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ORIGINAL ARTICLE

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A novel low molecular weight VEGF receptor-binding antagonist, VGA1102, inhibits the function of VEGF and in vivo tumor growth

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Abstract Vascular endothelial cell growth factor (VEGF) plays an important role in the processes of angiogenesis. Angiogenesis appears to be essential for the growth of solid tumors and their metastasis. VEGF plays a principal role in tumor angiogenesis. To identify a compound that inhibits the binding of VEGF to its receptor, we used a high-throughput screening method and found that oxydibenzoic acid derivatives inhibited VEGF binding to its receptors. Among the active compounds, 5-{3-[4-(octadecyloxy)phenyl]propionylamino}-2,4′-oxydibenzoic acid (VGA1102) was selected based on its potent binding inhibitory activity. VGA1102 inhibited [^{125}I]VEGF binding to both of two VEGF receptor-transfected cell lines, NIH-Flt-1 and NIH-KDR/Flk-1, in a concentration-dependent manner, with IC_{50} values of 0.66 ± 0.07 and 0.61 ± 0.16 μM, respectively. VGA1102 (10 μM) exhibited inhibitory activity against VEGF-induced receptor autophosphorylation. VGA1102 also inhibited VEGF-induced growth of rat liver sinusoidal endothelial cells ($IC_{50} = 0.89 \pm 0.16$ μM) as well as VEGF-induced tube formation of HUVEC in vitro. VGA1102 reduced intradermal VEGF-induced vascular permeability in guinea pigs. Treatment with VGA1102 (50 mg/kg, i.p., days 0–20) significantly increased the lifespan of MM2-bearing mice with an increase in lifespan of >195.8%, and all such mice were long-term survivors on day 71. Furthermore, VGA1102 (50 mg/kg, i.p.) administered daily suppressed the growth of nude mice transplanted with LC-6 human non-small-cell lung cancer. These results suggest that VGA1102 inhibits VEGF function resulting in inhibition of tumor angiogenesis, which led to suppression of growth of human tumors transplanted into nude mice.

Keywords VEGF binding antagonist · VGA1102 · Antitumor activity · Vascular endothelial cell · Vascular permeability

Abbreviations BSA: bovine serum albumin · DMEM: Dulbecco's modified Eagle's medium · FBS: fetal bovine serum · Flt-1: fms-like tyrosine kinase · HUVEC: human umbilical vein endothelial cells · ILS: increase in lifespan · KDR/Flk-1: kinase insert domain containing receptor/fetal liver kinase · MEM: minimum essential medium · MST: median survival time · MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · PBS: phosphate-buffered saline · VEGF: vascular endothelial growth factor

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, solid tumor growth, diabetic retinopathy, psoriasis, and rheumatoid arthritis [6, 7].

Vascular endothelial growth factor (VEGF) is essential for normal and abnormal angiogenic processes, including growth, mitogenesis, and tube formation of endothelial cells [4, 5, 19, 20]. VEGF binds to two tyrosine kinase receptors, Flt-1 and KDR/Flk-1, on the surface of endothelial cells to activate signal transduction and regulate both physiological and pathological angiogenesis. VEGF and VEGF receptors have been implicated in the angiogenesis that occurs in many human solid tumors. This importance of VEGF function

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suggests that blockade of this function may be useful for inhibiting angiogenesis and tumor growth.

To identify a compound that inhibits the binding of VEGF to its receptor, we used a high-throughput screening method using a VEGF receptor (Flt-1) binding scintillation proximity assay (SPA) kit (Amersham Biosciences, Uppsala, Sweden). We found that novel oxydibenzoic acid derivatives inhibited VEGF binding to receptors. Among VGA (VEGF binding antagonist) compounds, we selected VGA1102 based on its potent binding inhibitory activity. We report here the pharmacological profile of VGA1102, the first small molecular antagonist of VEGF–VEGF receptor binding.

Materials and methods

Materials

VGA1102 (5-{3-[4-(octadecyloxy)phenyl]propionylamino}-2,4′-oxydibenzoic acid; Fig. 1) was synthesized in our laboratory. For in vitro experiments, VGA1102 was dissolved in DMSO and diluted with medium before use. For in vivo tests, VGA1102 was dissolved in isotonic phosphate buffer (pH 9.0). VEGF (165 amino acid type) was obtained from Pepro Tech (London, UK). [^{125}I] VEGF and [methyl-^{3}H]thymidine was purchased from Amersham Biosciences.

Cells and animals

NIH3T3-Flt-1 cells and NIH3T3-KDR/Flk-1 cells overexpressing human Flt-1 and KDR/Flk-1, respectively, were established as described previously [17]. These cell lines were maintained in DMEM containing 10% calf serum and 200 μg/ml G418. KB human epidermoid carcinoma cells [14] were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FBS and gentamicin (80 μg/ml) at 37°C in a humidified atmosphere comprising 5% CO_2/95% air. MM2 mouse mammary tumors [12, 13] were passaged in the peritoneal cavities of syngeneic C3H/He mice. LC-6 human large-cell carcinoma of the lung [21] was obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). C3H/He mice, BALB/c-nu/nu mice, and Wistar rats were obtained from Charles River Japan (Kanagawa, Japan). All experiments involving animals were carried out under protocols approved by the Taisho Pharmaceutical Company Animal Care Committee.

