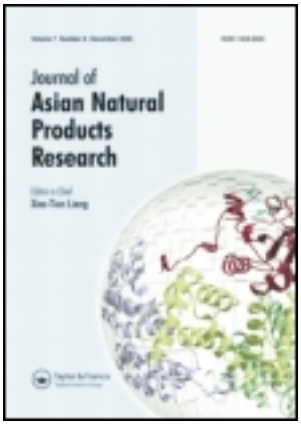

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Inhibitory effects of SY0916, a platelet-activating factor receptor antagonist, on the angiogenesis of human umbilical vascular endothelial cells

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SY0916 is a novel platelet-activating factor receptor antagonist. The objective of this study is to explore the anti-angiogenesis effects of SY0916 on human umbilical vascular endothelial cell (HUVEC) and to understand its possible mechanism. The effect of SY0916 on proliferation of HUVEC was measured by the MTT method, whereas the effect of SY0916 on HUVEC chemotaxis was carried out by Boyden chamber assay. The activities of metalloproteinase (MMP)-9 and MMP-2 were detected using gelatin zymography, and the expression of intercellular adhesion molecules-1 (ICAM-1) was measured by Western blot analysis. The 2D tube formation experiment of HUVEC with 10% fetal calf serum on Matrigel was also evaluated. It was shown that SY0916 had significant inhibitory effects on the proliferation and the chemotaxis of HUVEC induced by phorbol-12-myristate-13-acetate in a positive dose-dependent manner. Furthermore, SY0916 could significantly suppress the activity of MMP-2 and MMP-9 and decrease the expression of ICAM-1 in HUVEC. In 2D tube formation test, SY0916 could effectively inhibit the formation of vascular structure on Matrigel. The results showed that SY0916 could block the chemotaxis of HUVEC, and then inhibit the tube formation on Matrigel. Such anti-angiogenesis effect of SY0916 on HUVEC might relate to downregulate the expressions of MMP-2, MMP-9, and ICAM-1.

Keywords: PAF receptor; antagonist; angiogenesis; HUVEC

1. Introduction

Angiogenesis refers to the formation of new blood vessels from the existing capillaries. Under normal physiologic and pathologic conditions, it stays in a highly controlled and orderly regulated state to ensure its progression to adapt to the growing, metabolic, and repairing needs of tissues and organs [1,2].

During the course of angiogenesis, endothelial cell (EC) plays an extremely important role. The growth of new blood vessels is dependent on the migration and proliferation of EC, the formation of reticular interconnections, and the branching of new vessels. The vascular budding is tightly regulated by EC and its extracellular matrix (ECM) through the influences of angiogenesis-inducing or angiogenesis-inhibiting factors [3–5]. In normal condition, the inhibiting factors are predominant. But the angiogenic factors are at an advantage in the tissues which undergo neovascularization. Furthermore, the

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migration of EC is also dependent on the interactions of cells and ECM [6]. Besides the participation of matrix metalloproteinases (MMPs) in the degradation of ECM, adhesion molecules also play vital roles in the interactions of EC–ECM [7]. Protein kinase C (PKC) has been reported to have a pivotal role in angiogenesis. PKC-activating phorbol esters were reported to induce angiogenesis. Phorbol-12-myristate-13-acetate (PMA) enhanced the ability of human umbilical vascular EC (HUVEC) to organize into tubular networks when plated on Matrigel, which could be prevented by PKC inhibitors.

Platelet-activating factor (PAF) is a powerful inflammatory mediator, and it plays an important role in many angiogenesis-related diseases. Plenty of stimulators, such as PMA, can induce the synthesis of PAF in both remodeling and *de novo* pathway [8]. PAF has been demonstrated to have an effect on promoting angiogenesis in many *in vivo* and *in vitro* experiments [9,10]. As revealed by some studies, this effect seems to involve the enhancement of the proliferation and migration of EC and the formation of tubular structure. The promotion of angiogenesis might also relate to some biological molecules, such as EC-secreted MMPs and the adhesion molecules expressed by EC [11,12]. SY0916 is a new compound synthesized by our institute and the grade of purity exceeds more than 99%. The molecular formula of SY0916 is $C_{19}H_{24}ClNO_3$ and its molecular weight is 349.14. In our preliminary work, the present project group has confirmed that the new compound SY0916 (Figure 1) possesses the effects of antagonizing the proinflammatory action of PAF and competitively inhibiting the binding of [^{3}H]-PAF with its receptor. Employing the primarily cultured HUVEC as the study material, the present study was intended to explore the inhibiting effects of SY0916 on angiogenesis and to understand its possible acting mechanism.

