A novel low molecular weight antagonist of vascular endothelial growth factor receptor binding: VGA1155

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Abstract

Vascular endothelial growth factor (VEGF) plays a pivotal role in the processes of angiogenesis, which is essential for the growth of solid tumors and their metastasis. Because VEGF is a critical factor in tumor survival, inhibiting VEGF would provide significant benefits in tumor therapy. To identify a compound that inhibits the binding of VEGF to its receptor, we used a high throughput screening method, finding that small molecular compounds inhibited VEGF binding. Among active compounds, 5-[*N*-methyl-*N*-(4-octadecyloxyphenyl)acetyl]amino-2-methylthiobenzoic acid (VGA1155) was selected for its potent inhibition of binding. VGA1155 inhibited [^{125}I] VEGF binding to two cell lines, NIH3T3-fms-like tyrosine kinase-1 (VEGF receptor 1 transfected) cells and NIH3T3-kinase insert domain containing receptor/fetal liver kinase-1 (KDR/Flk-1; VEGF receptor 2 transfected), in a concentration-dependent manner. VGA1155 did not inhibit the binding of several other growth factors or cytokines to their receptors. Based on the results of surface plasmon resonance analysis using Biacore S51 system, it appears that this binding inhibitory property may be based on the association of VGA1155 with VEGF receptor 2 (KDR/Flk-1). Further, the interference in VEGF binding by VGA1155 in turn induces the inhibition of VEGF-induced KDR/Flk-1 autophosphorylation. VGA1155 also reduced intradermal VEGF-induced vascular permeability in guinea pigs. These findings indicate that VGA1155 inhibits not only VEGF binding to its receptors through association with KDR/Flk-1 but also VEGF function *in vivo*. These VGA1155 activities may provide a useful basis for the development of antiangiogenic and antitumor agents. (Mol Cancer Ther. 2003;2:1105–1111)

Introduction

Angiogenesis, the formation of new blood vessels sprouting from preexisting vessels, plays a crucial role in physiological and pathological phenomena, including embryonic development, wound healing, diabetic retinopathy, psoriasis, rheumatoid arthritis, and solid tumor growth and metastasis (1, 2).

In the process of angiognesis, vascular endothelial growth factor (VEGF) is essential for growth, mitogenesis, and tube formation of endothelial cells (3–6). This importance of the function of VEGF suggests that blocking this function may provide a promising new therapeutic strategy for inhibiting angiogenesis and tumor growths. Practically, several anti-VEGF agents have already been reported. One, SU5416, the first VEGF receptor tyrosine kinase inhibitor, has produced certain clinical benefits in clinical trials involving patients suffering from advanced malignancies (7).

VEGF binds to two tyrosine kinase receptors, fms-like tyrosine kinase-1 (Flt-1) and kinase insert domain containing receptor/fetal liver kinase-1 (KDR/Flk-1), on the surface of endothelial cells, thereby activating signal transduction and regulating physiological and pathological angiogenesis (8). We hypothesized that an agent that inhibits binding between VEGF and its receptors may serve as a valuable antiangiogenic and antitumor agent. Several VEGF binding antagonists, including anti-VEGF antibody (9, 10), anti-KDR/Flk-1 antibody (11), 2′-fluoropyrimidine RNA-based aptamers (12), various peptides (13, 14), and porphyrin analogues (15), have been reported. However, these antagonists may demand high cost of synthesis because of their large molecule weight. Furthermore, there is concern that antibodies and peptides may have antigenicity and *in vivo* instability. Therefore, we thought that it was worth finding a low molecular weight antagonist.

To identify the small compounds that inhibit the binding of VEGF to its receptor, we deployed a high throughput screening method using the VEGF receptor (Flt-1) binding scintillation proximity assay kit (Amersham, Biosciences, Uppsala, Sweden) and our chemical compound library. We found that novel benzoic acid derivatives inhibited VEGF binding to the two receptors. Among VEGF binding antagonist compounds, we selected 5-[*N*-methyl-*N*-(4-octadecyloxyphenyl)acetyl]amino-2-methylthiobenzoic acid (VGA1155) for its potent binding inhibitory action.