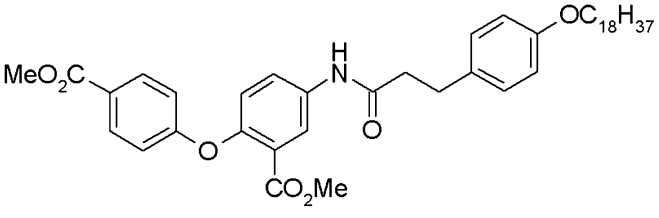

Fig. 1 Chemical structure of VGA1102 (5-{3-[4-(octadecyloxy)phenyl]propionylamino}-2,4′-oxydibenzoic acid). Molecular weight 673.9 Da

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 prior to experimental use. These cells were preincubated with medium A (DMEM containing 10 m *M* HEPES, pH 7.2, and 0.1% BSA) at 4°C for 30 min. Then the medium was replaced with 0.3 ml medium B (DMEM containing 10 m *M* HEPES, pH 7.2, and 0.5% BSA] containing 25 p *M* [^{125}I]VEGF (37.5–62.5 nCi/ml and various concentrations of VGA1102. The plates were incubated at 4°C for 90 min. All experiments were performed in triplicate wells. After incubation, cells were washed three times with ice-cold medium A. After the cells were solubilized with 0.5 ml 0.5 *N* NaOH for 30 min, each solution in the wells was transferred to test tubes. The wells were then washed with 0.5 ml PBS, and the washes were combined with the solutions in the tubes. The radioactivity of each tube was counted in a gamma counter. The percentage of radioactivity in each group against that in the control group was calculated. IC_{50} was calculated from the percentage by nonlinear regression analysis. Nonspecific binding was determined by incubation in the presence of 10 n *M* unlabelled VEGF.

Autophosphorylation of KDR/Flk-1 receptor

The effect of VGA1102 on VEGF-induced receptor autophosphorylation was assessed as described previously [17]. NIH3T3-KDR/Flk-1 cells were plated on 10-cm dishes, grown to near confluence, and serum-starved in 0.1% calf serum containing DMEM for 1 day. Dilutions of VGA1102 were added to culture dishes and incubated for 0 min or 60 min. KDR/Flk-1 autophosphorylation was stimulated by the addition of 10 ng/ml VEGF for 5 min, and cells were rinsed twice in ice-cold PBS containing 0.1 m *M* Na_3VO_4 and lysed in 1% Triton X-100 lysis buffer (50 m *M* HEPES, pH 7.4, 100 m *M* NaCl, 1.5 m *M* $MgCl_2$, 50 m *M* NaF, 10 m *M* $Na_4P_2O_7$, 10% glycerol, 1% Triton X-100, 2 m *M* PMSF, 2 m *M* 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 the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.) and the same amounts of protein from each sample were used for analysis. Cell lysates were incubated with anti-human KDR antibody (IBL, Fujioka, Japan) at 4°C overnight. The resulting immune complexes were collected by

precipitation with protein G plus protein A agarose beads (Calbiochem, La Jolla, Calif.). Immunoprecipitates were then washed five times with 1% Triton X-100 lysis buffer. Samples were then separated by electrophoresis on 7.5% polyacrylamide gel and transferred to PVDF membrane (Bio-Rad). After transfer, the filters were incubated in blocking buffer (1% BSA, 10 m *M* Tris, pH 7.5, 100 m *M* NaCl, 0.1% Tween 20) at 4°C overnight. The filters were then probed with anti-phosphotyrosine HRP-conjugated antibody (Amersham Biosciences). Chemiluminescence activity was determined by SuperSignal substrate Western blotting (Pierce Chemical Company, Rockford, Ill.).

