SHOCK, Vol. 34, No. 6, pp. 580–585, 2010

THE INDAZOLE DERIVATIVE YD-3 SPECIFICALLY INHIBITS THROMBIN-INDUCED ANGIOGENESIS *IN VITRO* AND *IN VIVO*

Chieh-Yu Peng,* Shiow-Lin Pan,*† Hui-Chen Pai,* An-Chi Tsai,* Jih-Hwa Guh,‡ Ya-Ling Chang,* Sheng-Chu Kuo,§ Fang-Yu Lee,‖ and Che-Ming Teng*

**Pharmacological Institute, College of Medicine, National Taiwan University; †Graduate Institute of Pharmacology, Taipei Medical University; and ‡School of Pharmacy, College of Medicine, National Taiwan University, Taipei; and §Graduate Institute of Pharmaceutical Chemistry, China Medical College; and ‖Yung-Shin Pharmaceutical Industry Co, Ltd, Taichung, Taiwan*

Received 30 Oct 2009; first review completed 24 Nov 2009; accepted in final form 9 Mar 2010

ABSTRACT—Angiogenesis is a process that involves endothelial cell proliferation, migration, invasion, and tube formation, and the inhibition of these processes has implications for angiogenesis-mediated disorders. The purpose of this study was to examine the antiangiogenic efficacy of YD-3 [1-benzyl-3(ethoxycarbonylphenyl)-indazole], a selective thrombin inhibitor, on thrombin-induced endothelial cell proliferation and neoangiogenesis in a murine Matrigel model. First, the effect of YD-3 on angiogenesis was evaluated *in vivo* using the mouse Matrigel implant model. Plugs treated with 1 and 10 μM of YD-3 inhibited neovascularization induced by thrombin, protease-activated receptor (PAR) 1, and PAR-4, but not by vascular endothelial growth factor, in a concentration-dependent manner over 7 days. These results indicate that YD-3 has specific antiangiogenic activity on thrombin. YD-3 also inhibited (in a concentration-dependent manner) the ability of thrombin, PAR-1, and PAR-4, but not PAR-2, to induce the proliferation of human umbilical vascular endothelial cells, using a [^{3}H]thymidine incorporation assay. YD-3 predominantly inhibited thrombin-induced vascular endothelial growth factor receptor 2 (Flk-1) expression, but not extracellular signal–regulated kinase 1/2 phosphorylation, using Western blot analysis. YD-3 may have benefit in elucidating pathophysiology induced by thrombin-induced angiogenesis.

KEYWORDS—YD-3, thrombin, human umbilical vein endothelial cells, angiogenesis, Flk-1

ABBREVIATIONS—YD-3-[1-benzyl-3(ethoxycarbonylphenyl)-indazole]; PARs — protease-activated receptors; VEGF — vascular endothelial growth factor; ERK1/2 — extracellular signal–regulated kinase 1/2; HUVECs — human umbilical vein endothelial cells; ECGs — endothelial cell growth supplements; PKC — protein kinase C; SLIGKV — PAR-2-activating peptide (SER-LEU-ILE-GLY-LYS-VAL)

INTRODUCTION

Angiogenesis is the formation of new blood vessels from preexisting endothelial vasculature (1). Physiologically, angiogenesis plays a crucial role in embryonic development, placental implantation, and wound healing. In contrast, it supports pathological conditions, such as solid tumor growth, diabetic retinopathy, psoriasis, and rheumatoid arthritis. Complex and diverse cellular actions, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes, have been implicated in angiogenesis (2). Although all these processes are regulated under normal conditions, abnormal vascularization is clearly implicated in tumor growth and metastasis. The extreme growth of tumors to sizes larger than a few cubic millimeters requires continuous recruitment of new blood vessels (3). These newly synthesized blood vessels also provide a route for cancer cells to enter the circulation and spread to other, distant organs (4). Because of the importance of angiogenesis, a simple and rapid *in vivo* method to determine the antiangiogenic potential of compounds is desirable to augment *in vitro* findings, and the murine Matrigel-plug assay has become the method of choice (5). Matrigel is extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. The major components of Matrigel are laminin, collagen IV, heparin sulfate proteoglycans, entactin, and nidogen. Matrigel is mixed with angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, or IL-8 and injected subcutaneously into the ventral region of mice, where it solidifies, forming a "Matrigel plug." Endothelial cells migrate into the plug and form vessels. Assessment of angiogenesis in the Matrigel plug can be achieved either by measuring hemoglobin or by scoring selected regions of histological sections for vascular density (6).

Thrombin, a serine protease derived from the precursor prothrombin, plays an important role in angiogenesis and is a mediator of cellular effects that contribute to inflammation reactions and the proliferation of endothelial cells in tumorigenesis (7, 8). Many of the functions of thrombin are mediated via activation of G protein–coupled protease-activated receptors, PAR-1, PAR-3, or PAR-4 (9, 10). Protease-activated receptors (PARs) are activated by an unusual, irreversible proteolytic mechanism in which the protease binds to and cleaves the amino-terminal exodomain of the receptor. This new amino terminus then binds intramolecularly to the body of the receptor to initiate transmembrane signaling (11). Recent studies have shown that thrombin has a significant stimulatory effect on angiogenesis in that it can induce VEGF

Address reprint requests to Che-Ming Teng, PhD, Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd, Sect. 1, Taipei, Taiwan. E-mail: cmteng@ntu.edu.tw.

