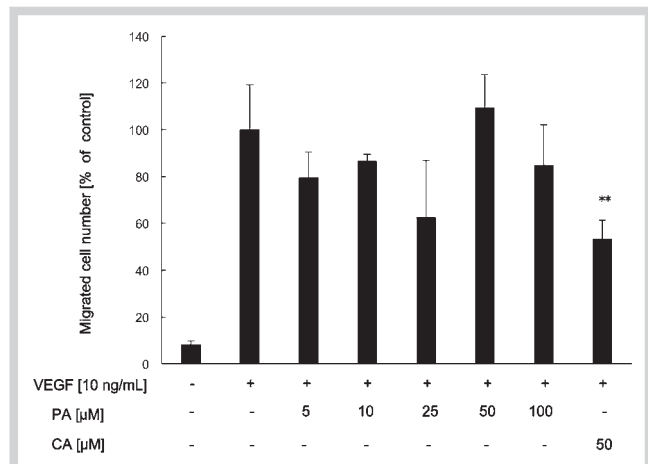s (data not shown), confirming the occurrence of lymphangiogenesis. PA completely inhibited lymphangiogenesis at 100 μM and strongly inhibited it at 50 μM in the model (○ **Fig. 6**). Since PA suppressed HUVEC tube formation and proliferation, we examined its effect on LEC tube formation and proliferation. PA exerted inhibitory effects in both assays (○ **Figs. 7** and **8**), suggesting that PA exerts inhibitory effects targeting the same molecules on both endothelial cells for angiogenesis and lymphangiogenesis. Recently, it has been demonstrated that FGF-2 (bFGF) and VEGF-C, an angiogenesis and lymphangiogenesis enhancing growth factor, respectively, collaboratively stimulate tumor growth, angiogenesis, lymphangiogenesis, and metastasis [18]. Thus, natural products suppressing both angiogenesis and lymphangiogenesis or the bFGF pathway would be useful as anticancer agents. Although elucidation of the mechanism by which PA exerts antilymphangiogenic activity is necessary, this study suggests that PA might be beneficial in suppressing both angiogenesis- and lymphangiogenesis-related diseases. In this study, we found that PA suppresses both angiogenesis and lymphangiogenesis. We previously reported the antiangiogenic
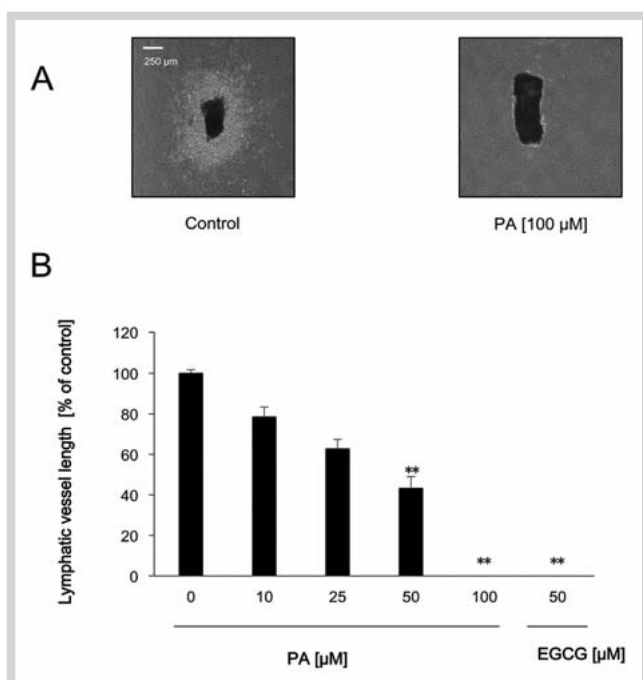
Fig. 6 Inhibitory effect of PA in the *ex vivo* lymphangiogenesis model. **A** Representative result of PA (100 μM). **B** Microvessel length was measured on day 7. Values are the means ± SEM (n = 3–4). Significant difference from the control: **p < 0.01. EGCG (50 μM) was used as a positive control.
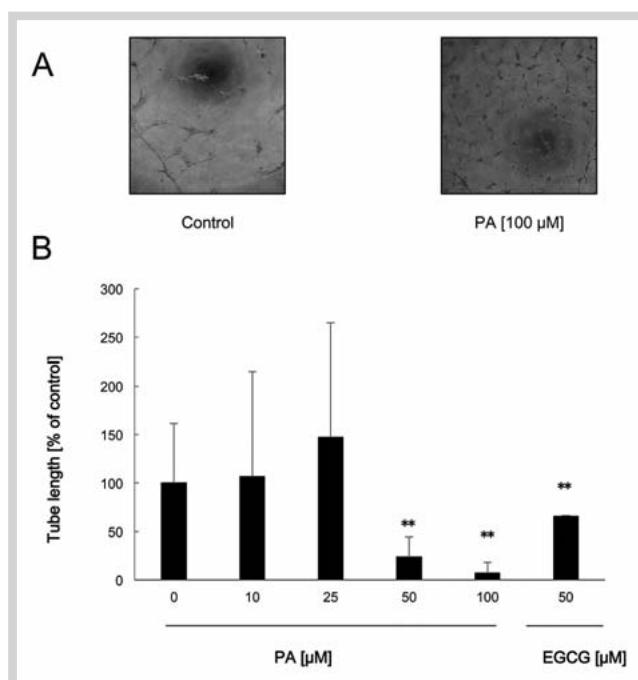