This report discusses the inhibitory action and mechanism of VGA1155 and its effects on signal transduction and the function of VEGF *in vivo*.

Materials and Methods

Materials

VGA1155 and 4-{*N*-[3-(4-octadecyloxyphenyl)propionyl]}aminophthalic acid (VGA1154) (Fig. 1, A and B) were synthesized in our laboratory. VGA1154 and VGA1155
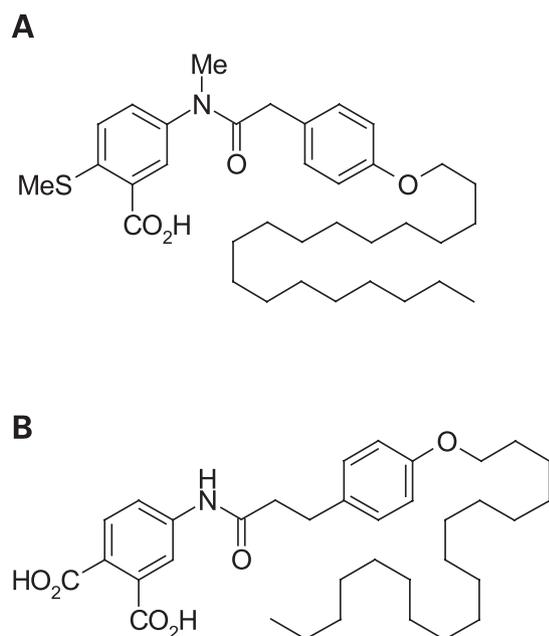
A

B

Figure 1. Chemical structure of VGA1155. **A,** VGA1155 (MW 583.87). **B,** VGA1154, an analogue of VGA1155 without inhibition of VEGF-KDR/Flk-1 binding (MW 581.79).

were dissolved in DMSO and diluted with the medium or PBS before use. VEGF (165-amino acid type) was purchased from Pepro Tech, EC, Ltd. (London, UK), KDR/Fc chimera protein (carrier free) was from R&D Systems, Inc. (Minneapolis, MN), $[^{125}I]$ VEGF was from Amersham, transforming growth factor (TGF)-β was from Genzyme/Techne (Minneapolis, MN), and $[^{125}I]$ TGF-β was from NEN Life Science Products, Inc. (Boston, MA).

Cells

NIH3T3-Flt-1 and NIH3T3-KDR/Flk-1 cells overexpressing human Flt-1 and KDR/Flk-1, respectively, were provided by Prof. Masabumi Shibuya (University of Tokyo). These cell lines were established as described previously (16) and maintained in DMEM containing 10% calf serum and 200-μg/ml G418 at 37°C in a humidified atmosphere of 5% CO_2/95% air. BALB/c-3T3 cells were maintained in DMEM containing 10% calf serum under the same conditions.

VEGF Receptor Binding Inhibition Assay

NIH3T3-Flt-1 and NIH3T3-KDR/Flk-1 cells were seeded at 7×10^4 cells/well in 24-well collagen-coated plates 24 h before experimental use. These cells were preincubated with medium A (DMEM containing 10-mM HEPES, pH 7.2, and 0.1% BSA) at 4°C for 30 min. The medium was then replaced with 0.3-ml medium B (DMEM containing 10-mM HEPES, pH 7.2, and 0.5% BSA) containing 25-pM $[^{125}I]$ VEGF (37.5–62.5 nCi/ml; Amersham) and varying concentrations of VGA1155. The plates were incubated at 4°C for 90 min. All experiments were performed in triplicate wells. After incubation, cells were washed thrice with ice-cold medium A. After cells were solubilized with 0.5 ml of 0.5-N NaOH at 37°C for 30 min, each solution in the wells was

transferred to test tubes. The wells were washed with 0.5-ml PBS, and the washing solution was combined with the solution in the tubes. The radioactivity of each tube was counted by a gamma counter. The percentage of radioactivity of each group relative to the control group was calculated, after which IC_{50} was calculated by nonlinear regression analysis.