Chieh-Yu Peng and Shiow-Lin Pan contributed equally to this work.

This study was supported by research grants from the National Science Council of the Republic of China (NSC 96-2628-B-002-109-MY3 and NSC 98-2321-B-002-022).
DOI: 10.1097/SHK.0b013e3181df00a3

(12), VEGF receptor 2 (Flk-1) (13), angiopoietin 2 (14), and αvβ3 integrin (15) in endothelial cells.

In previous studies (16, 17), we showed that YD-3 [1-benzyl-3(ethoxycarbonylphenyl)-indazole], a new synthetic indazole derivative, selectively inhibits rabbit platelet aggregation and vascular smooth muscle cell proliferation caused by thrombin. Alternatively, YD-3 selectively inhibits PAR-4–dependent platelet activation through blockade of PAR-4 and PAR-4–mediated thromboxane formation (18, 19). However, the role of YD-3 in thrombin-induced angiogenesis is unclear. In this study, we examined the ability of YD-3 to suppress angiogenesis *in vivo* and *in vitro* and elucidated its mechanism of action. Our data reveal that YD-3 specifically inhibits thrombin-induced angiogenesis in a murine Matrigel model but does not abolish the thrombin-induced angiogenic signal via extracellular signal–regulated kinase (ERK), which critically influences cell proliferation in endothelial cells. In the present study, we show that VEGF receptor 2 plays a predominant role in thrombin-mediated angiogenesis. Together, these data show that YD-3 inhibits thrombin-dependent endothelial cell proliferation *in vitro* and angiogenesis *in vivo*, by decreasing Flk-1 expression. Further studies will be needed to characterize the antiangiogenic effects of YD-3 more fully.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care Committee of College of Medicine, National Taiwan University, and care and handling of the animals were performed in accordance with the National Institutes of Health guidelines.

In vivo *matrigel plug assay*

The murine Matrigel-plug assay can be used to evaluate antiangiogenic effect. Matrigel, an extract of mouse Engelbreth-Holm-Swarm tumor, is liquid at 4°C, and forms a gel when warmed to 37°C. It provides the essential substrates for the development of angiogenesis. Male BALB/c-nu mice (20 g, 4 weeks of age) were obtained from National Laboratory Animal Center, Taiwan, and acclimated to laboratory conditions 1 week before tumor implantation. BALB/c-nu mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Nude mice were given s.c. injections of 500 μL of Matrigel (Becton Dickinson, Bedford, Mass) at 4°C with or without YD-3 (supplied by Yung-Shin Pharmaceutical Industry Co, Ltd, Taiwan) and thrombin, PAR-1–activating peptide (Ser-Phe-Leu-Leu-Arg-Asn, SFLLRN), PAR-4–activating peptide (Gly-Tyr-Pro-Gly-Lys-Phe, GYPGKF), and VEGF. After injection, the Matrigel rapidly formed a plug. After 7 days, animals were killed using an overdose injection of pentobarbital (150 mg/kg); the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. After quantitative differences were noted and photographed, hemoglobin was measured, as an indication of blood vessel formation, using the Drabkin method (Drabkin reagent kit 525; Sigma, St Louis, Mo). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical cord veins with collagenase and cultured in 75-cm^2 plastic flasks in M199 containing 20% inactivated fetal bovine serum (FBS), 15 μg/mL endothelial cell growth supplements. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Media were changed every 2 days, and cells were passaged after treatment with a solution of 0.05% trypsin/0.02% EDTA. Experiments were conducted on HUVECs that had been used in passages 2 to 5.

[^{3}H]thymidine incorporation assay

Confluent HUVECs were trypsinized, suspended in M199 supplemented with 20% FBS, and seeded at 5.0×10^3 cells per well into 96-well plates. After 24 h, the cells were starved with 2% FBS-M199 medium for 24 h. The cells were incubated with or without YD-3 and growth factors (thrombin, protease-activated receptors activating peptide, and VEGF) for 48 h and harvested. Before the harvest, cells were incubated with [^{3}H]thymidine (2 μCi/mL) for 16 h and harvested with Filter-Mate (Packard BioScience, Meriden, Conn), and incorporated radioactivity was determined.

Western blot analysis

After the exposure of cells to the indicated agents and time courses, cells were washed twice with ice-cold phosphate-buffered saline, and reaction was terminated by addition of 100 μL ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% Triton X-100). Protein (60 μg/lane) was separated by electrophoresis on a 5% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes, and blots were blocked with 5% nonfat milk for 1 h. The membrane was immunoreacted with the primary antibody to ERK1/2, phosphorylated-ERK1/2 (BD Biosciences, Rockville, Md), and Flk-1 (Santa Cruz biotechnology, Inc, Santa Cruz, Calif) for overnight incubation at 4C. After four washings with phosphate-buffered saline/0.1% Tween 20, the secondary antibody (dilute 1:2,000) was applied to membranes for 1 h at room temperature. The antibody-reactive bands were performed with an enhanced chemiluminescence kit (ECL; Amersham International, Little Chalfont, UK).