Rat sinusoidal endothelial cell growth assay

VEGF-induced rat liver sinusoidal endothelial cell growth was measured according to a previously described method [27]. Seven 12-week-old male Wistar rats were subjected to peritoneotomy under anesthesia and the liver tissue was perfused through the portal vein with liver perfusion medium (Invitrogen, Carlsbad, Calif.) for 10 min at 37°C and 0.05% collagenase solution for 15 min at 37°C. The isolated liver cells were separated to single cells by gentle pipetting in hepatocyte wash medium (Invitrogen). The cell suspension was centrifuged at 35 *g* for 2 min twice. The supernatant was centrifuged at 140 *g* for 3 min and the cell pellet containing sinusoidal endothelial cells was suspended in 0.8% NH_4Cl/Tris-HCl. After hemolysis for 10 min at 4°C, the cell suspension was further centrifuged at 150 *g* for 3 min and then washed with Hu-Media EG2 medium (Kurabo Industries, Osaka, Japan). The cell pellet was suspended in the medium and cultured in collagen-coated 48-well plates (Sumitomo Bakelite, Tokyo, Japan) for 4 h at 37°C in an atmosphere containing 5% CO_2. The wells were washed with PBS and Hu-media EG2 medium with 10 ng/ml VEGF and serial dilutions of test compounds were added. The cells were cultured for an additional 65 h and 1 μCi/ml [methyl-^{3}H]thymidine was then added. After a further 24-h culture, the cells were washed twice with PBS and trypsinized for 60 min at 37°C. They were then trapped with a glass filter on a cell harvester. The radioactivity of the glass filter was counted in a beta counter. Percentage radioactivity in each group in relation to that in the control group was calculated. IC_{50} values were calculated from the percentages by nonlinear regression analysis.

MTT assay

The effects of VGA1102 on the growth of KB cells and MM2 cells were determined by the MTT assay as previously described [26]. KB cells and MM2 cells (1×10^3 cells/100 μl) were cultured in 96-well plates and the cells were treated with graded concentrations of VGA1102 for 72 h. After this treatment, 50 μl of a 4-mg/ml solution of MTT in MEM was added to each of the culture wells. After 4 h, the fluid content of each well was removed, and 100 μl DMSO was added. The purple formazan product was solubilized, and absorbances were measured at 540 nm using an automatic microspectrophotometer. Background absorbance was subtracted from each well, and the percentage of control absorbance was considered to represent the surviving fraction of cells.

In vitro tube-formation assay

The effect of VGA1102 on in vitro angiogenesis was assessed as VEGF-induced tube formation of HUVEC using an in vitro angiogenesis kit (TCS Biologicals, Buckingham, UK) according to the manufacturer's instructions. Briefly, HUVEC and fibroblasts were cocultured in medium with 10 ng/ml VEGF and serial dilutions of VGA1102 for 13 days. The wells were then fixed and immunostained with anti-von Willebrand factor antibody, and tube formation was photographed.

Inhibitory effect on VEGF-induced vascular permeability

The effect of VGA1102 on VEGF-induced vascular permeability was determined by a method previously described [17]. Anesthetized guinea pigs were shaved on the back, and injected with 0.7 ml 1% trypan blue through the brachial vein. After 30 min, 100 μl of injection solutions (PBS, or 20 ng VEGF with or without 30 ng VGA1102) were injected intradermally into the back of the guinea pigs. After 30 min, leakage of dye was detected by the presence of a blue spot surrounding the injection site. In the case of histamine, leakage of dye was induced by 0.1 μg histamine.

Antitumor activity against ascites MM2 mammary tumor

MM2 ascites tumor cells are derived from a spontaneous mouse mammary adenocarcinoma and secrete moderate levels of VEGF [12, 13]. They were maintained in the peritoneal cavities of syngeneic C3H/He male mice. MM2 cells were intraperitoneally transplanted into C3H/He mice on day 0 (5×10^5 cells/mouse). Intraperitoneal doses of VGA1102 were given for 21 successive days from day 0 to day 20. The doses were dissolved in isotonic phosphate buffer (pH 9). The antitumor activity of VGA1102 was assessed by calculating T/C% from the median survival time (MST) of the mice using the following formula. Survival data were tested for statistical significance by log-rank analysis.

$$\text{T/C}\,(\%) = [(\text{MST of treated animals})((\text{MST of control animals})] \times 100$$

The effects of VGA1102 (50 mg/kg) on ascites fluid formation and growth of MM2 cells were determined on day 14. Mice were killed, 2 ml of cold PBS (pH 7.4) was injected intraperitoneally (i.p.) and the ascites fluid was harvested to the fullest extent possible. The exact volumes of the ascites fluid including the 2 ml of PBS injected were recorded. Total numbers of ascites cells were counted.

Antitumor activity against human tumor xenografts

LC-6 human non-small-cell lung cancer was maintained by subcutaneous transplantation into BALB/c-nu/nu mice [21]. The tumors were excised from the mice, the necrotic portions were removed and minced, and tumor fragments of about 2×2×2 mm were prepared and transplanted subcutaneously into the back of nude mice using a trocar (day 0). VGA1102 was given i.p. for 21 successive days from day 1 to day 21. VGA1102 was dissolved in isotonic phosphate buffer (pH 9). Tumor sizes were measured at least three times a week using a caliper, and tumor volumes were calculated using the following formula. Antitumor activity of the compounds was expressed as the T/C(%) of tumor volume.

$$\text{Tumor Volume}\,(\text{mm}^3) = 1/2 \times (\text{major diameter (mm)}) \times (\text{minor diameter (mm)})^2$$
$$\text{T/C}\,(\%) = [(\text{Tumor Volume of treated animals})((\text{Tumor Volume of control animals})] \times 100$$

Statistical analysis

Comparison of the results with and without treatment was performed using the Mann-Whitney *U*-test. The statistical significance of differences in the nude mice xenograft experiments was determined using Dunnett's test. Survival data were evaluated for statistical significance by log-rank analysis.