Nonspecific binding was determined by incubation in the presence of 10-nM unlabelled VEGF (Pepro Tech, EC).

TGF-β Receptor Binding Inhibition Assay

BALB/c-3T3 cells were seeded at 5×10^4 cells/well in 24-well collagen-coated plates at 48 h before experimental use. These cells were preincubated for 30 min in a binding buffer containing 50-mM HEPES (pH 7.5), 128-mM NaCl, 5-mM KCl, 5-mM $MgSO_4$, 1.2-mM $CaCl_2$, and 0.2% BSA at 4°C. The binding buffer was then refreshed and supplemented with 50-pM $[^{125}I]$ TGF-β (37.5–62.5 nCi/ml; Amersham) and varying concentrations of VGA1155. The plates were incubated at 4°C for 240 min. All experiments were performed in duplicate wells. After incubation, cells were washed four times with the ice-cold binding buffer. After cells were solubilized with the cell solubilization buffer containing 25-mM HEPES, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 1% BSA, each solution in the wells was transferred to test tubes. The wells were washed with 0.5-ml PBS, and the washing solution was combined with the solution in the tubes. The radioactivity of each tube was counted by a gamma counter, and the percentage of radioactivity of each group relative to the control group was calculated, after which IC_{50} was calculated by nonlinear regression analysis. Nonspecific binding was determined by incubation in the presence of 10-nM unlabelled TGF-β (Genzyme/Techne).

Receptor Binding Inhibition Assay in Cerep (Paris, France)

Receptor binding assays of several ligands [(epidermal growth factor (EGF), platelet-derived growth factor (PDGF), platelet-activating factor (PAF)-interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, MIP-1β, and insulin] were performed in Cerep. The membranes or cells included each receptor incubated with radioisotope-labeled ligands (0.02–1 nM) and VGA1155 (0.1–10 μM) for 0.5–4 h except insulin (20 h) at 4°C or 22°C. The membranes or cells in suspension were rapidly filtered under vacuum through glass fiber filters (GF/B or GF/C, Whatman, Inc., Clifton, NJ, or Packard, Meriden, CT). The filters were then washed several times with an ice-cold buffer using a cell harvester (Brandel, Gaithersburg, MD, or Packard). Cultured adherent cells were rinsed with an ice-cold buffer and then lysed. Bound radioactivity was measured with a scintillation counter (LS 6000 or LS 1701, Beckman Instruments, Fullerton, CA, or Topcount, Packard) using a liquid scintillation cocktail (Formula 989 or Microscint 0, Packard).

Interaction Study by Surface Plasmon Resonance in the Biacore S51 System

Human VEGF (66.7 μg/ml in 10-mM sodium acetate, pH 4.5) and human KDR/Fc chimera protein (5 μg/ml in 10-mM

sodium acetate, pH 4.0) were covalently immobilized on the Biacore sensorchips CM-5 (carboxylated dextran matrix) after activation with a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide according to the description of the manufacturer's instructions. The remaining active groups on the matrix were blocked with 1-M ethanolamine-HCl at pH 8.5. The corresponding molar quantities of immobilized VEGF and KDR/Fc chimera protein on the each sensor tip were 0.39 and 0.13 pmol, respectively. Studies of interactions were performed in a running buffer consisting of PBS containing 1% (v/v) DMSO and 0.05% (v/v) Tween 20. VGA1155 or VGA1154 (VGA1155 analogues without inhibitory activity against VEGF-KDR/Flk-1 binding) was injected onto immobilized VEGF and KDR/Fc chimera proteins at a flow rate of 30 μl/min.