Data analysis and statistics

Data are presented as the mean ± SE. Statistical significance was ensured by one-way ANOVA followed by the Tukey test for multiple comparisons. $P < 0.05$ was considered statistically significant.

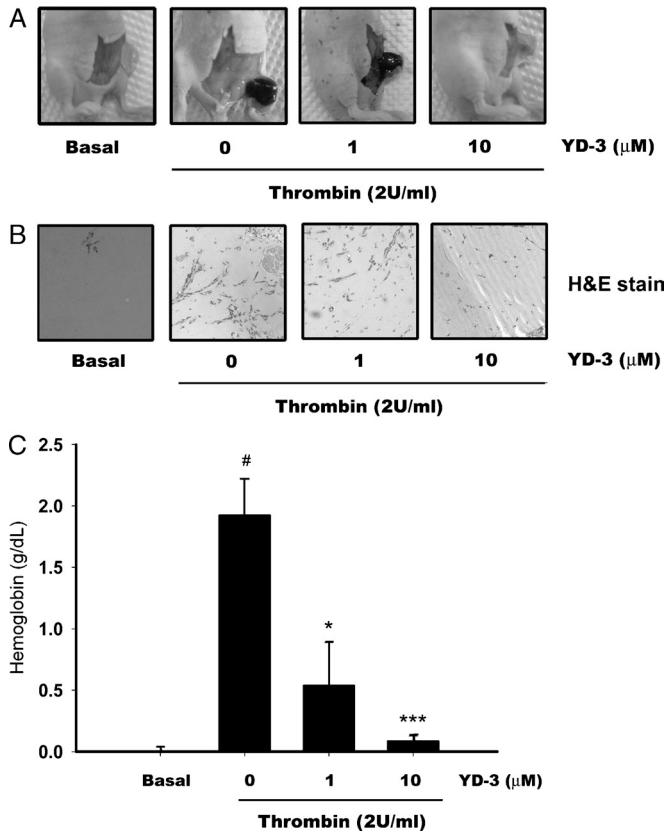

FIG. 1. **Effect of YD-3 on thrombin-induced neovascular formation *in vivo*.** A, Antiangiogenesis effect of YD-3 in *in vivo* mouse Matrigel-plug assay. The experimental procedures are described under Materials and Methods. Matrigel without growth factors (thrombin 2 U/mL) did not show any migration or invasion of endothelial cells. However, with Matrigel containing growth factor, many blood vessels appeared in the gel on mice subcutaneous. Note the significant concentration-dependent inhibition of the formation of blood vessel in the gel after coplug of YD-3 for 7 days. B, Histological analysis (hematoxylin and eosin staining) of the effect of YD-3 on *in vivo* angiogenesis. Matrigel containing thrombin in vehicle-treated mice demonstrated a high degree of cellularity and the presence of blood-containing vessels (original magnification ×100). C, Quantitation of active vasculature inside the Matrigel by measurement of hemoglobin content. Each value represents mean ± SE (n = 5 or 6). $^{\#}P < 0.001$ versus basal group; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control group.

RESULTS

Effect of YD-3 on thrombin-induced neovascularization in vivo

In a previous study, we showed that YD-3 specifically inhibited thrombin-induced vascular smooth muscle cell proliferation (17). Thus, we decided to determine whether YD-3 was capable of blocking thrombin-induced angiogenesis *in vivo*. Thrombin (2 U/mL) markedly increased the angiogenic response, compared with Matrigel alone (Fig. 1, A and B), and YD-3 (at 1 and 10 μM) significantly inhibited the angiogenic response in a concentration-dependent manner. Microscopic examination showed that the addition of thrombin to Matrigel induced cellularity and induced the formation of cords, tubules, and several blood-filled channels containing red blood cells. In contrast, Matrigel pellets with no angiogenic agent had only a few infiltrating, single, elongated cells. Thrombin-induced angiogenesis was significantly reduced in mice treated with thrombin plus YD-3, in a concentration-dependent manner. Quantification of angiogenesis, using the hemoglobin content, showed that the addition of thrombin to Matrigel induced an angiogenic response, compared with Matrigel alone (Fig. 1C). However, YD-3 also inhibited the thrombin-induced hemoglobin content, in a concentration-dependent manner. These results indicate that YD-3 is a potent antiangiogenic molecule *in vivo*.