Results

VEGF receptor binding inhibitory activity of VGA1102

To identify a novel VEGF receptor binding antagonist, we employed a high-throughput screening method using an Flt-1 SPA kit (Amersham Biosciences). This assay kit is constructed from an Flt-1-Fc chimera protein which includes the extracellular domain of Flt-1 end SPA beads. When [^{125}I]VEGF binds Flt-1-Fc linked to a SPA bead, the bead emits the radiation. We evaluated the inhibitory action of compounds against the binding between [^{125}I]VEGF and Flt-1-Fc by measurement of the radiation from the SPA beads. This assay showed that oxydibenzoic acid derivatives exhibited VEGF-Flt-1 binding inhibitory activity. We designed and synthesized several new oxydibenzoic acid derivatives based on the structures of the active compounds. In order to confirm VEGF receptor binding antagonistic activity, the effects of the oxydibenzoic acid derivatives on [^{125}I]VEGF (25 p *M*) binding to Flt-1 and KDR receptor-overexpressing cells (NIH-Flt-1 and NIH-KDR/Flk-1, respectively) were determined.

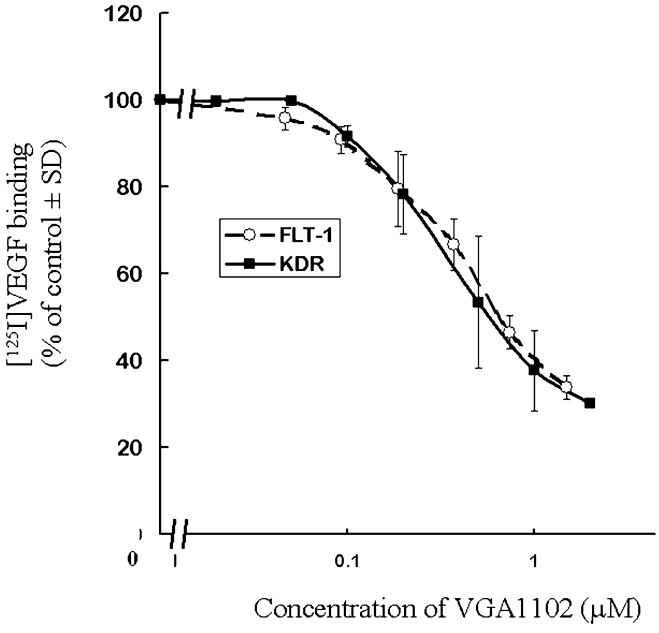

Fig. 2 VGA1102 inhibits VEGF binding to NIH/Flt-1 cells and NIH/KDR cells. NIH3T3-Flt-1 and NIH3T3-KDR/Flk-1 cells were incubated at 4°C for 90 min in medium containing 25 p *M* [^{125}I]VEGF and various concentrations of VGA1102. After incubation, cells were washed three times with ice-cold medium, and the radioactivity of each tube was counted in a gamma counter. The percentage of radioactivity in each group against that in the control group was calculated

Among the active compounds VGA1102 (Fig. 1) was selected based on its potent binding inhibitory activity. VGA1102 inhibited [^{125}I]VEGF binding to NIH-Flt-1 cells and NIH-KDR/Flk-1 cells in a concentration-dependent manner (Fig. 2), with IC_{50} values of 0.66 ± 0.07 and 0.61 ± 0.16 μ*M*, respectively. The inhibitory effects plateaued at 1 μ*M* of VGA1102, and more than 2 μ*M* VGA1102 increased VEGF binding, possibly due to its poor solubility in the binding buffer. Lineweaver-Burk plots showed the mode of action of VGA1102 to be competitive inhibition (data not shown).

To confirm whether VGA1102 inhibits the post-receptor signal transduction of VEGF receptor, we determined the effect of VGA1102 on VEGF-induced

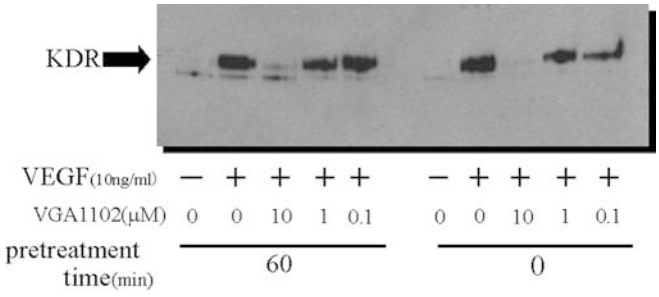

Fig. 3 Effect of VGA1102 on VEGF-induced autophosphorylation of KDR receptors. NIH3T3-KDR/Flk-1 cells were starved at 0.1% serum for 1 day, then dilutions of VGA1102 were added to the culture dishes which were incubated for 0 min or 60 min. KDR/Flk-1 autophosphorylation was stimulated by the addition 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 then analyzed by Western blotting with anti-phosphotyrosine antibody

KDR/Flk-1 receptor autophosphorylation using Western blot analysis (Fig. 3). Treatment with VEGF at 10 ng/ml induced autophosphorylation of the KDR/Flk-1 receptor. Both preincubation (60 min) and simultaneous VGA1102 (10 μ*M*) treatment inhibited receptor tyrosine autophosphorylation. These results indicate that VGA1102 inhibited VEGF receptor binding and its intracellular signal transduction without entering the cells.

