ABSTRACT

A small synthetic library of cyclohexapeptidomimetic calixarenes was prepared to identify disrupters of vascular endothelial growth factor (VEGF) binding to its receptor that inhibits angiogenesis. From this library, we discovered GFA-116, which potently inhibits 125I-VEGF binding to Flk-1 in Flk-1-overexpressing NIH 3T3 cells and human prostate tumor cells with an IC50 of 750 nM. This inhibition is highly selective for VEGF in that 125I-platelet-derived growth factor binding to its receptor is not affected. GFA-116 inhibits VEGF-stimulated Flk-1 tyrosine phosphorylation and subsequent activation of Erk1/2 mitogen-activated protein kinase families. Furthermore, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor-dependent stimulation of Erk1/2 phosphorylation are not affected at concentrations as high as 10 μM. In vitro, GFA-116 inhibits angiogenesis as measured by inhibition of migration and formation of capillary-like structures by human endothelial cells as well as suppression of microvein outgrowth in rat aortic rings and rat cornea angiogenesis. In vivo, GFA-116 (50 μg/day) inhibits tumor growth and angiogenesis as measured by CD31 staining of A-549 human lung tumors in nude mice. Furthermore, GFA-116 is also effective at inhibiting tumor growth and metastasis to the lung of B16-F10 melanoma cells injected into immunocompetent mice. Taken together, these results demonstrate that a synthetic molecule capable of disrupting the binding of VEGF to its receptor selectively inhibits VEGF-dependent signaling and suppresses angiogenesis and tumorigenesis.

INTRODUCTION

The development of tumors beyond a few mm3 requires the formation of new blood vessels. The angiogenic process is triggered in part by the tumor cells themselves and complex tumor microenvironment consisting of many different cell types (1–4). Today, the evidence favors a model where tumor cells under the harsh conditions of their microenvironment, such as hypoxia, are triggered to express and secrete several proangiogenic factors, such as a vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF; Refs. 1, 2, 5, and 6). These growth factors play important but distinct roles in the multistep angiogenesis process. VEGF is believed to be pivotal to the process of initiation of angiogenesis, whereas PDGF is believed to be important for vessel maintenance (2, 7–10). The key involvement of these growth factors in angiogenesis and hence tumor survival has prompted many researchers to develop antiangiogenic strategies as novel approaches for cancer therapy based on designing drugs that interfere with VEGF and PDGF-dependent angiogenesis (1, 4).

Although there are several members of the VEGF family (i.e., A, B, C, and D) and several VEGF receptors (VEGFR-1 or Flk-1, VEGFR-2 or Flk-1/KDR, VEGFR-3 or Flt-3, or Flt-4), the interaction between VEGF-A and Flk-1 is believed to be the most important for angiogenesis during tumor formation (1, 11–20). Several approaches have been used to target this interaction. Most common is the use of ATP mimics that are selective for the inhibition of the tyrosine kinase activity of Flk-1 (4, 21), e.g., Sugen’s SU5416 (21) and SU 6668 (23). AstraZeneca’s ZD4190 (24), and Norvatis’ PTK787/ZK2284 (25) are all ATP mimics that target Flk-1 tyrosine kinase and have been shown to inhibit angiogenesis and tumorigenesis in animal models, and some are presently in clinical trials. Another approach is targeting angiogenesis by treatment with antibodies against either VEGF or its receptors (21, 26), e.g., Genentech developed an anti-VEGF antibody that has antiangiogenic and antitumorigenic effects in animal models. Some success has been achieved through this approach in clinical trials of patients with colorectal cancer (27). Recently, a 17 amino acid cyclic peptide corresponding to amino acids 79–93 of the VEGF sequence was shown to block angiogenesis (28).

We have used an entirely novel approach to target angiogenesis. This consists of designing a relatively small library of calixarenes containing large surface areas with the potential to disrupt VEGF binding to its receptor. Here, we report the discovery of GFA-116 (growth factor antagonist-116), which inhibits VEGF binding to Flk-1, VEGF-stimulated Flk-1 tyrosine phosphorylation, Erk1/2 mitogen-activated protein kinase activation, angiogenesis both in vitro and in vivo, tumorigenesis, and metastasis.

MATERIALS AND METHODS

Inhibition of VEGF-Dependent Receptor Tyrosine Phosphorylation by Growth Factor Binders (GFBS). Starved Flk-1/KDR-overexpressing NIH 3T3 cells (Flk-1/NIH 3T3) were pretreated with GFBS (0–30 μM) for 5 min before stimulation with VEGF (50 ng/ml) for 10 min. The cells were then harvested and lysed; proteins from the lysates were separated by SDS-PAGE and transferred to nitrocellulose, and then membranes were immunoblotted with an antiphospho-VEGF receptor 2 antibody for activated Flk-1 (Tyr951; Cell Signaling Technology, Inc., Beverly, MA). The phosphoryrosine Flk-1 bands were scanned using a Bio-Rad Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA).

