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Synthesis and biological evaluation of vitamin K derivatives as angiogenesis inhibitor

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ABSTRACT

Ten vitamin K_3 derivatives were synthesized and screened for anti-angiogenic activity. Results indicated that amine derivatives (**1a–d**) exerted a stronger inhibition effect on angiogenesis compared to alkyl derivatives (**2a–d**). In addition to being the most potent inhibitor, **1b** also suppressed human umbilical vein endothelial cell tube formation and proliferation. These results suggest that vitamin K_3 amine derivatives with shorter alkyl chains, such as **1b**, could be useful for developing anti-angiogenic agents.

1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels, and it is involved in various pathological states including solid tumor growth, diabetic retinopathy, and age-related macular degradation.[1] Inhibition of angiogenesis is an important target for cancer treatment because not only solid tumor growth but also metastasis is facilitated through angiogenesis.[2] Interestingly, administration of drugs exhibiting anti-angiogenic activity has been shown to reduce the risk of brain diseases in older patients.[3] Angiogenesis-related diseases pose serious medical problems worldwide; therefore, much research has been devoted to developing effective angiogenesis inhibitors.

Natural products are important resources for new drug development. Many anti-angiogenic substances such as polysaccharides, phenolic compounds, carotenoids, and vitamins have been isolated from natural products.[4–8] Several derivatives of anti-angiogenic natural products have been assessed, and it was found that acylation of catechin and epicatechin, the phenolic compounds in green tea, enhances their anti-angiogenic effect.[9,10] The acylated derivatives of catechin and epicatechin inhibit angiogenesis process, endothelial cell tube formation (capillary formation), and DNA polymerase activity,[9,10] which is a molecular target of anti-cancer drugs. Thus, chemical modification of anti-angiogenic natural products could help develop more effective anti-angiogenic agents.

The importance of vitamins to human health is well established. Some vitamins, including B_6, D_3, and K_2, and the vitamin E derivative tocotrienol, exert anti-angiogenic activity.[11–15] We have also reported that vitamin K_3 and napthoquinone (a vitamin K_3-related compound) inhibit angiogenesis.[8,16] In addition, 1,4-naphthoquinone derivatives have been reported to exert anti-angiogenic activity.[17,18] These data suggest that vitamin K_3 structure is important to design new anti-anigonenic agents. In this study, we synthesized 10 vitamin K_3 derivatives (**1a–d**, **2a–d**, **3**, and **4**) (Fig. 1) and evaluated their anti-angiogenic effect on an ex vivo angiogenesis model using rat aortic rings. To determine the anti-angiogenic mechanism of the most potent inhibitor **1b**, its effect was evaluated using human umbilical vein endothelial cells (HUVECs).

2. Results and discussion

2.1. Effects of vitamin K derivatives on ex vivo angiogenesis

To investigate the relationship between chemical structure and anti-angiogenic activity, we examined the effect of vitamin K_3

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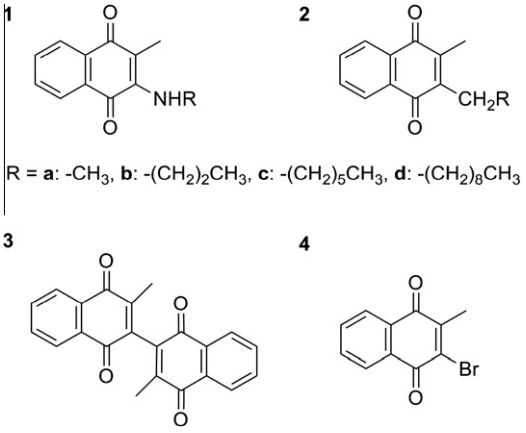

Figure 1. Structure of vitamin K_3 derivatives.

derivatives on angiogenesis at 100 μM in a rat aortic ring assay. We found that **1a**, **1b**, **2a**, and **4** suppressed angiogenesis, whereas the other compounds had no inhibitory effect on angiogenesis (Fig. 2).