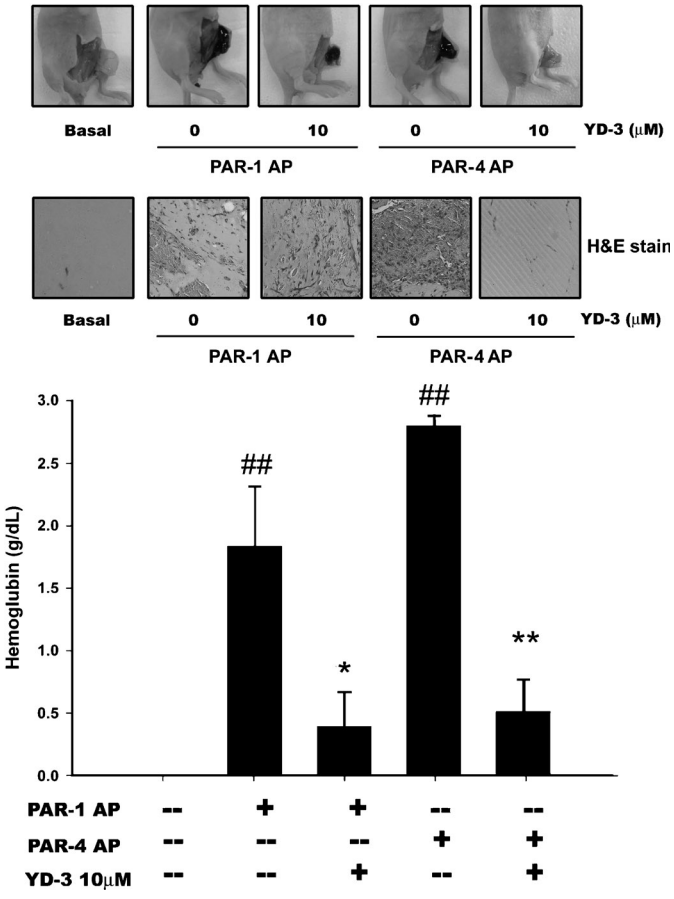

FIG. 2. **Effect of YD-3 on PAR-1– and PAR-4–induced neovascular formation *in vivo*.** Top, Antiangiogenesis effect of YD-3 in *in vivo* mouse Matrigel-plus assay. The experimental procedures are described under Materials and Methods. Matrigel without growth factors (100 μM PAR-1 AP and 500 μM PAR-4 AP) did not show any migration or invasion of endothelial cells. Matrigel containing growth factor, many blood vessels appeared in the gel on mice subcutaneous. Note the significant inhibition of the formation of blood vessel in the gel after coplug of YD-3 for 7 days. Middle, Histological analysis (hematoxylin and eosin staining) of the effect of YD-3 on *in vivo* angiogenesis. Matrigel containing thrombin in vehicle-treated mice demonstrated a high degree of cellularity and the presence of blood-containing vessels (original magnification ×100). Bottom, Quantitation of active vasculature inside the Matrigel by measurement of hemoglobin content. Each value represents mean ± SE (n = 5 or 6). $^{\#\#}P < 0.01$ versus basal group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus PAR-1 AP– and PAR-4 AP–treated group, respectively.

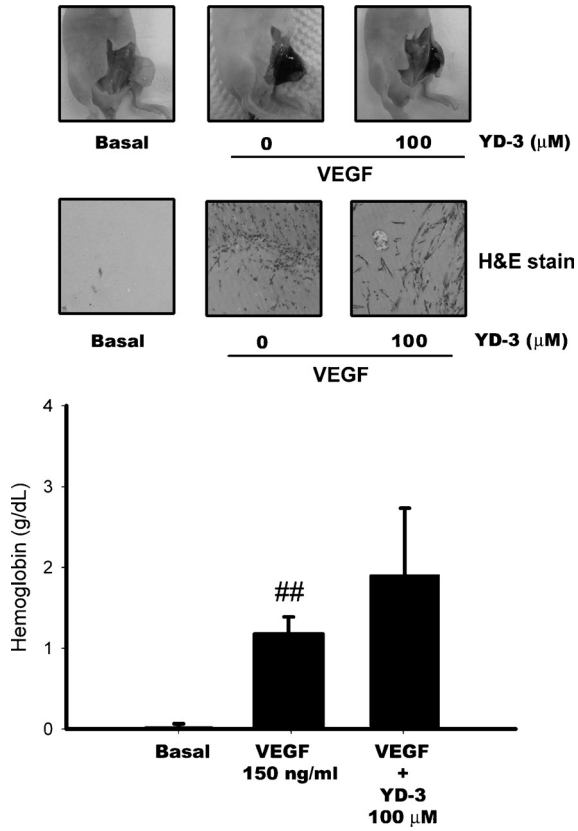

FIG. 3. **Effect of YD-3 on VEGF-induced neovascular formation *in vivo*.** Top, Antiangiogenesis effect of YD-3 in *in vivo* mouse Matrigel-plus assay. The experimental procedures are described under Materials and Methods. Matrigel without VEGF did not show any migration or invasion of endothelial cells. However, with Matrigel containing growth factor, many blood vessels appeared in the gel on mice subcutaneous. There is no significant inhibition of the formation of blood vessel between vehicle- and YD-3–treated groups. Middle, Histological analysis (hematoxylin and eosin staining) of the effect of YD-3 on *in vivo* angiogenesis. Matrigel containing VEGF in vehicle-treated mice demonstrated a high degree of cellularity and the presence of blood-containing vessels (original magnification ×100). Bottom, Quantitation of active vasculature inside the Matrigel by measurement of hemoglobin content. Each value represents mean ± SE (n = 5 or 6). $^{\#\#}P < 0.01$ versus basal group.