Autophosphorylation of KDR/Flk-1 Receptor

We assessed the effect of VGA1155 on VEGF-induced receptor autophosphorylation as described previously (16). NIH3T3-KDR/Flk-1 cells were plated on 10 cm dishes, grown to near confluence, and serum starved in serum-free DMEM for 1 day. Serial dilutions of VGA1155 were added to culture dishes and incubated for 0 or 60 min. KDR/Flk-1 autophosphorylation was stimulated by the addition of 10-ng/ml human VEGF (Pepro Tech, EC) for 5 min, and cells were rinsed twice in ice-cold PBS containing 0.1-mM Na_3VO_4 and lysed in 1% Triton X-100 lysis buffer (50-mM HEPES, pH 7.4, 100-mM NaCl, 1.5-mM $MgCl_2$, 50-mM NaF, 10-mM $Na_4P_2O_7$, 10% glycerol, 1% Triton X-100, 2-mM phenylmethylsulfonyl fluoride, 2-mM Na_3VO_4, 8-μg/ml benzamidine HCl, 5-μg/ml phenanthroline, 5-μg/ml aprotinin, 5-μg/ml leupeptin, 5-μg/ml pepstatin A). The lysates were clarified by centrifugation (15,000 rpm × 10 min). Protein concentrations were measured using Bio-Rad protein assay (Bio-Rad, Hercules, CA), and equal amounts of protein for each sample were used for analysis. The cell lysates were incubated overnight with anti-human KDR/Flk-1 antibodies (IBL, Fujioka, Japan) at 4°C. The resulting immune complexes were collected by precipitation with protein G + protein A agarose beads (Calbiochem, La Jolla, CA). Immunoprecipitates were then washed five times with a 1% Triton X-100 lysis buffer. Samples were separated by electrophoresis on 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After the transfer, the membrane was incubated overnight in a blocking buffer (1% BSA, 10-mM Tris, pH 7.5, 100-mM NaCl, 0.1% Tween 20) at 4°C. The membrane was then probed with anti-phosphotyrosine-horseradish peroxidase conjugate antibody (Amersham). Chemiluminescence activity was detected by SuperSignal substrate Western blotting (Pierce Chemical Co., Rockford, IL).

Inhibitory Effects on VEGF-Induced Vascular Permeability

We determined the effects of VGA1155 on VEGF-induced vascular permeability by the method described previously (16). After shaving the back of anesthetized guinea pig, we injected the animal with 0.7 ml of 1% trypan blue through the brachial vein. After 30 min, 100 μl of the injection solution (PBS, 20-ng VEGF with or without 30-ng VGA1155) were injected intradermally into the back of the guinea pig. After 30 additional minutes, dye leakage was detected by the presence of a blue spot surrounding the injection site. In the case of histamine, dye leakage was induced by 0.1-μg histamine.

Results

Inhibition of VEGF Receptor Binding by VGA1155

To identify a novel VEGF receptor binding antagonist, we employed a high throughput screening method using a Flt-1 scintillation proximity assay kit (Amersham). We found that the novel benzoic acid derivatives inhibited VEGF-Flt-1 binding. Among the active compounds, VGA1155 (Fig. 1A) was selected for its potent inhibition of binding.

To confirm activity antagonistic to VEGF receptor binding, we determined the effects of VGA1155 on [^{125}I] VEGF (25 pM) binding to Flt-1 receptor-overexpressing cells (NIH3T3-Flt-1). Additionally, given work in recent years that has clearly indicated the involvement of KDR/Flk-1, another VEGF receptor, in the process of angiogenesis (17, 18), we evaluated the antagonistic activity of VGA1155 against VEGF binding to KDR/Flk-1-overexpressing cells (NIH3T3-KDR/Flk-1). VGA1155 inhibited [^{125}I] VEGF binding to NIH3T3-Flt-1 cells and NIH3T3-KDR/Flk-1 cells in a concentration-dependent manner (Fig. 2), with IC_{50} values of 0.38 ± 0.07 and 0.14 ± 0.00 μM, respectively. VGA1155 (>3 μM) did not decrease the value of VEGF binding, which may be due to its poor solubility in the binding buffer.