Concerning the specificity of VGA1102, we investigated the effects of VGA1102 on the binding of several growth factors and cytokines and their receptors (Table 1). VGA1102 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 findings indicate the highly specific nature of the inhibitory effects of VGA1102 on VEGF binding to both receptors.

Table 1 VGA1102 has no inhibition effect on bindings of other ligands and receptors

Ligand	Receptor	IC_{50} (μg/ml)
EGF	EGFR	> 5
PDGF	PDGFR	> 5
IL-8	CXCR2	> 5
PAF	PAFR	> 5
IL-1β	IL-1bR	> 5
IL-2	IL-2R	> 5
IL-4	IL-4R	> 5
IL-6	IL-6R	> 5
IL-8	CXCR1	> 5
MIP-1α	CCR1	> 5
MIP-1β	CCR2	> 5
TNF-α	TNFR-α	> 5
Insulin	Insulin receptor	> 5

These experiments were demonstrated incarried out by Cerep (Paris, France)

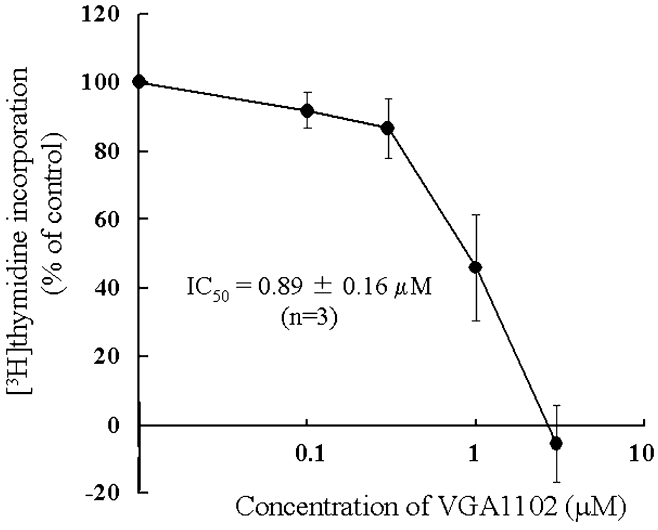

Fig. 4 VGA1102 inhibits VEGF-induced growth of rat liver sinusoidal endothelial cells. Rat liver sinusoidal endothelial cells were cultured in Humedia EG2 medium with 10 ng/ml VEGF and serial dilutions of VGA1102. The cells were cultured for an additional 65 h and 1 μCi/ml [methyl-^{3}H]thymidine was added after an additional 24 h of culture. The cells were then washed and harvested, and radioactivities were counted in a beta counter. The percentage of radioactivity in each group against that in the control group was calculated. IC_{50} was calculated from the percentage by nonlinear regression analysis

Effects of VGA1102 on endothelial cell proliferation

We investigated the effect of VGA1102 on VEGF-induced thymidine uptake by rat liver sinusoidal endothelial cells. As shown in Fig. 4, VGA1102 inhibited [methyl-^{3}H]thymidine uptake by sinusoidal endothelial cells in a concentration-dependent manner, with an IC_{50} of 0.89 ± 0.16 μ*M* ($n = 3$).

To eliminate the possibility that VGA1102 had direct cytotoxic effects, we determined the in vitro cytotoxicity of this compound against the growth of cultured human KB cells and mouse mammary tumor MM2 cells. VGA1102 did not inhibit the growth of either cell type (IC_{50} > 10 μ*M*). These results indicate that VGA1102 inhibited endothelial cell growth as a result of inhibition of VEGF function.

VEGF-induced tube formation of HUVEC

The effect of VGA1102 on VEGF-induced tube formation was investigated in vitro using an angiogenesis kit. HUVEC and fibroblasts were cocultured in medium with 10 ng/ml VEGF and serial dilutions of VGA1102 for 13 days. With this assay kit, VEGF (10 ng/ml) induced capillary-like structures in HUVEC (Fig. 5b), while unstimulated HUVEC did not form such structures (Fig. 5a). As shown in Fig. 5c, d, VEGF-dependent formation of tubular structures was inhibited by VGA1102 (0.3 and 3 μg/ml) in a concentration-dependent manner.