A general trend was observed for the anti-angiogenic effects of amine and alkyl derivatives. The inhibitory effect of amine derivatives (**1a**, **1b**) on angiogenesis was stronger than that of alkyl derivatives (**2a**, **2b**). Interestingly, although **1b** and **2b** have a similar structure, **1b** exerted a stronger anti-angiogenic effect (Fig. 2). In contrast, **1c**, **1d**, **2c** and **2d**, which have long alkyl chains, had no inhibitory effect on angiogenesis. These results show that the presence of long alkyl chains weakens the anti-angiogenic effect of vitamin K_3 derivatives. Although the molecular mechanism of the inhibitory effect on angiogenesis by vitamin K_3 derivatives has not been elucidated, the long alkyl side-chain might reduce the binding affinity of the quinone moiety with the target protein(s). Otherwise, the differences in electrochemical properties or electron affinities due to the substituents at the 3-position of vitamin K_3 might influence the enzyme-cofactor interactions.[19] However, these observations also suggest that some amine derivatives of vitamin K_3 could be effective angiogenesis inhibitors.

Among the 10 compounds tested, **1b** exerted the strongest anti-angiogenic activity (Fig. 2). As shown in Fig. 3, the inhibitory effect of **1b** was dose dependent and significantly stronger at values higher than 50 μM (p <0.01).

2.2. Compound 1b inhibits angiogenesis in vitro

Because **1b** exerted the strongest anti-angiogenic effect in the ex vivo angiogenesis model (Fig. 2), its effect in an in vitro angiogenesis model was examined using HUVECs in order to elucidate the anti-angiogenic mechanism. First, an HUVEC tube formation assay was performed. As shown in Fig. 4A, the HUVECs inoculated on reconstituted basement membranes (Matrigel) migrated, attached to each other, and finally formed tube structures. Compound **1b** strongly suppressed HUVEC tube formation at 100 μM and at the same concentration inhibited angiogenesis in a rat aortic ring assay (Fig. 4A and B). Next, the effect of **1b** on vascular endothelial growth factor (VEGF)-induced HUVEC migration was examined in a gelatin-coated Boyden chamber assay. While VEGF strongly stimulated HUVEC migration (Fig. 4C), **1b** had no statistically significant inhibitory effect on VEGF-induced HUVEC migration at the examined concentrations even though a weak suppressive effect was observed at 100 μM. In this assay, resveratrol, which is a major anti-angiogenic compound,[20,21] inhibited HUVEC migration. Finally, we examined the effect of **1b** on HUVEC proliferation. HUVECs were treated with various concentrations of **1b** for 72 h. As shown in Fig. 4D, there were significant differences (p <0.05) between control and **1b**-treated HUVECs (5–100 μM). Many angiogenesis inhibitors suppress endothelial cell proliferation in vitro. Similarly, **1b** exerted an anti-angiogenic effect mainly by suppressing endothelial cell proliferation. The IC_{50} value for the cytotoxicity of **1b** against cancer and normal cells, such as human colon carcinoma cells (HCT116) and human dermal fibroblasts (HDF), respectively, was more than 100 μM (data not shown), suggesting that the cytotoxicity might not account for the anti-angiogenic effects of **1b**. Vitamin K_3 and 1,4-naphthoquinone suppressed HUVEC proliferation, tube formation, and migration.[8,16] On the other hand, although **1b** suppressed HUVEC proliferation, its effects on HUVEC tube formation and migration were weaker than those of vitamin K_3 and 1,4-naphthoquinone. Thus, the anti-angio-

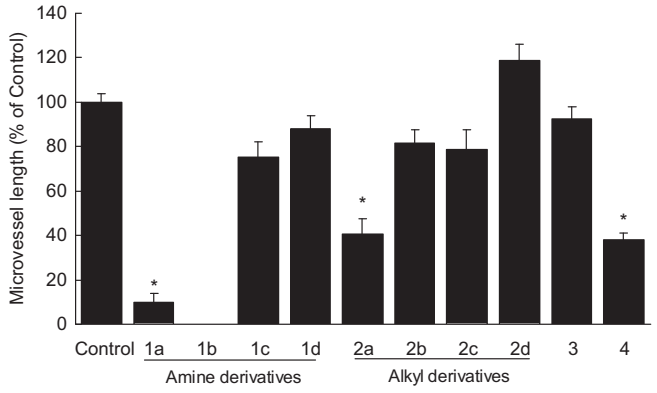

Figure 2. Effects of vitamin K_3 derivatives (100 μM each) on ex vivo angiogenesis using a rat aortic ring. Microvessel length was measured on day 7 of culture. Values are means ± SEM (n = 3–6). *Significantly different from control (p <0.01).

