An attempt to promote neo-vascularization by employing a newly synthesized inhibitor of protein tyrosine phosphatase

Shinji Soeda[a], Takuji Shimada[a], Satoru Koyanagi[a], Tsutomu Yokomatsu[b,*], Tetsuo Murano[b], Shiroshi Shibuya[b], Hiroshi Shimeno[a,*]

[a] *Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan*
[b] *School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

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Abstract Vascular endothelial growth factor (VEGF) and its receptors play a key role in angiogenesis. VEGF receptor-2 (VEGFR-2) has a tyrosine kinase domain, and, once activated, induces the phosphorylation of cytoplasmic signaling proteins. The phosphorylated VEGFR-2 may be a substrate for intracellular protein tyrosine phosphatases (PTPs) which prevent VEGF signaling. We synthesized a series of α,α-difluoro(phenyl)methylphosphonic acids (DFPMPAs) which inhibit the action of PTP. In this study, we test their effects on VEGF-induced angiogenesis. DFPMPA-3, the most effective inhibitor of human PTP-1B, promoted tube formation by human umbilical vein endothelial cells (HUVEC) on Matrigel more effectively than any other DFPMPAs. The inhibitor promoted the VEGF-induced proliferation and migration of HUVEC by inhibiting the dephosphorylation of VEGFR-2. Its effectiveness was proven through neo-vascularization in mice. The present findings suggest that targeting PTP to promote therapeutic neo-vascularization may be a potential strategy.

Key words: Angiogenesis; Vascular endothelial growth factor; Protein tyrosine phosphatase inhibitor; Human umbilical vein endothelial cell

1. Introduction

Vascular endothelial growth factor (VEGF) is a potent selective cytokine that acts on endothelial cells to promote the sprouting of blood vessels [1,2]. The angiogenic action of VEGF is mediated through VEGF receptor-2 (VEGFR-2) and the downstream phosphatidylinositol 3′-kinase (PI-3K)/Akt signaling pathway [3]. The binding of VEGF to VEGFR-2 induces conformational changes in the receptor, followed by dimerization and autophosphorylation of the tyrosine residues [4,5]. Thereafter, consecutive intracellular signal transduction events are initiated and culminate in VEGF-mediated angiogenesis.

Recently, successful VEGF gene therapy to treat patients with critical limb ischemia has been reported [6,7]. However, in some cases, such direct administration of the VEGF gene alone may not be sufficient to treat impaired neo-vascularization. It is known that older, diabetic, and hypercholesterolemic animals exhibit evidence of age-related endothelial dysfunction [8–10]. We hypothesize that the cause of this dysfunction is the presence of intracellular protein tyrosine phosphatase(s) (PTP), which switches off active VEGFR-2 through dephosphorylation of the tyrosine residues. Using a small molecule to block the dephosphorylation and thereby maintain the phosphorylated state of the tyrosine kinase domain would be useful as a 'support-side' strategy in neo-vascularization via gene therapy.

We synthesized a series of α,α-difluoro(phenyl)methylphosphonic acids (DFPMPAs) having a hydrophobic functional group and evaluated their inhibitory effects on human PTP-1B [11]. For the present study, we chose six DFPMPA derivatives with IC_{50} values varying from 57.94 to 718.1 μM [11]. We studied the effectiveness of these small-molecule stimulators on the angiogenic process by using a tube formation assay on Matrigel. We found that DFPMPA-3, the most effective PTP-1B inhibitor with an $IC_{50} = 57.94$ μM, is capable of enhancing VEGF-induced proliferation and migration of human umbilical vein endothelial cells (HUVEC) by prolonging the phosphorylated state of the kinase domain of VEGFR-2. We also show the efficacy of DFPMPA-3 on neo-vascularization in mice.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: Matrigel from Becton Dickinson Labware; fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL; porcine type I and type IV collagens from Nitta Gelatin, Osaka, Japan; human plasma fibronectin from Boehringer; human plasminogen from American Diagnostica; recombinant human $VEGF_{165}$ from Oncogene Research Products; wortmannin from Sigma. A series of DFPMPAs were prepared from 2-iodonaphthalene and iodobenzene derivatives as described previously [11].