YD-3 selectively inhibits PAR-1– and PAR-4–induced neovascularization in vivo

Thrombin stimulates cellular functions that are mediated through the proteolytic activation of PAR-1 and PAR-4 (20). Thus, we decided to determine the effects of YD-3 on the PAR-1 activating-peptide (AP)–mediated (100 μM) and PAR-4 AP–mediated (500 μM) angiogenic functions. As shown in Figure 2, YD-3 (10 μM) significantly inhibited the PAR-1 AP– and PAR-4 AP–induced angiogenic effects (Fig. 2). The thrombin-antagonizing action of YD-3 (100 μM) was verified by its failure to inhibit angiogenesis stimulated by VEGF (Fig. 3), a strong mitogen that induces angiogenesis. These

results suggest that YD-3 is a specific thrombin inhibitor that decreases angiogenesis *in vivo*.

Effect of YD-3 on thrombin- and PAR-induced endothelial cell proliferation

The effect of YD-3 on thrombin (2 U/mL) and PAR-mediated HUVEC growth was assessed using [^{3}H]thymidine incorporation. As shown in Figure 4A, YD-3 significantly inhibited the thrombin-induced increase of DNA synthesis, in a concentration-dependent manner ($IC_{50} = 1.1 \times 10^{-5}$ M). On the other hand, YD-3 specifically suppressed cell proliferation induced by PAR-1 AP (Fig. 4B) and PAR-4 AP (Fig. 4C) in a concentration-dependent fashion ($IC_{50} = 1.1 \times 10^{-5}$ M and 6.9×10^{-7} M, respectively), but did not affect the cell proliferation induced by PAR-2–AP (SLIGKV, Fig. 4D).

Effect of YD-3 on ERK1/2 phosphorylation induced by thrombin and PARs

It has been established that mitogen-activated protein kinases (MAPKs), components in the signaling pathway, are activated during the stimulation of cell proliferation (21).

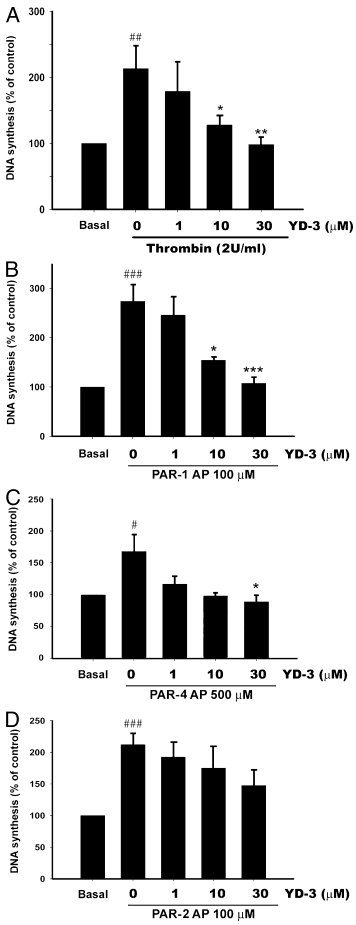

FIG. 4. **Effect of YD-3 on thrombin- and PAR-induced endothelial cell proliferation**. Effects of YD-3 (1–30 μM) on (A) thrombin (2 U/mL), (B) PAR-1 AP (100 μM), (C) PAR-4 AP (500 μM), and (D) PAR-2 AP (100 μM) HUVEC growth were examined using [^{3}H]thymidine incorporation to assess proliferation. Data represent the mean ± SEM of six independent experiments (each performed in triplicate). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus basal group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group. Without YD-3, only mitogen and vehicle-treated cells assigned as control group.