Interaction between VGA1155 and KDR/Flk-1

To understand how VGA1155 inhibits interactions between VEGF and its receptors, we investigated whether the compound selectively bind to the receptor (KDR/Flk-1), the ligand (VEGF), or both. We used the Biacore S51 system to examine the binding affinity of VGA1155 to both proteins.
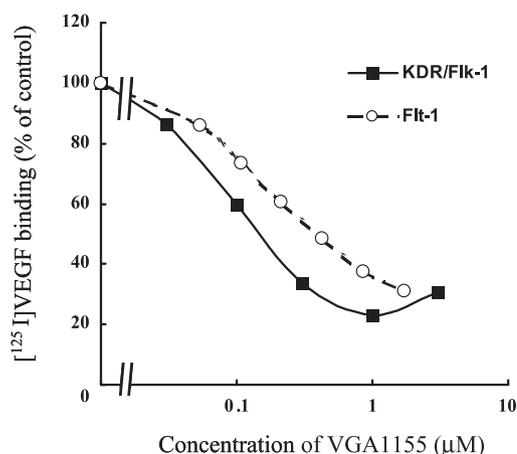

Figure 2. VGA1155 inhibits the binding to NIH3T3-Flt-1 cells and NIH3T3-KDR cells. NIH3T3-Flt-1 and NIH3T3-KDR/Flk-1 cells were incubated at 4°C for 90 min in the medium containing 25-pM [^{125}I] VEGF and varying concentrations of VGA1155. After incubation, cells were washed thrice with ice-cold medium, and the radioactivity of each tube was counted in a gamma counter. The percentage of radioactivity of each group relative to the control group was calculated.

In recent years, use of Biacore biosensor technology has become common in investigations of drug-protein interactions (19–22). The principle underlying this technology relies on the surface plasmon resonance (SPR) phenomenon, which transforms the specific incident angle of the light reflected from a metallic surface in response to the solute bound to the surface. Biacore S51 measurements are arbitrary expressed in resonance units (RU) proportional to the concentration of the solute bound to the surface. Because the change of mass of 1 ng/mm² corresponds to 1000 RU, we cannot directly observe how many compounds are bound to each protein. Thus, we converted RU to the average number of compounds bound to each protein.

Fig. 3, A and B, shows the average number of molecules bound to VEGF or KDR/Flk-1 based on the RU, as given by the following formula:

$$[\textit{Average number of molecules bound to VEGF or KDR/Flk} - 1]$$
$$= ([RU]/[\textit{Molecular weight of compounds}])$$
$$\times (1/[\textit{Amount of immobilized VEGF or KDR/Flk} - 1$$
$$\textit{on the sensor tip (pmol)}])$$