2.2. Cell cultures

Cryo HUVEC and the culture medium EGM-2 were purchased from Sanko Junyaku, Tokyo, Japan. EGM-2 is composed of modified MCDB 131 medium and supplements that contain FBS, VEGF, epidermal growth factor, basic fibroblast growth factor, insulin-like

*Corresponding authors. Fax: (81)-426-76 3239; (81)-92-863 0389.
E-mail addresses: yokomatu@ps.toyaku.ac.jp (T. Yokomatsu), shimeno@fukuoka-u.ac.jp (H. Shimeno).

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2 (KDR); PTP, protein tyrosine phosphatase; DFPMPA, α,α-difluoro(phenyl)methylphosphonic acid derivative; HUVEC, human umbilical vein endothelial cells; PI-3K, phosphatidylinositol 3′-kinase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium

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growth factor, heparin, ascorbic acid, hydrocortisone, amphotericin B and gentamicin. HUVEC were grown in EGM-2 at 37°C in a humidified 5% CO_2 atmosphere. All experiments were performed with HUVEC at passages 3–5.

2.3. Tube formation assays

For the assay using Matrigel, diluted Matrigel (350 μl) was placed into each well of a 24-well culture plate at 4°C and allowed to polymerize by incubation at 37°C for 30 min. HUVEC (5×10^4 cells) were seeded on the Matrigel in 1 ml of DMEM/2% FBS alone or with DFPMPA, and incubation followed at 37°C for 24 h. Three different phase-contact microscopic fields (×100) per well were photographed. The total length of tube structures in each photograph was scanned and quantified using NIH Imaging software. For the tube formation assay using collagen gel, 350 μl of a type I collagen solution (2.4 mg/ml) containing VEGF (50 ng/ml), fibronectin and plasminogen (20 μg/ml each) was placed into each well of a 24-well culture plate at 4°C and polymerized at 37°C for 30 min. The subsequent procedures were the same as those described above for the assay using Matrigel.

2.4. Assay of cell proliferation

HUVEC (5×10^3 cells) in EGM-2 containing 100 μl of VEGF (10 ng/ml) were seeded into each well of 96-well culture plates and incubated at 37°C for 24 h. Cell proliferation was assayed with a MTT Cell Proliferation kit I (Roche Diagnostics), following the manufacturer's instructions.

2.5. Assay of cell migration

HUVEC (1×10^3 cells) were seeded on type IV collagen-coated Transwell chambers (Falcon), and the chamber was inserted into each well of 24-well culture plates containing VEGF (50 ng/ml), fibronectin, plasminogen (20 μg/ml each) and, where indicated, DFPMPA-3 in a total volume of 700 μl. After 24 h incubation, HUVEC remaining on the upper side of the membrane were removed with cotton swabs. The cells were then fixed with a 5% glutaraldehyde solution and stained with Giemsa's reagent (Merck). The stained cells were microscopically counted in three fields (×100).

2.6. Western blot analysis of phosphorylated VEGFR-2

Confluent cultures of HUVEC in 175-cm^2 culture flasks were incubated for 3 h in DMEM/0.05% FBS and further for 30 min in DMEM/2% FBS that contained VEGF (10 ng/ml) and, where indicated, DFPMPA-3. The cells were harvested and pelleted by centrifugation. The pellets were washed with cold phosphate-buffered saline, and extraction followed for 10 min in 100 μl of a lysis buffer, 10 mM HEPES buffer (pH 7.5)/50 mM KCl/3 mM MgCl/0.3 mM EDTA/10% glycerol/0.5% Nonidet P-40/leupeptin (10 μg/ml)/antipain (10 μg/ml). After centrifugation at $12\,000\times g$ for 15 min, the resulting supernatants were assayed to detect phosphorylated VEGFR-2. Supernatants (30 μg protein) were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane. The transferred proteins were reacted with antibodies against phospho-VEGFR-2 (Oncogene Research Products). The resulting immune complex was further reacted with peroxidase-conjugated secondary antibodies and made visible with 4-chloro-1-naphthol as the peroxidase substrate.