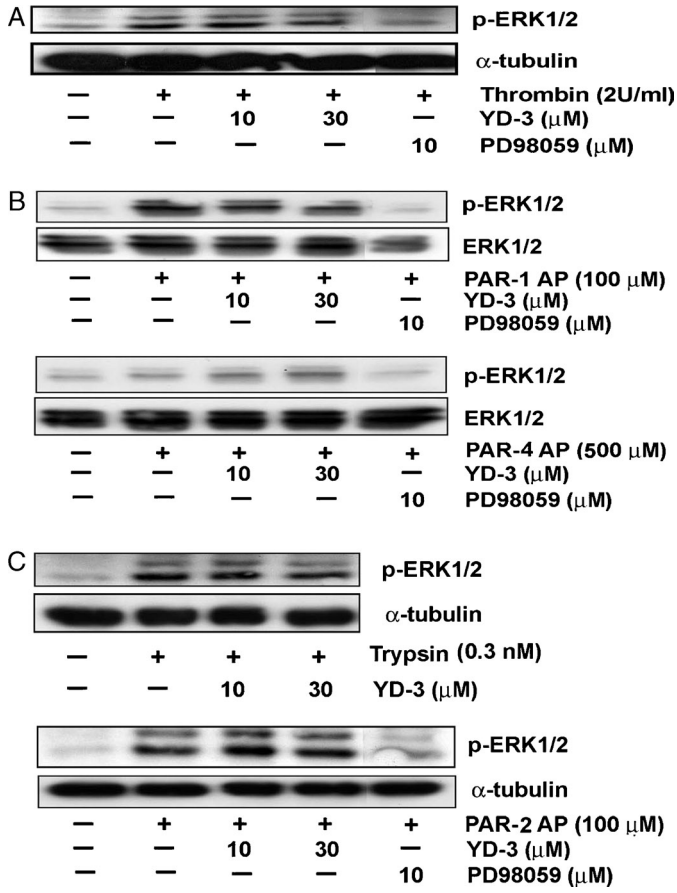

FIG. 5. **Effect of YD-3 on ERK1/2 phosphorylation induced by thrombin and protease-activated receptors activating peptide**. Human umbilical vein endothelial cells were incubated in the absence or presence of YD-3 for 1 h, and vehicle or angiogenic growth factors (A) thrombin (2 U/mL), (B) PAR-1 AP (100 μM), PAR-4 AP (500 μM), and (C) trypsin (0.3 nM), PAR-2 AP (100 μM) were added to the cells for another 15 min. PD98059, a MEK inhibitor, was used as positive control. Cells were harvested for the detection of phosphorylated-ERK1/2 and total ERK1/2 using Western blotting. Thrombin, PAR-1 AP, PAR-4 AP, PAR-2 AP, and trypsin induced a profound increase in ERK1/2 phosphorylation, and no significant inhibition was observed in YD-3–treated groups.

Thus, we determined whether YD-3 inhibits thrombin- and PAR-induced activation of ERK1/2 in HUVECs. As shown in Figure 5, thrombin, PAR-1 AP, PAR-2 AP, and PAR-4 AP induced a profound increase in ERK1/2 activation. YD-3 did not suppress thrombin-induced ERK1/2 phosphorylation in HUVECs (Fig. 5A). On the other hand, PD98059, a selective MAPK inhibitor (it inhibits MEK), markedly inhibited the effects of thrombin. No inhibition was observed with YD-3 on PAR-1 AP, PAR-4 AP, or PAR-2 AP. Moreover, trypsin, a specific PAR-2 agonist, stimulated ERK1/2 activation (Fig. 5, B and C), indicating that ERK1/2 does not plays a major role in YD-3–medicated inhibition of thrombin-induced endothelial cell proliferation.

Effect of YD-3 on thrombin-induced VEGF receptor 2 upregulation

The VEGF receptor, which drives endothelial cell proliferation, is also highly expressed in these cells. In a previous study, thrombin was shown to mediate upregulation of the VEGF receptor in endothelial cells (13). To investigate whether

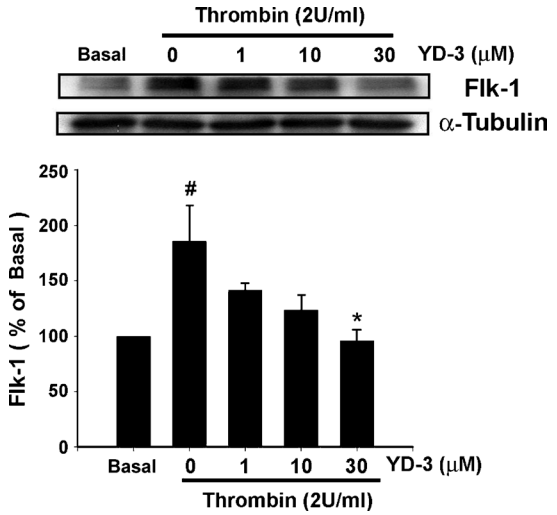

FIG. 6. **Effect of YD-3 on thrombin-induced Flk-1 upregulation**. Quiescent HUVECs were pretreated with dimethyl sulfoxide (CTL) or YD-3 (1, 10, 30 μM) for 1 h and untreated (basal) or treated with thrombin (2 U/mL) for another 24 h. Cell extracts were prepared, and equal amounts of protein were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with antibodies specific for Flk-1. The quantitative data are shown for ratio between basal and thrombin-treated groups. $^{\#}P < 0.05$ versus basal group; $^{*}P < 0.05$ versus control group.

YD-3 inhibits thrombin-stimulated VEGF receptor upregulation, HUVECs were stimulated with thrombin (2 U/mL), and Flk-1 expression was evaluated by Western blot analysis. Thrombin increased the expression of Flk-1 (Fig. 6), which was involved in the mechanism of activation of angiogenesis by thrombin. YD-3 significantly inhibited thrombin-induced Flk-1 upregulation in a concentration-dependent manner.

DISCUSSION

The purpose of this study was to examine the ability of YD-3 to suppress angiogenesis *in vivo* and *in vitro* and to determine its specificity and mechanism of action. Our data revealed that YD-3 significantly inhibited thrombin-induced angiogenesis in a murine Matrigel model. In contrast, YD-3 had no or little inhibitory effect on the angiogenesis elicited by VEGF, a highly angiogenic growth factor.