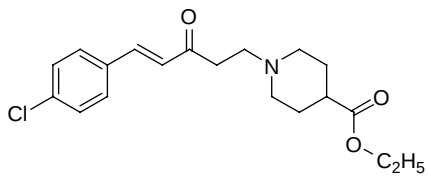

Figure 1. Chemical structure of PAF receptor antagonist SY0916.

2. Results

2.1 Isolation and identification of HUVEC

After isolation, purification, and culturing, HUVEC grew confluent with a typical characteristic of cobblestone shape in a culture flask (Figure 2A). As confirmed by immunofluorescent staining, the isolated HUVEC expressed the typical marker of EC, i.e. vWF (Figure 2B, a typical cytoplasmic distribution with a negative nucleus) and CD31 (Figure 2C, cell margin distribution) and functional characteristics of Dil-Ac-LDL uptake by EC (Figure 2D, cytoplasmic distribution after uptake).

2.2 Inhibitory effect of SY0916 on the proliferation of HUVEC

The light absorbance of surviving HUVEC was detected with the MTT method. The result showed that, as compared with the control group, PMA at 10 nM could stimulate a marked proliferation of HUVEC. And within a dose range of 100 nM–10 μM, SY0916 could markedly inhibit the PMA-induced proliferation of HUVEC (Figure 3).

2.3 Inhibitory effect of SY0916 on the chemotaxis of HUVEC

Under a micrometer microscope, three viewing fields were selected randomly to calculate the number of migratory EC after fixation. The result showed, as compared with the control group, PMA could effectively induce the chemotaxis of HUVEC. Within a dose range from 100 nM to 10 μM,

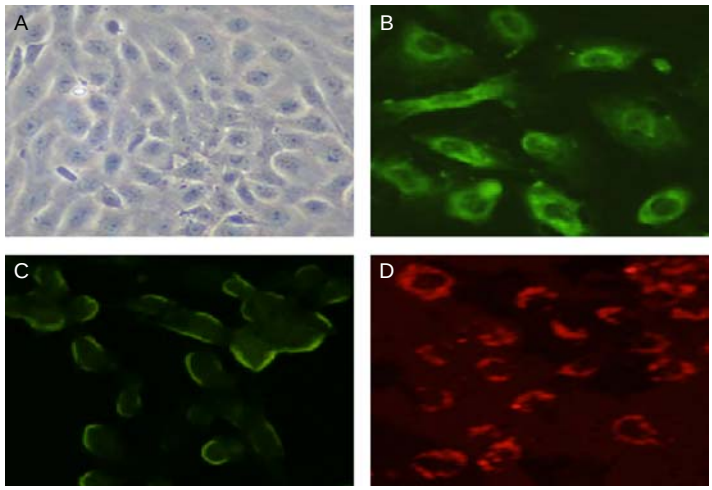

Figure 2. Identification of primary HUVEC. (A) Cultured confluent HUVEC, showing a typical cobblestone shape and contact inhibition characteristics under light microscope (LM × 200); immunofluorescent staining of (B) vWF and (C) CD31 on HUVEC; and (D) uptaking high level of Dil-Ac-LDL as a functional characteristic of EC.