Growth Factor-Mediated Activation of Mitogen-Activated Protein Kinase. Starved NIH 3T3 cells (PDGF-BB, basic fibroblast growth factor) and NIH 3T3 cells overexpressing EGF receptor (EGF) and Flk-1 (VEGF) were pretreated with the indicated concentration of GFA-116 for 5 min before 10-min stimulation with PDGF-BB (10 ng/ml), EGF (100 ng/ml), and VEGF (50 ng/ml). Cell lysates were run on SDS-PAGE gels, then transferred to nitrocellulose, and Western blotted with antiphosphorylated Erk1/Erk2 (Cell Signaling).

Binding of 125I-VEGF to Flk-1/NIH3T3 and PC3 Cells. The binding assay of 125I-VEGF to Flk-1 was carried out as described previously (28). Briefly, Flk-1/NIH 3T3 and human prostate carcinoma PC3 cells were incubated with 125I-VEGF (50,000 cpm/well; 40 μM) and increasing concentrations of GFA-116. Cells were incubated at 4°C for 2 h and then washed three times with PBS and three times with 25 mM Tris (pH 8.0), 1% Triton X-100, 1% glycerol, and 1% SDS before determination. A 2,500-fold excess of cold VEGF was used to determine nonspecific binding levels (29), and 93–97% total binding was found to be specific.

Antitumor Activity in the Nude Mouse Tumor Xenograft Model. Nude mice (Charles River, Wilmington, MA) were maintained in accordance with the
Institutional Animal Care and Use Committee procedures and guidelines. A-549 and B16-F10 cells were harvested and resuspended in PBS. A-549 cells were injected s.c. into the right and left flanks (10 × 10^6 cells/flank) of 8-week-old female nude mice as reported previously (30). B16-F10 melanoma cells were injected s.c. into both flanks of 8-week-old female C57BL/6 mice (1 × 10^6 cells/flank). When tumors reached ~100 mm^3 (A-549) or 200 mm^3 (B16-F10), animals were dosed i.p. with 0.2 ml once daily. Control animals received a vehicle, whereas treated animals were injected with GFA-116 (50 mg/kg/day). The tumor volumes were determined by measuring the length (l) and width (w) and calculating the volume (V = l w^2/2), as described previously (30). Statistical significance between control and treated animals in A-549 and B16-F10 groups were evaluated using Student’s t test.

Capillary Network Formation. Two-hundred µl of Matrigel were placed into each well of a 24-well culture plate at 4°C and allowed to polymerize by incubation at 37°C as described previously (31). Human middle cerebral artery endothelial cells (5 × 10^5) were seeded on the Matrigel in 1 ml of endothelial cell-based medium containing VEGF (20 ng/ml). The cells were incubated in the presence or absence of GFA-116 at the concentrations indicated in the figure legend. For each culture, three randomly chosen fields were photographed using a ×4 objective lens. An experimenter unaware of the different treatments measured the total length of tube structures in each photograph using the Image Pro Plus software (Media Cybernetic, Inc., Silver Spring, MD).

Microvessel Outgrowths in Rat Aortic Rings. Twenty-four-well plates were covered with 250 µl of Matrigel and allowed to gel for 30 min at 37°C, 5% CO_2. Thoracic aortae were excised from 9-month-old Sprague Dawley rats. After removing the fibroadipose tissue, arteries were sectioned into 1-mm-long cross-sections, rinsed five times with endothelial cell-based medium, and placed on the Matrigel-coated wells as described previously (32). Artery rings were covered with an additional 250 µl of Matrigel. The rings were cultured for 24 h in 2 ml of EGM medium (Cambrex Biosciences, Rockland, ME). After the 24-h incubation, the medium was replaced with 2 ml of endothelial cell-based medium, containing 4% FBS with vehicle or with GFA-116. After 3 days, the medium was replaced with medium of the exact composition as described above. The microvessel growth was quantitated in the rat aortic ring assay by taking photographs of aortic ring cultures using a digital video camera linked to an Olympus BX60 microscope. The outgrowth area was delineated and measured with the Image Pro Plus software (Media Cybernetic). Pictures of the rings were taken at day 4 with a ×2 objective lens. The quantification of the whole area of microvessel outgrowths showed a significant difference between GFA-116-treated groups and control (ANOVA analysis; P < 0.005).

Human Brain Endothelial Cell Migration Assay. Migration of adult human brain endothelial cells was evaluated using a modified Boyden chamber assay (BD BioCoat Matrigel Invasion Chamber; Ref. 33). The cells were plated at 8 × 10^4/ml onto an 8-µm pore size membrane coated with a thin layer of Matrigel basement membrane matrix. GFA-116 (5 µM) was added to the medium in the outer chamber, and the cells were cultured for 18 h. Non-adherent cells were removed from the top surface with a cotton swab. Membrane inserts were then fixed with 4% paraformaldehyde and stained with cresyl-Violet dye. The number of cells which migrated to the undersurface of the filter was quantified by counting the cells in at least three randomly selected microscopic fields (×10). Samples were analyzed for significant differences using Student’s t test for independent samples.