Angiogenesis, the process of new blood-vessel growth, involves complex molecular signaling (22). Proliferation of endothelial cells, a crucial event in angiogenesis, is regulated by growth factors such as VEGF and basic fibroblast growth factor (23). Thrombin also modulates endothelial cell proliferation and angiogenesis (8, 24, 25).

YD-3, a low-molecular-weight nonpeptide thrombin antagonist, has an advantage over a direct thrombin inhibitor because it does not inhibit the enzymatic action of thrombin in the coagulation cascade, with minimal bleeding adverse effects. Compared with peptide-mimic thrombin antagonists, YD-3 is also advantageous because the instability of peptides often restricts their medical application. Moreover, YD-3 had good oral availability in a previous study (17) and dual effects on both PAR-1 and PAR-4.

It is well established that thrombin activates PAR-1, PAR-3, and PAR-4 receptors (9). However, on the basis of studies with vascular smooth muscle cells and platelets, it seems that YD-3 specifically blocks the action of PAR-1 and PAR-4 (17, 18). To date, there is much functional evidence about PAR-1 and PAR-4 protein expression in endothelial cells (26, 27), and thus PAR-1 and PAR-4 were considered to be the major thrombin receptor in these cells. In this study, we demonstrated that the addition of YD-3 significantly inhibited the proliferative effect of thrombin, PAR-1 AP, and PAR-4 AP in HUVECs. These results reveal that YD-3 acts via PAR-1 and PAR-4 to inhibit thrombin-induced endothelial cell proliferation and then blocks angiogenesis *in vivo*.

Many studies have revealed that thrombin-induced endothelial cell proliferation involves activation of protein kinase C (PKC) (28, 29). Protein kinase C is found primarily in the cytosol of unstimulated cells and becomes firmly associated with the cell membrane after stimulation. In this study, we found that YD-3 did not affect thrombin-stimulated PKC translocation (data not shown). Additionally, ERK1/2 MAPK is a key regulator of cell proliferation; it regulates gene expression and cell cycle reentry. Many growth factors and G protein–coupled receptor agonists induce cell proliferation via activation of ERK1/2 MAPK (30). Several lines of evidence show that thrombin activates MAPK in a variety of cell types. In the present study, we demonstrated that thrombin-induced cell proliferation was mediated via activation of ERK1/2. However, ERK1/2 phosphorylation induced by thrombin and PAR peptides was not abolished by YD-3.

As previously noted, thrombin-induced angiogenesis is associated with upregulation of VEGF (12) and the major VEGF receptor, Flk-1 (13). Thrombin also upregulates αvβ3 integrin (15) and matrix metalloproteinase 2 (31) in endothelial cells. Recent evidence indicates a pivotal role for chemokine growth-regulated oncogene α in thrombin-induced angiogenesis (32). All of these proteins contribute to thrombin-induced angiogenesis. We demonstrated that thrombin-induced expressions of VEGF and αvβ3 integrin were not affected by YD-3 treatment (data not shown). Moreover, YD-3 does not alter the upregulation of chemokine growth-regulated oncogene α and activation of MMP-2 stimulated by thrombin (data not shown), using reverse transcriptase–polymerase chain reaction and zymography. In this study, compared with the basal group, protein expression of the VEGF receptor (Flk-1) was significantly increased at 24 h after thrombin stimulation and was significantly inhibited in YD-3–treated cells, suggesting that upregulation of the VEGF receptor may play a key role in the thrombin-stimulated angiogenic response.

In conclusion, YD-3–mediated Flk-1 suppression may be important for the inhibition of angiogenesis stimulated by thrombin in endothelial cells. There are currently no effective treatments for some angiogenesis-related diseases, such as cancer, restenosis, and age-related macular degeneration. Thrombin-induced angiogenesis may be involved in the pathological process of these diseases. YD-3 used alone or in combination with other agents may potentially be the treatment for angiogenic disorders. Further investigation is required to characterize the detailed molecular mechanism(s) and to identify the molecular target(s) associated with the antiangiogenic activities of YD-3.