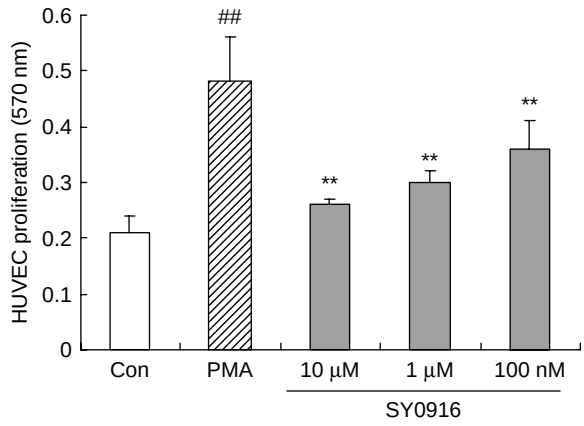

Figure 3. HUVEC proliferation stimulated with PMA and inhibitory effects of SY0916 by the MTT assay. All the data shown are representative of three independent experiments. Mean ± SD, $n = 6$, ##$P < 0.01$ vs. control; **$P < 0.01$ vs. PMA.

SY0916 could markedly inhibit the PMA-caused chemotaxis of HUVEC (Figure 4).

2.4 Inhibitory effect of SY0916 on the activity of MMPs in HUVEC

As demonstrated by the result of gelatin zymography, the non-stimulated HUVEC almost excreted no MMP-9. 10 nM PMA could promote the release of MMP-9 from HUVEC. SY0916 could markedly inhibit the PMA-induced release of MMP-9. And there was a positive correlation with doses. At 100 nM, 1 μM, and 10 μM, the inhibiting rates of SY0916 for the activity of MMP-9 were 13.03, 63.57, and 98.70%, respectively (Figure 5A). As demonstrated by the detection result of MMP-2 activity, the non-PMA-stimulated HUVEC almost secreted little MMP-2, whereas PMA could markedly boost the secretion of MMP-2 from

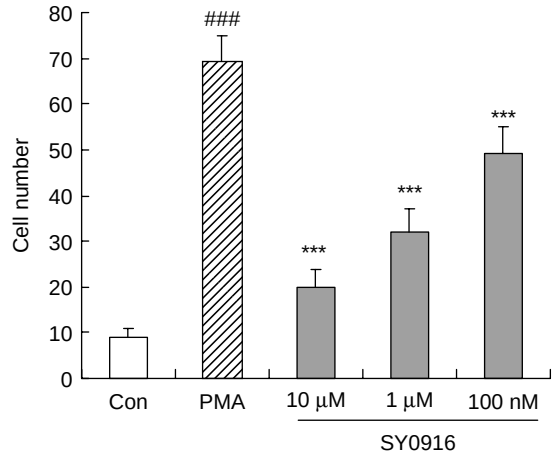

Figure 4. Inhibitory effects of SY0916 on the chemotaxis of HUVEC stimulated with PMA. All the data shown are representative of three independent experiments. Mean ± SD, $n = 3$, ###$P < 0.001$ vs. control; ***$P < 0.001$ vs. PMA.

HUVEC. SY0916 could markedly inhibit the PMA-induced release of MMP-2 and such an effect had a positive correlation with the doses. The inhibiting rates of SY0916 at 100 nM, 1 μM, and 10 μM on the activity of MMP-2 in HUVEC were 16.59, 20.65, and 84.15%, respectively (Figure 5B).

2.5 Inhibitory effect of SY0916 on the expression of intercellular adhesion molecules-1

As demonstrated by the gray scale scanning result of GelPro3.1 software, compared with the control group, PMA could markedly stimulate the expression of intercellular adhesion molecules-1 (ICAM-1) in HUVEC. And SY0916 could effectively inhibit the PMA-induced expression of ICAM-1 and such an effect had a positive correlation with doses. The inhibiting rates of SY0916 at the concentration of 100 nM, 1 μM, and 10 μM on the expression of ICAM-1 were 63.31, 71.13, and 98.97%, respectively (Figure 6).

2.6 Effect of SY0916 on the formation of tubular EC structure

As demonstrated by the tube forming result of 2D on Matrigel, the control group could form a relatively complete vascular structure under the culture condition of 10% fetal calf serum (FCS). Various concentrations of SY0916 could effectively inhibit the formation of tubular structure. And there was a positive correlation with the doses of SY0916 (Figure 7).