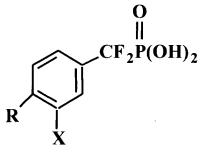
2.7. Animal studies

Male ICR mice (5 weeks old) were purchased from Charles River Japan, Kanagawa, Japan. Animal treatment followed the animal care guidelines of the Japanese Government, Law No. 105 and Notification No. 6. Neo-vascularization in mice was assessed by the method of Asano et al. [12]. Briefly, diluted Matrigel alone or with DFPMPA-3 was packed into a diffusion chamber (Millipore), and the chamber was implanted into the dorsal air sac of mice. After 7 days, the blood vessels newly formed in the subcutaneous regions adjacent to the implanted chamber were photographed under a dissection microscope.

2.8. Statistical analysis

All values are expressed as mean ± S.D., and significant levels between groups were assessed by Student's *t*-test.

A



B

Compound	Structure	IC_{50}	Ratio of tube lengths (% of control)
DFPMPA	R=H, X=H	NI	105±25.4
DFPMPA-1	Benzene nuclei→naphthalene	718	133±31.6
DFPMPA-2	R=(E)-styryl X=H	450	193±27.7*
DFPMPA-3	R=(E)-styryl $X=N(SO_3Me)_2$	58	335±30.6***
DFPMPA-4	R=(E)-styryl $X=NHSO_3Me$ (di-ammonium salt)	176	276±41.0**
DFPMPA-5	R=(E)-styryl $X=N(SO_3Ph)_2$	387	273±31.3**

Fig. 1. Characterization of DFPMPAs: chemical structures, IC_{50} values, and their effects on tube formation by HUVEC on Matrigel. IC_{50} values are quoted from our previous paper [11]. The enzyme assay was performed with *p*-nitrophenyl phosphate as the substrate. The effects of DFPMPAs on tube formation were evaluated at a concentration of 10 μM. Each value represents the mean ± S.D. ($n=3$). *$P<0.05$; **$P<0.01$; ***$P<0.005$, compared to the control. NI, no inhibition.

3. Results and discussion

3.1. The effect of DFPMPAs on HUVEC tube formation

Fig. 1A shows the chemical structure of DFPMPA. Fig. 1B presents the structure of each DFPMPA derivative we used, its IC_{50} values for human PTP-1B, and its effect on HUVEC tube formation. It has been reported that DFPMPA itself has no inhibitory effect on PTP-1B, but the naphthyl derivative, DFPMPA-1, can bind to the catalytic site of PTP-1B and competitively inhibit enzyme activity [11]. The inhibitory effect of DFPMPA-2, which has an (*E*)-styryl group on the benzene nucleus, is superior to that of DFPMPA-1. The most effective derivative is DFPMPA-3, which contains a bis-methylsulfonamide group. DFPMPAs 4 and 5 also contain these groups, but are not as effective as DFPMPA-3. The data show that the effectiveness of DFPMPAs in promoting HUVEC tube formation corresponds in inhibiting PTP-1B. The screening assay identified DFPMPA-3 as the most effective stimulator of HUVEC tube formation. Okadaic acid, a potent inhibitor of serine-threonine protein phosphatase [13], had little or no effect on HUVEC tube formation at a concentration of 10 μM (data not shown). This result suggests that inhibition of PTP is an important factor in HUVEC tube formation on Matrigel.

To determine whether the target of DFPMPAs is VEGFR-related PTP, we examined the effect of DFPMPA-3 on VEGF-induced tube formation by HUVEC on type I collagen gel (Fig. 2). Under experimental conditions, control HUVEC failed to form the capillary-like networks (panel A). However, as shown in panels B and C, DFPMPA-3 promoted the formation of capillary-like networks by HUVEC. However, the addition of 25 or 50 μM DFPMPA-3 did not cause additional enhancement of the effect. These results indicate that DFPMPA-3 produces its best effect on VEGF-induced tube formation by HUVEC at a concentration of 10 μM and that higher concentrations of the inhibitor may have some cytotoxic action on HUVEC, although cell death was not observed.