Fig. 7 Effect of PA on LEC tube formation on a reconstituted basement membrane. (**A**) LEC tube formation on reconstituted gel was observed at 12 h, and the capillary length (**B**) was measured. Values are the means ± SD (n = 3). Significant difference from the control: **p < 0.01. EGCG (50 μM) was used as a positive control.

effect of CA, whose chemical structure is very similar to PA. The inhibitory effects of PA on angiogenesis are similar to that of CA, but its effect on HUVECs is different. CA suppresses HUVEC tube formation, proliferation, and migration, whereas PA has no effect on HUVEC migration. This difference in biological activity could be attributed to the difference in their targets on endothelial cells. To reveal the difference, our study focused on their effects on cell signaling pathways. CA is a unique compound because it has various biological effects, especially in the neural system. PA is used for the synthesis of CA [7]. If PA exerts similar effects as CA in the neural system, PA may be used instead of CA. Thus, research is underway, and we have found that PA affects neural cell function similar to CA, but the mechanisms seem to be different (unpublished data). The difference would be related to their mechanisms of antiangiogenic and antilymphangiogenic activities.

Materials and Methods
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PA (purity > 99%), isolated as previously described [1], was dissolved in DMSO to 20 mM as a stock solution, and kept at − 25 °C until use. As positive control inhibitors, CA (purity > 91%) from Sigma-Aldrich and (−)-epigallocatechin gallate (EGCG; purity > 99%) from Nacalai Tesque were used for angiogenesis [11] and lymphangiogenesis [19], respectively. The purity of all compounds was determined by HPLC. All animal experiments were approved by the animal experiment committee of Hiroshima University Animal Research Committee; the approval numbers were C11-1 (2011/04/12) and C13–2 (2013/04/11). The *ex vivo* angiogenesis assay was performed according to a previously reported method [20, 21]. The *ex vivo* lymphangiogenesis assay was performed according to a previously described method, with
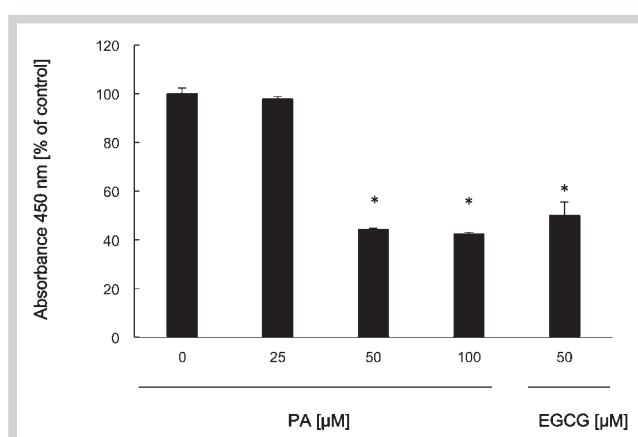

Fig. 8 Effect of PA on LEC proliferation. Values are the means ± SD (n = 5). Significant difference from the control: *p < 0.05. EGCG (50 μM) was used as a positive control.

some modifications [17]. Rat thoracic ducts were excised from the rats and cut into approximately 1 mm sections under a stereomicroscope. The lymphatic duct rings were embedded in type I collagen gel in a 6-well culture plate as described in the *ex vivo* angiogenesis assay (Supporting Information). After 30 min to form a gel at 37 °C in a CO_2 incubator, 2 mL of RPMI 1640 medium (Gibco) containing 10% FBS (Lonza), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) were added to the wells. PA or EGCG solution or DMSO was added, and the plates were incubated for 7 days at 37 °C in a CO_2 incubator. The images of the

lymphatic rings were taken with a digital camera (OLYMPUS DSE330-A system), and the length of the capillaries was measured using Adobe Photoshop software.

Supporting information

A detailed description of the *ex vivo* angiogenesis assay and *in vitro* assays using HUVEC and LEC and statistical analysis are available as Supporting Information.

Conflict of Interest
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The authors declare no conflict of interest.

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