3. Discussion

This study exhibited that after isolation and purification, the harvested HUVEC showed a typical morphological feature of cobblestone. With a high grade of purity, it proliferates rapidly. At confluence, cell culture demonstrated the EC characteristics of monolayer growth and contact inhibition. After propagation, excellent morphologies and characteristics have been maintained. First, as proved by immunofluorescent staining, the resulting HUVEC expressed its classical markers of vWF and CD31. And then vWF staining presented a marked cytoplasmic distribution, whereas CD31 was mainly expressed at the cellular margins and connecting zones. Furthermore, it was shown that the isolated HUVEC possessed the function of uptaking DiL-Ac-LDL at a high level. All the above results confirmed that the harvested HUVEC after isolation, purification, and

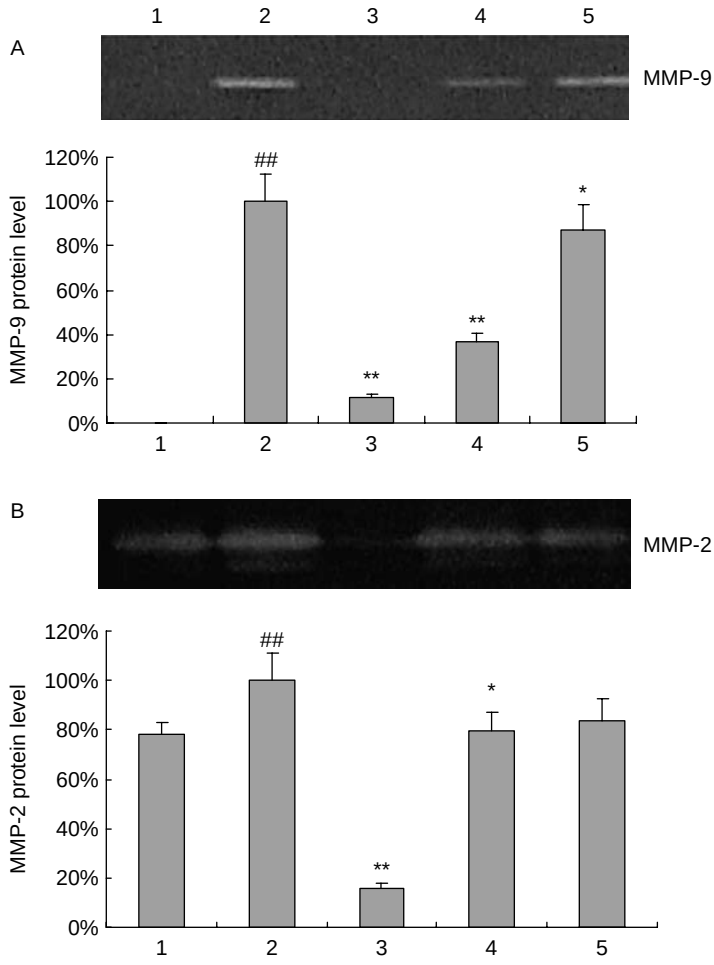

Figure 5. Inhibitory effects of SY0916 on MMPs activity of HUVEC induced with PMA. (A) MMP-9 activity of HUVEC stimulated with PMA and inhibitory effect of SY0916. Lane 1, control; 2, PMA; 3, PMA + 10 μM SY0916; 4, PMA + 1 μM SY0916; 5, PMA + 100 nM SY0916; (B) MMP-2 activity of HUVEC stimulated with PMA and inhibitory effect of SY0916. Lane 1, control; 2, PMA; 3, PMA + 10 μM SY0916; 4, PMA + 1 μM SY0916; 5, PMA + 100 nM SY0916. Mean ± SD, $n = 3$. ##$P < 0.01$ vs. control; *$P < 0.05$, **$P < 0.01$ vs. PMA.

amplification was indeed derived from vascular EC. As detected by flow cytometry, the positive cell rates of vWF and DiL-Ac-LDL surpassed 95% (result not shown).

It was reported in the literature that PAF could be synthesized by stimulating with PMA in EC, and it had an inherent proliferative effect on EC during angiogenesis [13,14]. As revealed by the present study, the compound SY0916 had no marked effect on the non-stimulated proliferation of HUVEC (result not shown). However, SY0916 could significantly inhibit the PMA-induced proliferation of HUVEC. This result appeared to be related with the antagonism of PAF receptor. It suggested that the blocking effect of SY0916 on PAF receptor may contribute to the inhibition of HUVEC proliferation induced by PMA.