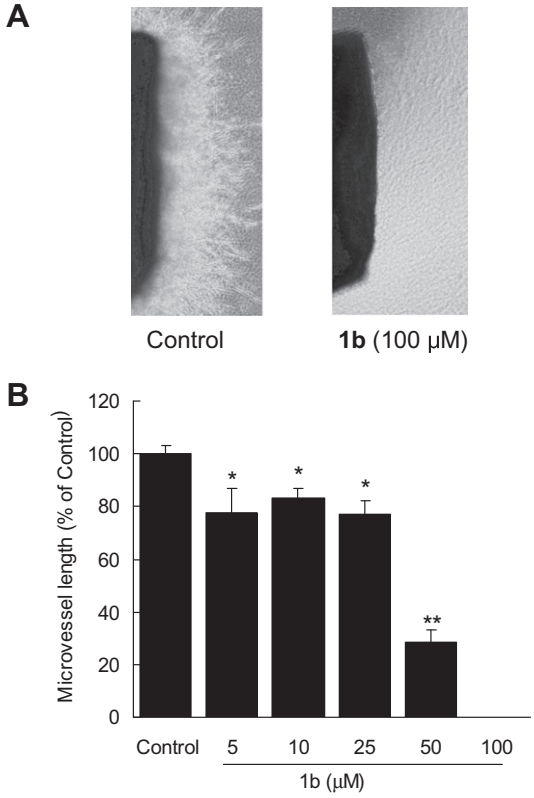

Figure 3. Suppressive effect of **1b** in ex vivo angiogenesis model. (A) Representative result of the inhibitory effect of **1b** (100 μM). (B) Microvessel length was measured on day 7 of culture. Values are means ± SEM (n = 3). Significantly different from control: *p <0.05 and **p <0.01.