A

Degree of Binding to VEGF

B
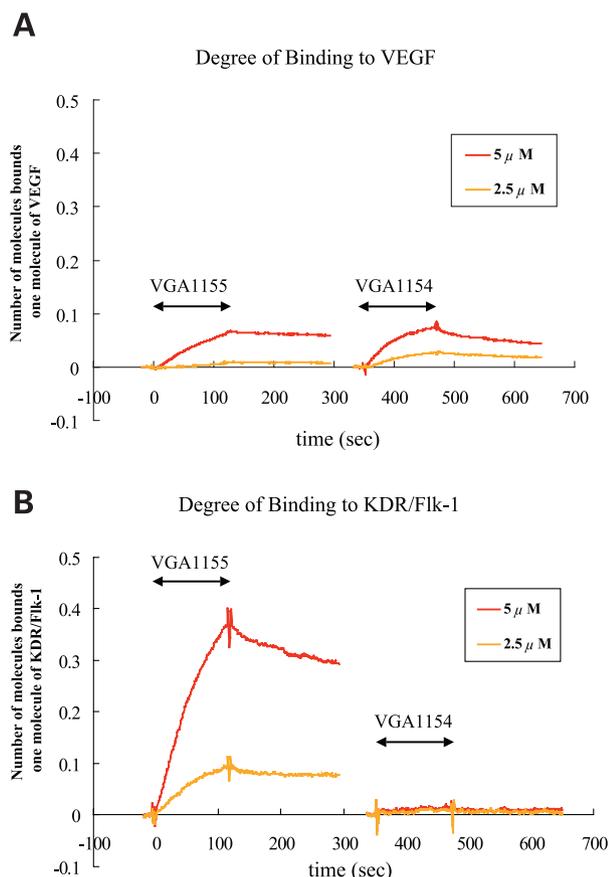
Degree of Binding to KDR/Flk-1

Figure 3. Binding of VGA1155 to KDR/Flk-1 indicated by analysis of SPR. VGA1154 and VGA1155 at concentrations of 2.5 or 5 μM in PBS containing 1% (v/v) DMSO and 0.05% (v/v) Tween 20 were injected (30 μl/min) over immobilized VEGF (**A**) and KDR/Fc chimera protein (**B**) on the sensorchip CM-5. *Arrows,* the beginning and the end of injections.
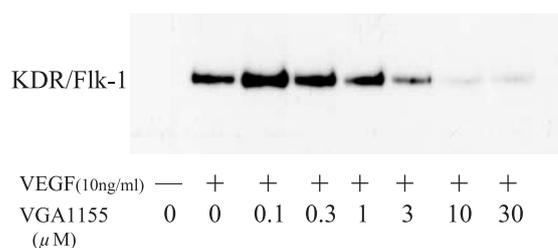
Figure 4. Effects of VGA1155 on the VEGF-induced autophosphorylation of KDR receptor. NIH3T3-KDR/Flk-1 cells were serum starved for 1 day; then, dilutions of VGA1155 were added to culture dishes and incubated for 0 or 60 min. KDR/Flk-1 autophosphorylation was stimulated by the addition of 10-ng/ml human VEGF for 5 min. The cell lysates were immunoprecipitated with anti-KDR/Flk-1 antibody. The samples were separated by electrophoresis on 7.5% polyacrylamide gel and analyzed by Western blotting with anti-phosphotyrosine antibody.

After the circulation of compounds in the Biacore S51, VGA1155 bound to the KDR/Flk-1 immobilized sensor tip in a concentration-dependent manner, whereas VGA1154 (Fig. 1B), the analogue of VGA1155 (Fig. 1A) without inhibition of VEGF-KDR/Flk-1 binding (data not shown), did not bind to the receptor. On the other hand, almost equal amounts of both compounds bound to the VEGF immobilized sensor tip. These results indicate that VGA1155 inhibits VEGF binding to KDR/Flk-1 through specific association with the receptor (KDR/Flk-1) but not with the ligand (VEGF).

VGA1155 Inhibits Post-Receptor Signal Transduction

KDR/Flk-1 is a member of the VEGF receptor tyrosine kinase family. To confirm whether VGA1155 can inhibit post-receptor signal transduction of VEGF receptors based on binding inhibition, we examined the effects of VGA1155 on VEGF-induced autophosphorylation of KDR/Flk-1 using Western blot analysis (Fig. 4). Treatment of NIH3T3-KDR/Flk-1 cells with VEGF (10 ng/ml) induced autophosphorylation of KDR/Flk-1. VGA1155 (10 μM) inhibited receptor tyrosine autophosphorylation, indicating that VGA1155 inhibited intracellular signal transduction of VEGF by inhibiting receptor binding.

VGA1155 Specifically Inhibits VEGF Binding to Its Receptors

To evaluate the specificity of the binding inhibitory effects of VGA1155, we investigated the effects of VGA1155 on the binding of several growth factors or cytokines and their receptors (Table 1). VGA1155 exhibited potent inhibition of VEGF binding to both Flt-1 and KDR/Flk-1 but did not inhibit the binding of other ligands to their receptors, such as EGF, PDGF, IL-8, PAF, IL-1β, IL-2, IL-4, IL-6, MIP-1α, MIP-1β, TNF-α, and insulin. These data indicate the highly specific nature of the inhibitory effects of VGA1155 on VEGF binding to both receptors.