MMPs are a group of important protein hydrolase during angiogenesis. Especially, MMP-2 and MMP-9 can degrade the ECM. The migration of EC occurs after losing

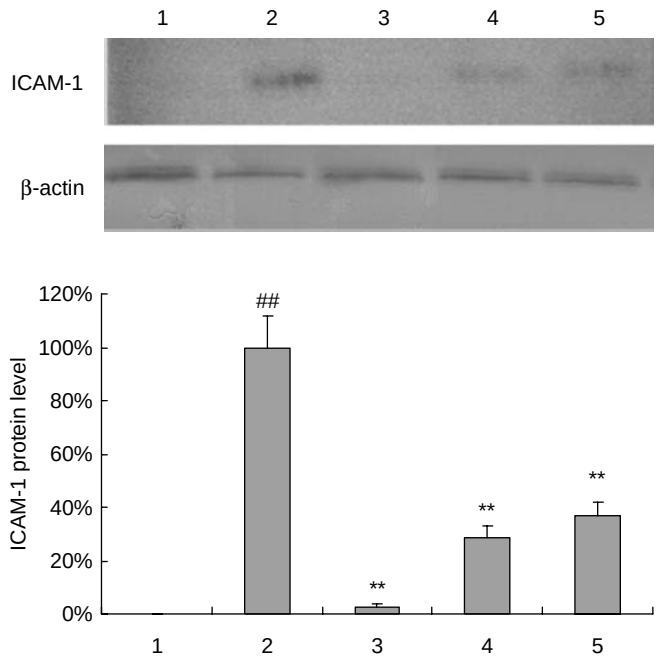

Figure 6. ICAM-1 expression of HUVEC stimulated with PMA and inhibitory effect of SY0916. Lane 1, control; 2, PMA; 3, PMA + 10 μM SY0916; 4, PMA + 1 μM SY0916; 5, PMA + 100 nM SY0916; The inhibiting rates of SY0916 at 100 nM (lane 5), 1 μM (lane 4), and 10 μM (lane 3) on the activity of ICAM-1 were 63.31, 71.13, and 98.97%, respectively. Mean ± SD, $n = 3$. ##$P < 0.01$ vs. control; **$P < 0.01$ vs. PMA.

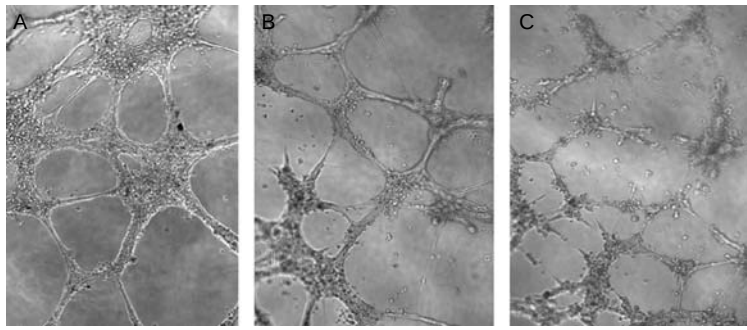

Figure 7. 2D tube-formation assay of HUVEC on Matrigel and inhibitory effect of SY0916. This result is typical of two independent performances. Magnification, LM × 100. (A) control; (B) 1 μM SY0916; and (C) 10 μM SY0916.

cell–cell and cell–matrix connections. It is a vital step in the process of angiogenesis [15]. Furthermore, MMPs can promote the release of growth factors, such as TGF-β, M-GSF, and IGF from ECM and transform them into active forms [16]. All of these positive regulating factors (e.g. TGF-β, IGF, and so on) are essential for the angiogenesis. PAF could upregulate the expression of MT1-MMP and TIMP2 which combined to pro-MMP2, resulting the activation of MMP2 [17]. The secretion of MMP9 was also promoted by PAF via disturbing the balance of MMPs and TIMPs. The present result showed that SY0916 could markedly inhibit the

elevated activity of MMP-9 and MMP-2 under the induction of PMA in HUVEC. Furthermore, the expressions of MMP-9 and MMP-2 were restrained at the protein level. The lowered activity of MMPs was probably due to its directly inhibited secretions of MMP-9 and MMP-2.