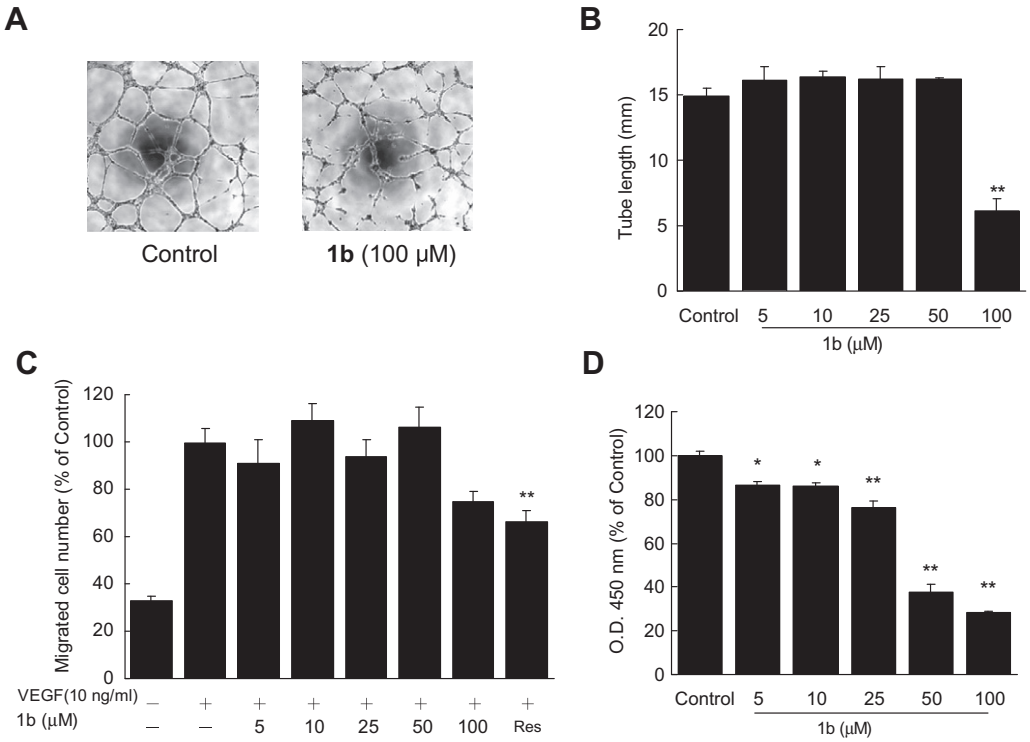

Figure 4. Effects of **1b** on HUVEC functions. (A and B) Effect of **1b** on HUVEC tube formation on reconstituted basement membrane; (A) cells were plated on reconstituted gel and observed after 12 h and (B) capillary length was measured. Values are means ± SEM (n = 3). (C) Effect of **1b** on HUVEC chemotaxis. HUVECs migrated after 6 h incubation to the lower surface of the filter was counted in five 200× filter fields. Means of fields of three filters ± SEM are shown (n = 9). Control was VEGF containing medium without sample. Resveratrol (100 μM) was used as a positive control inhibitor (Res). (D) Effect of **1b** on HUVEC proliferation. Values are means ± SEM (n = 3). Significantly different from control: $^{*}p$ <0.05 and $^{**}p$ <0.01.

genic mechanisms of **1b** were different from those of vitamin K_3 and 1,4-naphthoquinone, with the differences being attributed to chemical modification. To clarify the anti-angiogenic mechanism of vitamin K_3 amine derivatives, further study will be undertaken.

3. Conclusion

We synthesized 10 vitamin K_3 derivatives and investigated the relationship between chemical structure and anti-angiogenic activity. Our results indicated that amine derivatives (**1a–d**) exerted a stronger inhibition effect on angiogenesis compared to alkyl derivatives (**2a–d**). Alkyl chain length might be important for these vitamin K_3 derivatives to exert anti-angiogenic activity. Thus, vitamin K_3 amine derivatives with shorter alkyl chains could be useful for developing anti-angiogenic agents.

4. Experimental

4.1. Vitamin K_3 derivatives

All reactions were monitored by TLC, which was carried out on Silica Gel 60 F_{254} plates (Merck, Germany). Flash chromatography separations were performed on PSQ 100B (Fuji Silysia Co., Ltd, Japan). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL 270 MHz spectrometer (EX-270 W), using $CDCl_3$ (with TMS for ^{1}H NMR and chloroform-*d* for ^{13}C NMR as the internal reference) solution, unless otherwise noted. Chemical shifts were expressed in δ (ppm) relative to Me_4Si or residual solvent resonance, and coupling constants (J) were expressed in hertz. Melting points were determined with Yanaco MP-3S and were uncorrected. Infrared spectra (IR) were recorded on a Jasco FT/IR-410 spectrometer using NaCl (neat) or KBr pellets (solid) and reported in wave numbers (cm^{-1}). Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar I) under conditions as high resolution, using poly (ethylene glycol) as internal standard.

Compounds **1a** and **1b** were prepared by coupling of vitamin K_3 with methylamine or *n*-propylamine.[22] Compounds **2a**, **2b**, and **2d** were prepared by radical alkylation of vitamin K_3 with carboxylic acids in the presence of silver nitrate and ammonium persulfate.[23,24] Bivitamin K_3 (**3**) was prepared by our original method, which will be reported in due course. 2-Bromovitamin K_3 (**4**) was prepared by bromination of vitamin K_3.[25] Menaquinones were prepared according to the procedure reported by Mayer and Isler with a slight modification.[26] The structures of known compounds were confirmed by comparison of the spectral data with those reported.

4.1.1. 2-(*n*-Hexylamino)-3-methyl-1,4-naphthoquinone (1c)

A solution of vitamin K_3 (173 mg, 1.0 mmol) in *n*-hexylamine (3.0 ml) was stirred for 24 h at room temperature. The mixture was evaporated and the residue was purified by silica gel chromatography (hexanes/EtOAc = 20:1) to yield **1c** (125 mg, 46%) as a red solid. Mp = 40–44 °C. IR (KBr): 3347, 2927, 2856, 1666, 1608, 1567, 1515, 1340, 1272, 1228, 1085, 973, and 727 cm^{-1}. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 8.09–7.96 (m, 2H), 7.69–7.53 (m, 2H), 5.73 (s, 1H), 3.54 (q, J = 6.75 Hz, 2H), 2.23 (s, 3H), 1.66–1.56 (m, 2H), 1.44–1.25 (m, 6H), 0.90 (t, 3H). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ: 183.3, 182.3, 146.0, 134.1, 133.4, 131.6, 130.1, 126.0, 125.8, 111.8, 45.5, 31.5, 30.9, 26.4, 22.6, 14.1, 11.3. HRMS: calcd for $C_{17}H_{21}NO_2Na$ ($[M+Na]^+$) 294.1464, found 294.1470.