Effects of VGA1155 on VEGF-Induced Vascular Permeability Assay

Senger *et al.* (23) reported that VEGF increases the permeability of blood vessels as detected by the Miles assay. To confirm that the inhibition of binding with VGA1155 suppresses VEGF functions *in vivo*, we evaluated

Table 1. VGA1155 has no inhibition effects on bindings of other cytokines

Ligands	Receptors	IC$_{50}$ (μM, mean ± SE)
VEGF	Flt-1	0.38 ± 0.07
	KDR/Flk-1	0.14 ± 0.00
TGF-β	TGFR	10.1
EGF	EGFR	>10[a]
PDGF	PDGFR	>10[a]
IL-8	CXCR2	>10[a]
PAF	PAFR	>10[a]
IL-1β	IL-1βR	>5[a]
IL-2	IL-2R	>5[a]
IL-4	IL-4R	>5[a]
IL-6	IL-6R	>5[a]
IL-8	CXCR1	>5[a]
MIP-1α	CCR1	>5[a]
MIP-1β	CCR5	>5[a]
TNF-α	TNFR-α	>5[a]
Insulin	Insulin receptor	>5[a]

[a]These experiments were demonstrated in Cerep.

the effect of VGA1155 on VEGF-induced vascular permeability using the Miles assay. As shown in Fig. 5A, intradermal injection of VEGF in guinea pigs induced dye leakage from dermal microvessels. Coinjection of VEGF with VGA1155 (30 ng/spot) reduced dye leakage (Fig. 5B). On the other hand, VGA1155 (30 ng/spot) did not reduce the dye leakage induced by histamine (0.3 μg/spot; Fig. 5, C and D). These results demonstrate that VGA1155 specifically inhibits the vascular permeability induced by VEGF.

Discussion

VEGF is a critical factor responsible for promoting the survival of immature endothelial cells in normal and oncogenic contexts. Its withdrawal induces apoptotic death in cultured human umbilical vein endothelial cells and in tumor endothelia. VGA1155, one of the first synthetic small molecular antagonists, was selected for its high demonstrated ability to inhibit VEGF binding to its receptors, which suggests that it may function as a potent antiangiogenic and antitumor agent.

This present study demonstrated that VGA1155, having low molecular weight (MW 583.87), inhibited interaction between a large protein ligand (VEGF) and its receptor (Fig. 2). VGA1155 was particularly effective in inhibiting the binding of VEGF to its receptors (Table 1). SPR analysis using the Biacore S51 system indicated that the inhibition of VEGF receptor binding is due to the binding of VGA1155 to
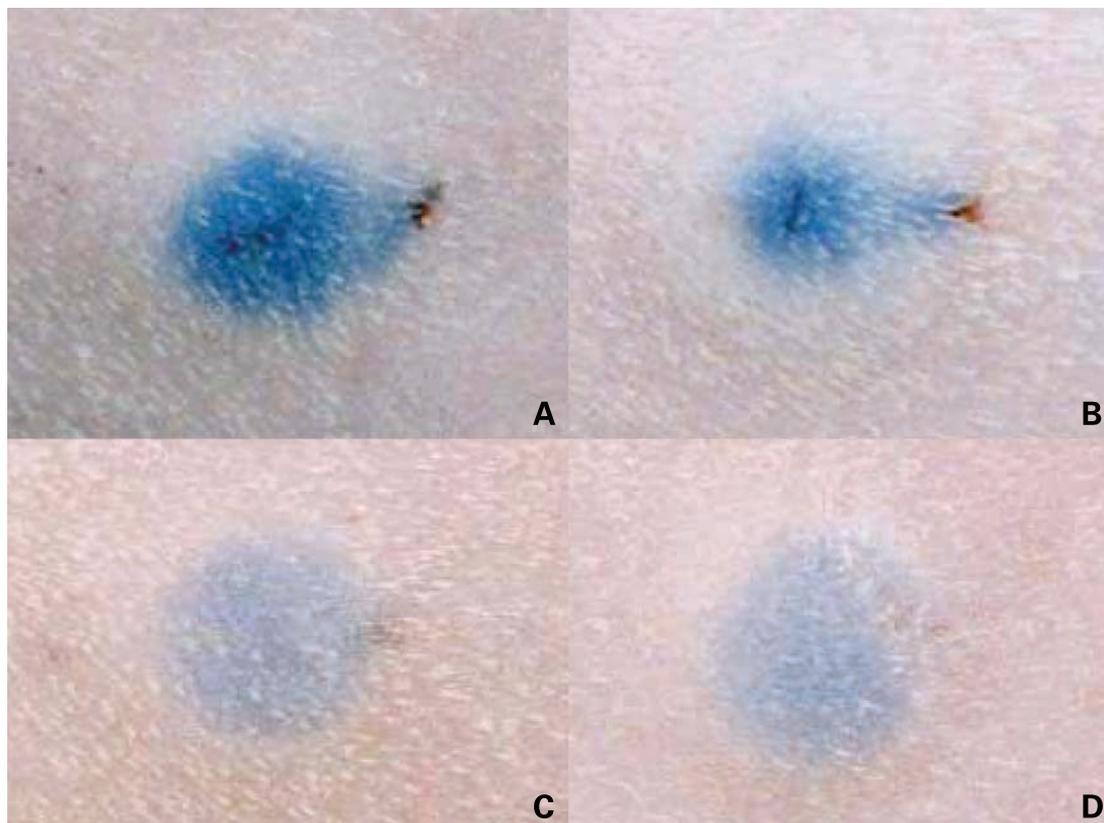