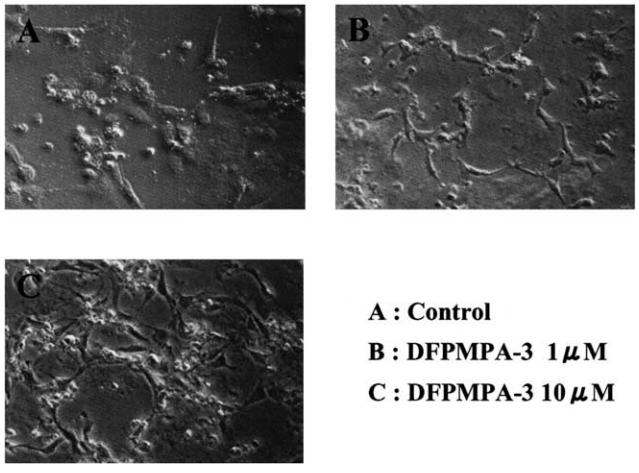


Fig. 2. The effect of DFPMPA-3 on VEGF-induced tube formation by HUVEC on type I collagen gel. The photographs show results from one of three experiments that produced very similar results.

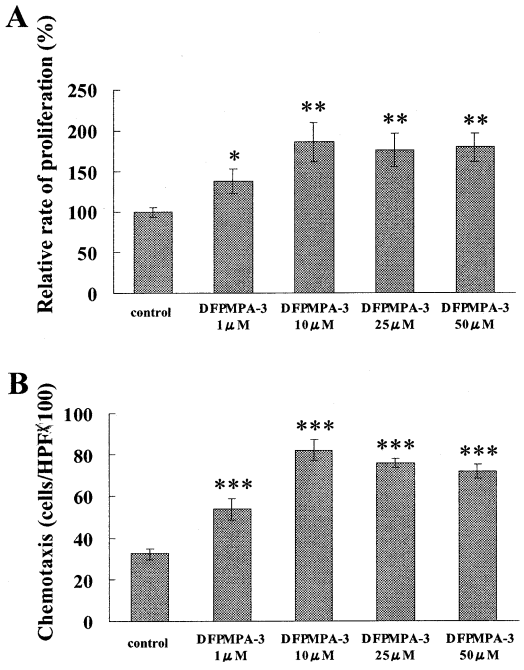


Fig. 3. The effect of DFPMPA-3 on VEGF-induced proliferation (A) and migration (B) of HUVEC. Each bar represents the mean ± S.D. (proliferation, $n=3$; chemotaxis, $n=6$). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$, compared to the control.

3.2. The effect of DFPMPA-3 on the VEGF-induced proliferation, migration and receptor phosphorylation of HUVEC

Neo-vascularization in adults has been thought to result exclusively from proliferation, migration, and remodeling of preexisting endothelial cells [14,15]. Because the most important mediator in these processes is VEGF [1,2], we next tested the effect of DFPMPA-3 on VEGF-induced proliferation and migration of HUVEC on type IV collagen gel. As shown in Fig. 3A,B, DFPMPA-3 significantly promoted these VEGF-induced angiogenic processes. DFPMPA-3's best effects were obtained at a concentration of 10 μM (1.9-fold and 2.5-fold increases, respectively, for cell proliferation and migration). Tyrosine phosphorylations induced by receptor tyrosine kinases promote cellular proliferative responses, and protein tyrosine phosphatases counterbalance the activities of tyrosine kinase receptors [16]. Recent studies provide evidence that a low molecular weight PTP exists in the cytoplasm of HUVEC and prevents VEGF-induced cell migration, proliferation, and the resulting angiogenesis [17]. An earlier report indicates that the tyrosine kinase domain of VEGFR-2 is a substrate for the cytoplasmic PTP [18]. Thus, DFPMPA-3 may increase the amount of autophosphorylated VEGFR-2 by inhibiting the cytoplasmic PTP present in HUVEC. Western blot analysis using antibodies against phosphorylated VEGFR-2 demonstrates that the phosphorylated protein migrating at ~210 kDa during SDS–PAGE represents VEGFR-2, since VEGF increased the phosphorylation of the protein to approximately 1.4 times that of the control (Fig. 4A,B). The presence of 10 μM DFPMPA-3 further increased the phosphorylated VEGFR-2 levels, compared to VEGF alone (approximately 1.3 times the VEGF level, $P<0.005$). These results suggest that VEGF-mediated signaling in HUVEC is regulated by a PTP, and that the inhibition of PTP activity can enhance the