4.1.2. 2-Methyl-3-(*n*-nonylamino)-1,4-naphthoquinone (1d)

A solution of vitamin K_3 (172 mg, 1.0 mmol) in *n*-nonylamine (1.0 ml) was stirred for 24 h at room temperature. The mixture

was evaporated and the residue was purified by silica gel chromatography (hexanes/EtOAc = 20:1) to yield **1d** (111 mg, 35%) as a red solid. Mp = 54–55 °C. IR (KBr): 3301, 2921, 2852, 1668, 1600, 1565, 1506, 1342, 1274, 1228, 1078, 973, 890, 723, 559 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 8.08–7.96 (m, 2H), 7.69–7.53 (m, 2H), 5.73 (s, 1H), 3.53 (q, J = 6.75 Hz, 2H), 2.23 (s, 3H), 1.65–1.60 (m, 2H), 1.27 (s, 12H), 0.88 (t, 3H). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ: 183.1, 182.1, 145.9, 134.0, 133.3, 131.5, 130.1, 125.9, 125.7, 111.7, 45.4, 31.8, 30.9, 29.4, 29.3, 29.2, 26.7, 22.6, 14.1, 11.2. HRMS: calcd for $C_{20}H_{27}NO_2Na$ ($[M+Na]^+$) 336.1934, found 336.1946.

4.1.3. 2-(*n*-Heptyl)-3-methyl-1,4-naphthoquinone (2c)

A degassed solution of ammonium persulfate (430 mg, 1.89 mmol) in H_2O (5 ml) was added slowly to a degassed solution of vitamin K_3 (109 mg, 0.63 mmol), *n*-octanoic acid (0.12 ml, 0.76 mmol), and silver nitrate (54 mg, 0.32 mmol) in acetonitrile (6.7 ml) and H_2O (5 ml). The mixture was stirred at 60 °C for 1 h. Then, the crude solid was collected by filtration and purified by silica gel chromatography (hexanes/EtOAc = 30:1) to yield **2c** (132 mg, 77%) as a yellow solid. Mp = 77–79 °C. IR (KBr): 3448, 2919, 2854, 1654, 1590, 1457, 1378, 1292, 1187, 1122, 925, 892, 792, 717 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 8.09–8.04 (m, 2H), 7.70–7.67 (m, 2H), 2.63 (t, J = 6.75 Hz, 2H), 2.19 (s, 3H), 1.47–1.29 (m, 10H), 0.88 (t, J = 6.48 Hz, 3H). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ: 185.2, 184.5, 147.4, 142.9, 133.2, 133.1, 132.1, 132.0, 126.2, 126.0, 31.8, 30.0, 29.2, 28.8, 27.2, 22.7, 14.2, 12.7. HRMS: calcd for $C_{18}H_{22}O_2Na$ ($[M+Na]^+$) 293.1512, found 293.1529.

4.2. Ex vivo angiogenesis assay

Male Wistar rats (6 weeks old; Charles River Laboratories, Kanagawa, Japan) were housed in metal cages in a room with controlled temperature (24 ± 1 °C) and a 12 h light–dark cycle (lights on, 08:00–20:00 h). These rats had free access to food and deionized water, and were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University.