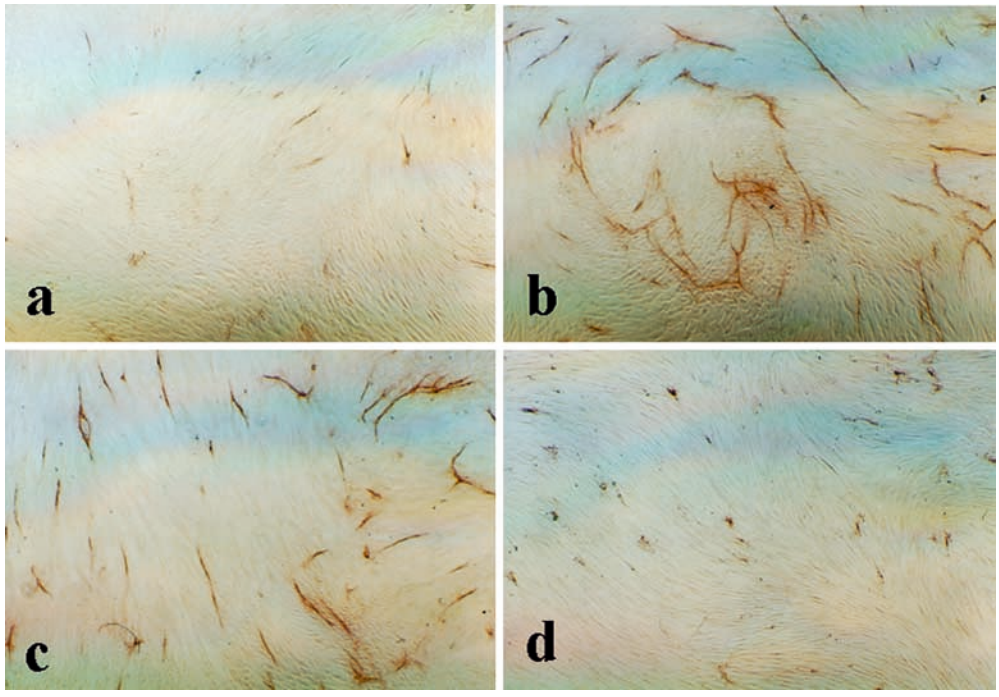

Fig. 5a–d Effect on VEGF-induced tube formation of HUVEC. Formation of capillary-like structures of HUVEC cultured with fibroblasts was observed after 13 days. **a** Control: cells were cultured without VEGF. No tubular structures were observed. **b** VEGF (10 ng/ml) induced capillary-like structures in the in vitro angiogenesis assay. **c** VGA1102 (0.3 μg/ml) slightly decreased the formation of capillary-like structures induced by VEGF (10 ng/ml). **d** In the presence of VEGF plus VGA1102 (3 μg/ml) no capillary-like structures were formed

Effect of VGA1102 on VEGF-induced vascular permeability assay

Senger et al. [18] have reported that VEGF increases permeability of blood vessels as detected by the Miles assay. We next evaluated the effect of VGA1102 on VEGF-induced vascular permeability activity using the Miles assay. As shown in Fig. 6a, intradermal injection of VEGF into guinea pigs induced leakage of dye from dermal microvessels. Coinjection of VEGF with VGA1102 (30 ng/spot) reduced the leakage of dye (Fig. 6b). On the other hand, VGA1102 (30 ng/spot) did not reduce dye leakage induced by histamine (0.3 μg/spot, Fig. 6c, d). These results demonstrate that VGA1102 specifically inhibited the vascular permeability induced by VEGF.

Effect of VGA1102 on MM2 mouse ascites tumor

Luo et al. [12] have reported that MM2 mammary tumor cells grow in ascites form, and that such ascites formation depends mainly on secretion of VEGF by the tumor cells. Furthermore, they demonstrated that anti-VEGF