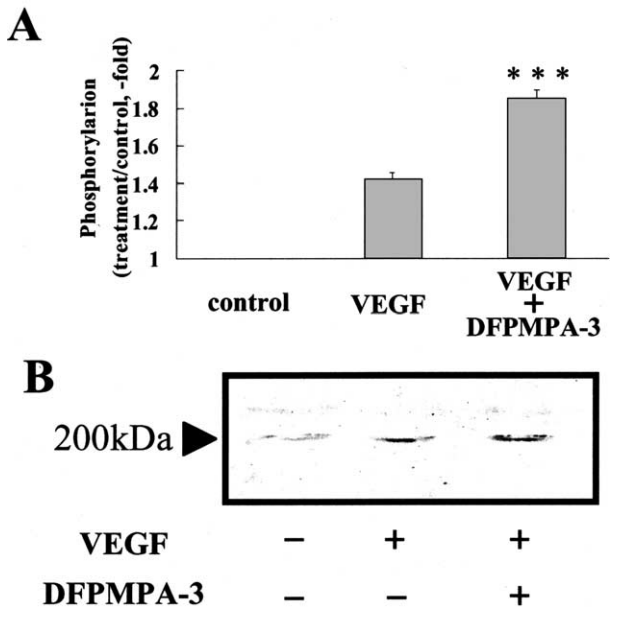


Fig. 4. The effect of DFPMPA-3 on VEGF-induced autophosphorylation of VEGFR-2. The phosphorylated VEGFR-2 was made visible by Western blotting (B), and the bands were quantified using an NIH Imager (A). VEGFR-2 in HUVEC incubated in the absence of both VEGF and DFPMPA-3 was used as the control. The data are results from one of two experiments that produced very similar. $***P < 0.005$, compared to VEGF alone.

VEGFR-2 signal transduction that leads to the proliferation, migration and tube formation by HUVEC. However, it remains to be determined whether the PTP activity is mediated by the cytoplasmic PTP [17].

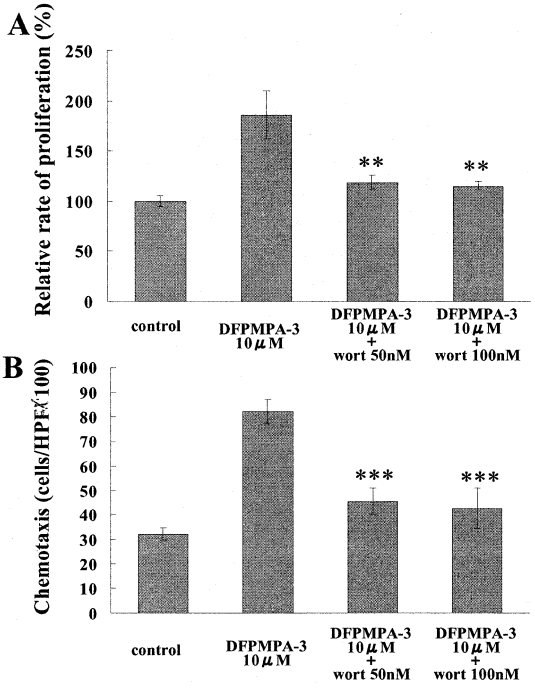


Fig. 5. The effect of wortmannin on the ability of DFPMPA-3 to enhance VEGF-induced proliferation (A) and migration (B) of HUVEC. Each bar represents the mean ± S.D. (proliferation, $n = 3$; chemotaxis, $n = 6$). $**P < 0.01$; $***P < 0.005$, compared to DFPMPA-3 alone.