The ex vivo angiogenesis assay was performed according to slightly modified methods as described before.[27,28] A male Wistar rat (body weight ~200 g) was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. To avoid contamination with blood, the thoracic aorta was removed and washed with RPMI 1640 medium (Gibco, NY, USA). The artery was then turned inside out and cut into short segments of about 1–1.5 mm. A collagen gel (gel matrix solution) was then prepared with 8 vol of porcine tendon collagen solution (3 mg/ml) (Cellmatrix Ia, Nitta Gelatin Co., Osaka, Japan), 1 vol of 10× Eagle's MEM (Gibco), and 1 vol of reconstitution buffer (80 mM NaOH and 200 mM HEPES). These solutions were mixed gently at 4 °C. Each aortic segment was placed in the center of a well on a six-well culture plate and covered with 0.5 ml of gel matrix solution, which was reconstituted as described above. The solution was allowed to gel at 37 °C for 20 min. RPMI 1640 medium containing 1% of ITS+ (Becton, Dickinson and Company, Tokyo, Japan) with vitamin K_3 derivatives or vehicle (DMSO) was prepared. The collagen gel was then overlaid with 2 ml of culture medium. Incubation was carried out for 7 days in a humidified atmosphere containing 5% CO_2 in air, at 37 °C. Capillary length was estimated by phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate mid-point of the capillary. Microscopic fields were photographed using a digital camera (OLYMPUS DSE330-A system). Capillary length was measured using Adobe Photoshop software CS3. Each reported value represents the average of 3–6 culture samples.

4.3. Endothelial cell functions

4.3.1. Cell culture

HUVECs were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in HuMedia EG2 medium (Kurabo Industries), which is modified MCDB 131 medium containing 2% fetal bovine serum, 10 ng/ml recombinant human epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml recombinant human basic fibroblast growth factor, and 10 μg/ml heparin, in a humidified incubator with 5% CO_2 at 37 °C. Subcultures were obtained by treating the HUVEC culture with Hanks'-based enzyme-free cell dissociation buffer solution (Gibco). We used HUVECs in passages 3–7 in this experiment.

4.3.2. HUVEC tube formation assay

The tube formation assay was performed using BD Matrigel™ (Becton, Dickinson and Company). Briefly, solid gels were prepared according to the manufacturer's manual on a 96-well tissue culture plate. HUVECs (1×10^5 cells/ml) in HuMedia EG2 medium containing **1b** (5–100 μM) or vehicle (DMSO) were seeded 100 μl per well onto the surface of BD Matrigel™. The cells were incubated for 12 h at 37 °C in a CO_2 incubator. Tube formation was observed under an inverted light microscope at 40× magnification. Microscopic fields were photographed using a digital camera. The total length of tube structures in each photograph was measured using Adobe Photoshop software CS3. Each reported value represents the average of three samples.

4.3.3. HUVEC chemotaxis assay

The chemotaxis assay was carried out in a modified Boyden chamber.[29] A microporous membrane (8 μm) of 24-well cell culture inserts (Becton, Dickinson and Company) was coated with 0.1% gelatin. HUVECs were suspended in Medium 199 (Gibco) with 0.1% bovine serum albumin (BSA) and then seeded in the chamber (2.5×10^5 cells/ml). The well was filled with 400 μl of Medium 199 containing 0.1% BSA and 10 ng/ml of human recombinant VEGF (R&D systems, MN, USA) with or without **1b** (5–100 μM). Resveratrol (Cayman Chemical, MI, USA), the anti-angiogenic compound that inhibits endothelial cell migration[20,21], was used as a positive control inhibitor (100 μM). The assembled chamber was incubated for 6 h in a humidified 5% CO_2 at 37 °C. Non-migrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol and stained with Diff-Quik stain (Sysmex, Kobe, Japan). Migrated cells were counted in three fields of each membrane under the microscope at 200× magnification, and the average number of cells in each field was calculated. The experiment was performed in triplicate.

4.3.4. HUVEC proliferation assay

A HUVEC suspension (1.5×10^4 cells/ml) was plated onto 96-well plates (100 μl/well), cultured in HuMedia EG2 medium, and incubated for 24 h in a humidified incubator with 5% CO_2 at 37 °C. The medium was replaced with fresh HuMedia EG2 medium containing **1b** (5–100 μM). After 72 h, 10 μl of WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) was added into each well of a 96-well plate and incubated for 4 h. Absorbance was measured at 450 nm using a microplate spectrophotometer (BIO-RAD model 680). Each reported value represents the average of three wells.

5. Statistical analysis

Values are presented as means ± SEM. Data were analyzed by one-way analysis of variance followed by the Dunnet test. Differences with p <0.05 were considered significant.

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