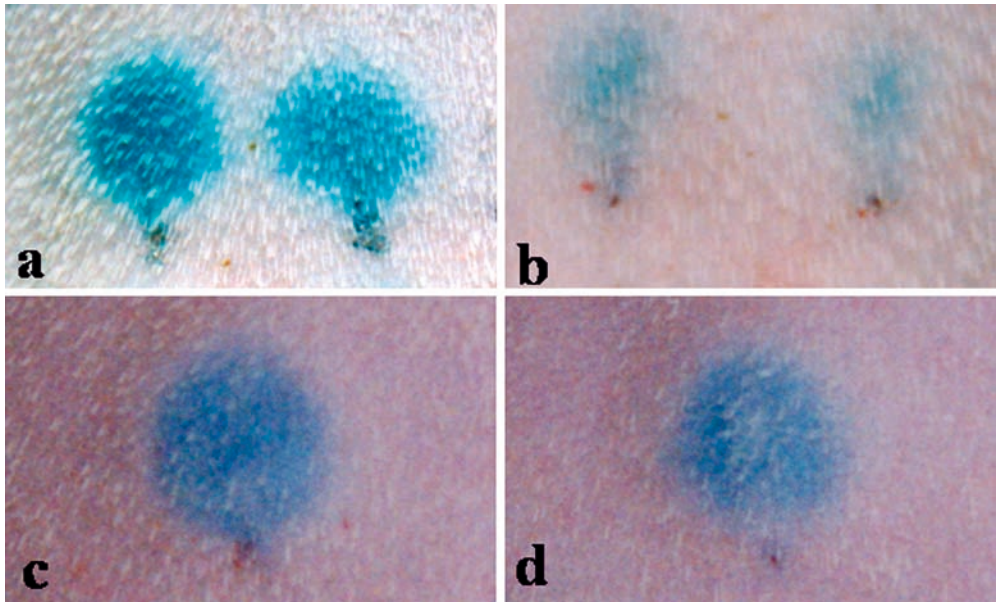

Fig. 6a–d Effect of VGA1102 on vascular permeability. Anesthetized guinea pigs were shaved on the back and injected with 0.7 ml 1% trypan blue through the brachial vein. After 30 min, 100 μl of injection solutions (PBS, or 20 ng VEGF with or without 30 ng VGA1102) were injected intradermally into the back of the guinea pigs. After 30 min, leakage of dye was detected by the presence of a blue spot surrounding the injection site. In the case of histamine, leakage of dye was induced by 0.1 μg of histamine (**a** VEGF plus 0.5% DMSO, **b** VEGF plus 0.5% DMSO plus VGA1102, **c** histamine plus 0.5% DMSO, **d** histamine plus 0.5% DMSO plus VGA1102)

Table 2 Antitumor activity of VGA1102 against i.p.-implanted MM2 mouse mammary carcinoma. MM2 cells were transplanted i.p. into C3H/He mice on day 0 (5×10^5 cells/mouse). VGA1102 and vehicle (isotonic phosphate buffer) were administered i.p. for 21 successive days from days 0–20

Compound	Dose (mg/kg)	Median survival (days)	ILS (%)	71-day survivors	Ascites fluid (ml/mouse, day 13)	Ascites cells ($\times10^6$/mouse, day 13)	Survival (*P*-value, log-rank test)
Vehicle	–	21.3	–	–	8.09 ± 0.99	554.1 ± 69.4	
VGA1102	30	24.0	12.5	3/8	–	–	NS
	50	>71.0	>195.8	8/8	1.49 ± 0.03	24.91 ± 3.75	<0.001

neutralizing antibody treatment inhibits ascites formation and prolongs the survival time [13]. Using this VEGF-producing ascites tumor model, we evaluated the therapeutic effects of VGA1102 on MM2-induced ascites formation and survival time. We confirmed that VGA1102 did not directly inhibit the growth of MM2 in vitro using the MTT assay (IC_{50} >10 μ*M*). Moreover, since VGA1102 is a dimethyl ester and therefore may be metabolized quickly, we confirmed that VGA1102 was retained at over 1 μg/ml in mouse serum after 24 h from i.p. administration (data not shown). Treatment with VGA1102 (50 mg/kg, i.p., days 0–20) significantly increased the lifespan of MM2-bearing mice with an ILS of >195.8% (Table 2), and all mice were long-term survivors on day 71 (the final day of evaluation). VGA1102 also inhibited ascites formation of mice. Volumes of ascites (each value including 2 ml PBS) in nontreated and vehicle-treated animals on day 13 were 9.23 ± 0.75 and 8.09 ± 0.99 ml, respectively. VGA1102 treatment (50 mg/kg, i.p., days 0–13) resulted in a statistically significant decrease in ascites fluid volume (1.49 ± 0.03 ml) to a level comparable to that in normal mice (1.47 ± 0.09 ml). The total number of ascites cells per vehicle-treated mouse was $554.1\pm69.4\times10^6$. VGA1102 treatment simultaneously decreased the total number of ascites cells to $24.91\pm3.75\times10^6$ cells per mouse.

The above results indicate that the VEGF-receptor binding inhibitory activity of VGA1102 contributed to the antitumor activity of this compound against MM2 ascites tumor models.

Growth-inhibitory activity of VGA1102 in human tumor xenografts

To evaluate the antitumor activity of VGA1102, we examined the growth-inhibitory activity of VGA1102 against a tumor xenograft model of human non-small-cell lung cancer LC-6 subcutaneously transplanted into nude mice. Daily i.p. administration of VGA1102 at doses of 30 and 50 mg/kg for 21 days suppressed tumor growth dose-dependently, with inhibitions of 54.1% and 71.1% ($P<0.01$) on day 21, respectively (Fig. 7). Furthermore, VGA1102 was also effective against human colon carcinoma cells (HT29, Col-1) and breast cancer cells (MX-1) transplanted into nude mice (data not shown). In these experiments, no toxicity or body weight loss was observed in VGA1102-treated mice (data not shown).