ICAM-1 is a key adhesion molecule implicated in the development of inflammatory vascular disease, and evidence suggests that it may also play an important role in angiogenesis as well [18,19]. The present study revealed that SY0916 could markedly inhibit the expression of ICAM-1 under the inductions of PMA. It might facilitate the effects of anti-inflammation and anti-angiogenesis.

HUVEC has the characteristic of forming a tube-like 2D structure on Matrigel. This phenomenon is mostly indicative of the migratory capacity of vascular EC [20]. It was also reported in the literature that a physiologic concentration of PAF could induce the migration of EC in a dose-dependent fashion. After the addition of PAF into the cultured EC, there were the disappearances of intercellular gap and F-actin [21]. The migratory capacity of the cells intensified and the cells tended to be in a moving status. As demonstrated by the present study, the compound SY0916 could inhibit the formation of 2D tube-like HUVEC structure under the induction of 10% FCS in a dose-dependent manner.

In conclusion, the present results showed that SY0916 could inhibit the proliferation, chemotaxis, tubular structure formation, activity, and expressions of MMPs and expression of ICAM-1 of HUVEC induced by PMA, which closely associated with angiogenesis. Since PMA is related to the synthesis of PAF and SY0916 is an antagonist of PAF receptor, blocking the binding between PAF and its receptors maybe one of the important mechanisms for anti-angiogenic action of SY0916. But it remains to be clarified whether there is another PAF-independent pathway during this course.

4. Materials and methods

4.1 Isolation, culture, and identification of HUVEC

Under a sterile condition, the neonate umbilical cord was placed into a Petri dish with a diameter of 90 mm and injected with 0.1% type I collagenase. After a 20 min digestion in a 37°C water bath, the cell suspension was centrifuged for 8 min at 1000*g*. After discarding the supernatant, 5 ml M199 culture medium containing 20% FCS (GIBCO, Carlsbad, CA, USA) was added into cell pellet. Then the specimens were inoculated into a T25 plastic culture flask embedded with 2% gelatin. Finally, EC growth factor (St Louis, MO, USA; 100–200 $\mu g\,ml^{-1}$), glutamine (2 mM), and sodium heparin (100 $\mu g\,ml^{-1}$) were added into the culture medium. There was a change of medium at 12 h. During propagation, 0.1% pancreatin (containing 0.1% ethylenediaminetetraacetic acid) was used for cell digestion. The human HUVEC of the second to fifth generation was used for the present study. The cover slides were placed into a disposable Petri dish and extracted until an 80% growing confluence of HUVEC. After fixing with cold acetone at -20°C, the routine detection method of immunofluorescence was employed to examine the typical EC molecules of vWF and CD31 (antibody from Santa Cruz, CA, USA). Also the functional assessments of EC were conducted to observe the uptake of an acetylated low-density cell lipoprotein (Dil-Ac-LDL, St Louis, MO, USA) by isolated EC.

4.2 Detection of HUVEC proliferation by the MTT method

For each hole of a 96-hole plate, 2×10^3 HUVEC were inoculated. After a 24-h incubation in a 5% CO_2 incubator at 37°C, the supernatant was discarded.

After washing twice with an FCS-free M199 culture medium, 160 μl M199 culture medium containing 5% FCS and 20 μl conditional culture medium containing various concentrations of SY0916 were added consecutively. After 30 min, VEGF was titrated until a final concentration of 10 ng ml^{-1}. After further culturing for 48–72 h, 5 mg ml^{-1} methylthiazolyldiphenyl-tetrazolium bromide (St Louis, MO, USA) was loaded into each hole. After incubation for another 4 h, the supernatant was carefully decanted. One hundred fifty microliters of dimethyl sulfoxide was loaded into each hole. After a thorough mixing with a micro-amount vortex for 10 min, the values of light absorbance were measured at a wavelength of 570 nm.