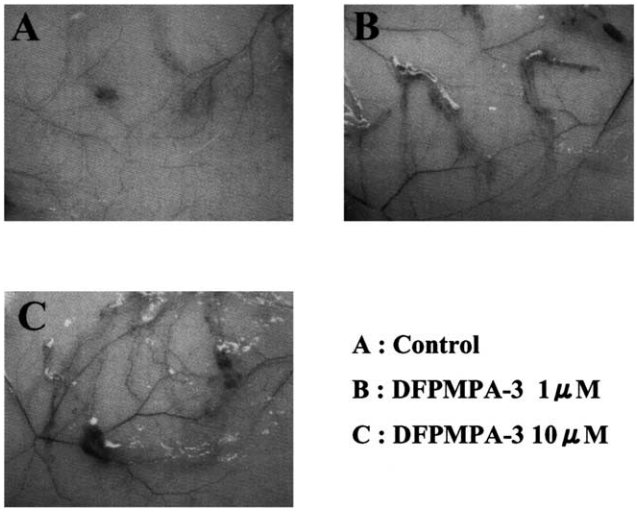


Fig. 6. The effect of DFPMPA-3 on angiogenesis in mice. Photographs show neo-vascularization induced by a chemoattractant, Matrigel, alone (A) or with DFPMPA-3 (B,C) packed in the membrane chamber.

3.3. PI-3K is involved in DFPMPA-3's enhancement of VEGF-mediated angiogenic responses

PI-3K is known to affect the ability of VEGF to enhance the survival of HUVEC [3]. PI-3K is thought to resist cell death by activating the serine-threonine protein kinase, Akt, which enhances the activity of anti-apoptotic proteins through the transcription factor NF-κB and inhibits pro-apoptotic signaling by Bad, caspase-9, and other effectors [19]. To assess whether DFPMPA-3's enhancement of VEGF-mediated angiogenic responses of HUVEC involves PI-3K signaling, we used a specific PI-3K inhibitor, wortmannin, in cell proliferation and migration assays of HUVEC. As shown in Fig. 5, the PI-3K inhibitor (50 or 100 nM) diminished the ability of DFPMPA-3 to promote VEGF-induced proliferation (panel A) and migration (panel B). These results suggest that the inhibition of dephosphorylation of VEGFR-2 by a PTP inhibitor, DFPMPA-3, contributes to amplification of the PI-3K/Akt signaling that plays an important role in anti-apoptosis of endothelium [3,19] and ischemic neurons [20].

3.4. DFPMPA-3 promotes angiogenesis in mice

Since DFPMPA-3 promoted the VEGF-mediated signaling and cellular responses in cultured HUVEC, we next studied whether DFPMPA-3 was able to promote angiogenesis in vivo. A diffusion chamber containing diluted Matrigel (as the chemoattractant) alone or with DFPMPA-3 was implanted into the dorsal air sac of mice. After 7 days, neovascularization in the subcutaneous region adjacent to the implanted chamber was observed under a dissection microscope. The chamber containing Matrigel alone produced few vascular sprouts (Fig. 6A). In contrast, the presence of DFPMPA-3 in the chamber produced a luxuriant development of vascular sprouts in a dose-dependent manner (Fig. 6B,C).

We conclude that DFPMPA-3 is a small-molecule stimulator of VEGFR-2 signaling. A possible target molecule for DFPMPA-3 may be the cytoplasmic PTP [17]. The potential importance of therapeutic angiogenesis has been proved [21]. Successful therapeutic angiogenesis has been achieved by de-

livery of the VEGF gene [6,7], basic fibroblast growth factor protein and other growth factors [21] and more recently, transplantation of ex vivo expanded endothelial progenitor cells [22]. In these clinical treatments, VEGF receptor-related tyrosine kinase signaling may play a crucial role in the development of new blood vessels. The present study suggests PTP has an important role as the downstream inhibitor of VEGF signaling and thus in angiogenic responses. Therefore, methods to prevent PTP's functions may be useful in achieving ideal therapeutic angiogenesis.

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