Figure 5. Effects of VGA1155 on vascular permeability assay. Anesthetized guinea pig was shaved on the back and was injected with 0.7 ml of 1% trypan blue through the brachial vein. After 30 min, 100 μl of injection solution (PBS, 20-ng VEGF and with or without 30-ng VGA1155) were injected intradermally into the back of the guinea pig. After 30 min, dye leakage was detected by the presence of blue spot surrounding the injection site. For histamine, dye leakage was induced by 0.1-μg histamine. (**A**) VEGF + 0.5% DMSO, (**B**) VEGF + 0.5% DMSO + VGA1155, (**C**) histamine + 0.5% DMSO, and (**D**) histamine + 0.5% DMSO + VGA1155.

KDR/Flk-1 rather than to VEGF (Fig. 3, A and B). In contrast, VGA1154, which bears a strong structural resemblance to VGA1155, did not bind to KDR/Flk-1 at all. In our preliminary study, it was observed that demethylation of the amino group or single-carbon insertion between keto and phenyl groups did not affect inhibition of VEGF binding (data not shown). Therefore, another difference of VGA1155 from VGA1154 is that the charge neutrality at *ortho*-position of this benzoic acid derivative may significantly affect how successfully compounds fit to the structure of KDR/Flk-1, although further investigation is required to clarify the binding site of VGA1155 in the KDR/Flk-1 molecule.

The inhibitory effect of VGA1155 on VEGF and KDR/Flk-1 interaction resulted in the suppression of the post-receptor signal transduction of KDR/Flk-1 (Fig. 4), suggesting to inhibit the functions of VEGF through KDR/Flk-1. In the present study, VGA1155 treatment exhibited inhibitory action against vascular permeability, one of the functions of VEGF, *in vivo* (Fig. 5). The antiangiogenic and antitumor properties of VGA1155 are currently being investigated.

Recently, several VEGF antagonists such as SU5416 (24), SU6668 (25), ZD4190 (26), and PTK787 (27) have emerged as antitumor agents. All of these agents are tyrosine kinase inhibitors. In contrast, VGA1155, an agent that inhibits the binding of VEGF receptors on the cell surfaces, may be able to exert this action without entering the target cells. We speculate that this inhibitory mechanism may be related to the potentially lower toxicity of VGA1155 compared with tyrosine kinase inhibitors.

Several molecules have previously been reported to function as VEGF binding antagonists, including anti-VEGF antibody (9, 10), anti-KDR/Flk-1 receptor antibody (11), 2′-fluoropyrimidine RNA-based aptamers (12), various peptides (13, 14), and porphyrin analogues (15). Compared with the above molecules, VGA1155, with its low molecule weight, offers certain advantages, including low cost of synthesis, low antigenicity, and *in vivo* stability.

In the present study, VGA1155 inhibited VEGF binding to its receptors. If the potential of VGA1155 relates suppression of VEGF function *in vivo*, it may prove to be useful in the treatment of VEGF-related diseases like tumor, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, endometriosis, and brain edema (1, 3–6, 28).

Acknowledgments

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