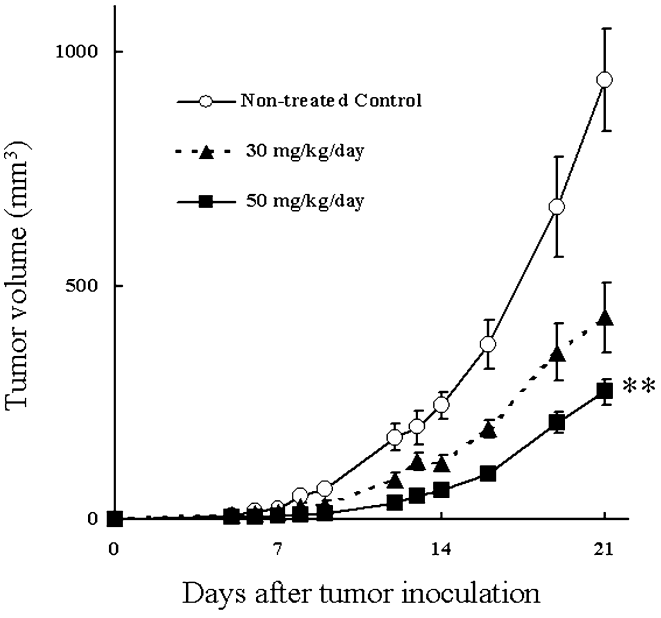

Fig. 7 Antitumor effects of VGA1102 administered i.p. on LC-6 xenografts in nude mice. LC-6 tumor was inoculated into Balb/c nude mice on day 0. Animals were treated once daily with i.p. administration of VGA1102 (30 or 50 mg/kg per day) for 21 days beginning 1 day after tumor inoculation. The values shown are the means ± SE from six animals. $^{**}P<0.001$, nontreated control animals vs animals treated with 50 mg/kg VGA1102 (Dunnett's test)

Discussion

In the present study, we showed VGA1102 to be one of the first synthetic small molecular antagonists to inhibit VEGF receptor binding (Fig. 2). We thus demonstrated that a low molecular weight compound (MW 673.9 Da) inhibited the interaction between the protein ligand and its receptor. VGA1102 inhibited post-receptor signal transduction (Fig. 3), endothelial growth (Fig. 4) and tube formation (Fig. 5) in vitro. VGA1102 treatment exhibited in vivo VEGF inhibitory activity in the Miles assay (Fig. 6), MM2 ascites tumor model (Table 2), and human tumor xenograft models (Fig. 7). On the other hand, VGA1102 did not inhibit binding of other protein ligands or receptors (Table 1). These results indicate the highly specific nature of the inhibitory effects of VGA1102 on VEGF binding to its receptors.

Several VEGF antagonists are being developed as antitumor agents including SU5416 [8], SU6668 [11],

ZD4190 [23], and PTK787 [24]. All of those compounds are VEGF receptor tyrosine kinase inhibitors. On the other hand, VGA1102 is a receptor binding antagonist, and may be able to exhibit activity without entering target cells. This suggests that VGA1102 may have less toxicity than other tyrosine kinase inhibitors. Several molecules are also reported to be VEGF binding antagonists, including anti-VEGF antibody [9, 10] anti-KDR/Flk-1 receptor antibody [15], 2′-fluoropyrimidine RNA-based aptamers [16], various peptides [2, 3], and porphyrin analogues [1]. Compared to those molecules, VGA1102 may have certain advantages, including low cost of synthesis, low antigenicity and in vivo stability, since it is a small molecule.

VEGF is a potent inducer of vascular permeability, and is thought to contribute to clinical ascites formation [12, 13, 25] and pleural effusion [28]. Using the VEGF-producing ascites tumor MM2 cells, we demonstrated that VGA1102 inhibited ascites formation and growth of mouse MM2 tumor cells (Table 2). VGA1102 treatment exhibited a long-term (more than 71-day) life-prolonging effect in MM2-transplanted mice. This result indicates that VGA1102 treatment completely cured MM2-transplanted mice. No cytotoxicity of VGA1102 on MM2 was found in the in vitro growth (MTT) assay ($IC_{50} > 10\ \mu M$). These results indicate that VGA1102 reduced ascites formation mainly by inhibiting vascular permeability induced by VEGF, suggesting that VGA1102 could be used to treat malignant ascites formation and pleural effusion in cancer patients.

We also showed that VGA1102 suppressed the growth of nude mice transplanted with LC-6 human non-small-cell lung cancer (Fig. 7). This is important as it is the first report that a low molecular weight compound which inhibits the binding between VEGF and it receptors suppresses tumor growth in vivo. VEGF is produced by most human solid tumor cells including lung cancers, colon cancers, and breast cancers [4, 5]. Although, the antitumor effect was growth delay rather than cure, VGA1102 may thus be useful for cancer treatment in combination with cytotoxic chemotherapeutic agents.

Furthermore, VEGF and its receptors play an important role not only in tumor angiogenesis but also in other diseases such as diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, endometriosis, and brain edema [4, 5, 7, 19, 20, 22]. VGA1102 may thus also be useful for treatment of these diseases.

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