4.3 Detection of HUVEC chemotaxis by the method of Boyden chamber

The concentration of HUVEC was adjusted with a culture medium containing 0.1% bovine serum albumin (BSA) until 1×10^6 cells ml^{-1}. Various concentrations of compound SY0916 or control solvent were added, respectively. The specimens were incubated for 10 min at 37°C. The stimulant was diluted into the required concentration with a culture medium containing 0.1% BSA. The specimens were loaded into the lower part of a 48-hole Boyden chamber at 27 μl each hole. A PVDF filtration membrane with a diameter of 8 μm was applied gently. The upper plate of chemotactic apparatus was installed. The cell suspension was loaded into the upper chamber between the upper plate and membrane. The loading volume was 55 μl each hole. After 8–24 h culturing in a 5% CO_2 incubator at 37°C, the upper chamber fluid was discarded. The filtration membrane was extracted and a cotton swab used to scrape the adhered cells off its surface. The following staining steps were followed: methanol, 4 s; hematoxylin staining solution, 10 min; water rinsing; 0.1% hydrochloric acid 30s; water rinsing; blue staining solution 2 min; eosin staining solution, 1 min; and water rinsing. The extracted specimen was air-dried. The number of chemotactic cells was counted under a micrometer microscope. Three viewing fields were randomly selected from each hole to obtain the numbers of the chemotactic cells [22].

4.4 Detection of MMP activity for HUVEC by gelatin zymography

HUVEC was adjusted to 1×10^5 cells ml^{-1} and inoculated into a 48-hole plate at an aliquot of 500 μl each. After a 6-h culturing in a 5% CO_2 incubator at 37°C, the primary cultured medium was discarded and the plates were washed twice with serum-free Dulbecco's modified Eagle's medium. Then various concentrations of SY0916 and control solvent were added and the incubation continued for 30 min. After the addition of a stimulant, the cells were incubated for another 24 h. Later, the supernatant was collected after cell harvesting. The impurities were removed by an 8-min centrifuge at 12,000 rpm. The supernatant was divided into tiny portions for preservation and storage at −20°C. The levels of MMPs were measured by gelatin zymography according to the literature [23].

4.5 Detection of ICAM-1 expression by Western blot

HUVEC was adjusted to 1×10^5 cells ml^{-1} and inoculated into a 6 cm Petri dish. Upon a confluent growth of 90%, the supernatant was discarded. After washing the plate twice, various concentrations of SY0916 and control solvent were added and the incubation continued for 30 min. Then a stimulant was added and the incubation was extended for 6–8 h. The cellular proteins were extracted for routine electrophoresis. After membrane transferring, Tris-buffered saline Tween (TBS/T) containing 5% BSA was used for blocking at 4°C overnight. The mouse anti-human

ICAM-1 monoclonal antibody (Santa Cruz, CA, USA) diluted with blocking solution at 1:100 was added. After incubation at room temperature for 2–3 h, a 10-min wash of TBS/T was carried out thrice. The horseradish peroxidase-tagged goat anti-mouse IgG was diluted with blocking solution at 1:100. After incubation at room temperature for 1–2 h, a 10-min wash of TBS/T was carried out thrice. Staining was carried out in accordance with the instructions of 3,3′-diaminobenzidine reagent kit. The results were photographed and their gray scales were scanned and analyzed with the computer.

4.6 Microscopic observation of formed tubular HUVEC structure on Matrigel

An aliquot of 100 μl Matrigel (BD, MA, USA) was loaded into each hole of a 96-hole cell culturing plate. At 37°C, it was let to stand for 15 min until solidification. The cell density was adjusted until 1×10^5 cells ml^{-1}. For each hole, various concentrations of SY0916 or control solvent were added into 100 μl Matrigel. The stimulant was added after reacting for 30 min at 37°C. After incubation in a 5% CO_2 incubator for 12–24 h, the specimens were observed microscopically and photographed.

4.7 Statistical analysis

The results were expressed as mean ± SD. All the data were processed with the software SPSS 3.0. One-factor variance analysis was used in the comparison of several groups, and *t*-test was used to compare two groups. $P < 0.01$ was considered as statistically significant.

Acknowledgements

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