REFERENCES

1. Folkman J, Merler E, Abernathy C, Williams G: Isolation of a tumor factor responsible on angiogenesis. *J Exp Med* 133:275–288, 1971.
2. Bussolino F, Mantovani A, Persico G: Molecular mechanisms of blood vessel formation. *Trends Biochem Sci* 22:251–256, 1997.
3. Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82:4–6, 1990.
4. Fidler IJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185–188, 1994.
5. Malinda KM: *In vivo* matrigel migration and angiogenesis assays. *Methods in Mol Med* 46:47–52, 2001.
6. Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N: Angiogenesis assays: a critical overview. *Clin Chem* 49:32–40, 2003.
7. Tsopanoglou NE, Maragoudakis ME: Role of thrombin in angiogenesis and tumor progression. *Semin Thromb Hemost* 30:63–69, 2004.
8. Nierodzik ML, Karpatkin S: Thrombin induces tumor growth, metastasis, and angiogenesis: evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell* 10:355–362, 2006.
9. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R: Proteinase-activated receptors. *Pharmacol Rev* 53:245–282, 2001.
10. Barnes JA, Singh S, Gomes AV: Protease activated receptors in cardiovascular function and disease. *Mol Cell Biochem* 263:227–239, 2004.
11. Traynelis SF, Trejo J: Protease-activated receptor signaling: new roles and regulatory mechanisms. *Curr Opin Hematol* 14:230–235, 2007.
12. Ollivier V, Chabbat J, Herbert JM, Hakim J, de Prost D: Vascular endothelial growth factor production by fibroblasts in response to factor VIIa binding to tissue factor involves thrombin and factor Xa. *Arterioscler Thromb Vasc Biol* 20:1374–1381, 2000.
13. Tsopanoglou NE, Maragoudakis ME: On the mechanism of thrombin-induced angiogenesis. Potentiation of vascular endothelial growth factor activity on endothelial cells by up-regulation of its receptors. *J Biol Chem* 274:23969–23976, 1999.
14. Huang YQ, Li JJ, Hu L, Lee M, Karpatkin S: Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells. *Blood* 99:1646–1650, 2002.
15. Tsopanoglou NE, Andriopoulou P, Maragoudakis ME: On the mechanism of thrombin-induced angiogenesis: involvement of alphavbeta3-integrin. *Am J Physiol Cell Physiol* 283:C1501–C1510, 2002.
16. Wu CC, Huang SW, Hwang TL, Kuo SC, Lee FY, Teng CM: YD-3, a novel inhibitor of protease-induced platelet activation. *Br J Pharmacol* 130: 1289–1296, 2000.
17. Peng CY, Pan SL, Guh JH, Liu YN, Chang YL, Kuo SC, Lee FY, Teng CM: The indazole derivative YD-3 inhibits thrombin-induced vascular smooth muscle cell proliferation and attenuates intimal thickening after balloon injury. *Thromb Haemost* 92:1232–1239, 2004.
18. Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Lee CY, Teng CM: Selective inhibition of protease-activated receptor 4-dependent platelet activation by YD-3. *Thromb Haemost* 87:1026–1033, 2002.
19. Wu CC, Hwang TL, Liao CH, Kuo SC, Lee FY, Teng CM: The role of PAR4 in thrombin-induced thromboxane production in human platelets. *Thromb Haemost* 90:299–308, 2003.
20. Coughlin SR: Thrombin signalling and protease-activated receptors. *Nature* 407:258–264, 2000.
21. Seger R, Krebs EG: The MAPK signaling cascade. *FASEB J* 9:726–735, 1995.
22. Ahmed Z, Bicknell R: Angiogenic signalling pathways. *Methods Mol Biol* 467:3–24, 2009.
23. Suhardja A, Hoffman H: Role of growth factors and their receptors in proliferation of microvascular endothelial cells. *Microsc Res Tech* 60:70–75, 2003.
24. Maragoudakis ME, Tsopanoglou NE, Andriopoulou P: Mechanism of thrombin-induced angiogenesis. *Biochem Soc Trans* 30:173–177, 2002.
25. Caunt M, Huang YQ, Brooks PC, Karpatkin S: Thrombin induces neoangiogenesis in the chick chorioallantoic membrane. *J Thromb Haemost* 1:2097–2102, 2003.
26. Kataoka H, Hamilton JR, McKemy DD, Camerer E, Zheng YW, Cheng A, Griffin C, Coughlin SR: Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* 102:3224–3231, 2003.
27. Hirano K, Nomoto N, Hirano M, Momota F, Hanada A, Kanaide H: Distinct Ca^{2+} requirement for NO production between proteinase-activated receptor 1 and 4 (PAR1 and PAR4) in vascular endothelial cells. *J Pharmacol Exp Ther* 322:668–677, 2007.
28. Bogatkevich GS, Gustilo E, Oates JC, Feghali-Bostwick C, Harley RA, Silver RM, Ludwicka-Bradley A: Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. *Am J Physiol Lung Cell Mol Physiol* 288:L190–L201, 2005.
29. Webb ML, Taylor DS, Molloy CJ: Effects of thrombin receptor activating peptide on phosphoinositide hydrolysis and protein kinase C activation in cultured rat aortic smooth muscle cells: evidence for tethered-ligand activation of smooth muscle cell thrombin receptors. *Biochem Pharmacol* 45:1577–1582, 1993.
30. Rozengurt E: Mitogenic signaling pathways induced by G protein–coupled receptors. *J Cell Physiol* 213:589–602, 2007.
31. Lafleur MA, Hollenberg MD, Atkinson SJ, Knäuper V, Murphy G, Edwards DR: Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63 kDa active species. *Biochem J* 357:107–115, 2001.
32. Caunt M, Hu L, Tang T, Brooks PC, Ibrahim S, Karpatkin S: Growth-regulated oncogene is pivotal in thrombin-induced angiogenesis. *Cancer Res* 66: 4125–4132, 2006.