Aortic Cornea Assay. This assay was performed as described previously (34). Briefly, Wistar rats were anesthetized with ketamine/acepromazine intraperitoneal; eyes were propitosed, and tetracaine was applied topically to the cornea. A small slit was made into the center of the cornea to the depth of the stroma. Using a corneal spatula, a pocket was formed reaching 1 mm from the limbus. A hydroxyl pellet, containing either VEGF (200 ng) alone, or in combination with GFA-116 as indicated in the figure legend, was added to the corneal pocket with PBS and inserted into each pocket. Corneal vascular response was quantitated 24 h postimplantation by using Student’s t test for independent samples.

RESULTS

Identification of GFA-116, a Calixarene-Based Protein Surface-Binding Agent that Disrupts VEGF Binding to its Receptor Flk-1 and VEGF-Stimulated Flk-1 Tyrosine Phosphorylation and Erk1/2 Activation

Our approach to target angiogenesis for cancer drug discovery has focused on disrupting protein–protein interaction between proangiogenic growth factors and their receptors. The approach consists of designing a relatively small library of calixarene derivatives containing four peptide loop domains whose sequences can be varied to optimize binding to a protein surface. The peptide loops consist of four amino acids that are cyclized through a 3-aminoo-5-aminomethyl-benzoic acid spacer (Fig. 1). This strategy provides a large number of diverse structures which present at their surfaces different charges and hydrophobic characteristics. Previously, we identified GFB-111, which contains four peptide loops containing the amino acid sequence GDGY (Fig. 1) as a potent inhibitor of PDGF binding to its receptor (29). In this study, we evaluated this library for compounds capable of interfering with VEGF binding to its receptor Flk-1. To this end, we initially screened the library for agents that inhibit VEGF-stimulated Flk-1 tyrosine phosphorylation using human Flk-1-overexpressing NIH 3T3 (Flk-1/NIH 3T3) cells as described in “Materials and Methods.” This initial rapid screen resulted in the identification of GFA-116, which contains four peptide loops with the amino acid sequence GKGK linked to a calixarene scaffold (Fig. 1), as a potent inhibitor of VEGF-stimulated tyrosine phosphorylation of Flk-1.

We next evaluated whether GFA-116 inhibits VEGF binding to its receptor. To this end, the ability of GFA-116 to inhibit 125I-VEGF binding to Flk-1 in Flk-1/NIH 3T3 or human prostate cancer cells (PC3) that naturally express Flk-1 was determined as described in “Materials and Methods.” Fig. 2A shows that in both cell lines, 125I-VEGF binding to Flk-1 was inhibited with IC_50 of 750 and 500 nM, respectively. The ability of GFA-116 to inhibit VEGF binding to Flk-1 was selective in that binding of 125I-PDGF to its receptor PDGFB receptor was not affected at concentrations as high as 10 µM (Fig. 2A). Fig. 2B shows that VEGF-stimulated Flk-1 tyrosine phosphorylation in Flk-1/NIH 3T3 cells was inhibited by GFA-116 in a dose-dependent fashion with an IC₅₀ of 1.5 µM. The concentration of GFA-116 to inhibit Flk-1 tyrosine phosphorylation (IC₅₀ = 1.5 µM) was twice as high as that required to inhibit VEGF/FLK-1 binding (IC₅₀ = 0.75 µM). Fig. 2C also shows that GFA-116 blocked VEGF but not PDGF, EGFR, or fibroblast growth factor stimulation of Erk1/2 phosphorylation, further demonstrating the selectivity of GFA-116 to disrupt VEGF but not other growth factor-driven signaling.

GFA-116 Inhibits Angiogenesis in Vitro

The ability of GFA-116 to inhibit VEGF binding to its receptor and subsequently to suppress Flk-1-dependent signaling prompted us to evaluate whether GFA-116 could inhibit VEGF-dependent angiogenesis pro-
GFA-116 inhibits angiogenesis and tumorigenesis in mice

Inhibition of Microvessel Outgrowth in Rat Aortic Rings. We also further confirmed the in vitro antiangiogenic effects of GFA-116 by measuring microvessel outgrowth in a rat aortic ring ex vivo model (32). Aortic rings (1-mm long) were preincubated for 24 h with Matrigel containing VEGF and treated with either vehicle or 1 μM GFA-116. Fig. 3C shows that in the absence of GFA-116, VEGF stimulated efficiently microvessel outgrowth from the rings, whereas in the presence of GFA-116, the outgrowth was inhibited by 65.4 ± 7.2% (P < 0.0005).

GFA-116 inhibits angiogenesis in vivo

Inhibition of Rat Cornea Angiogenesis in vivo. Rat cornea angiogenesis was assayed by inserting into each corneal pocket hydron pellets containing either VEGF (200 ng) plus vehicle or VEGF plus GFA-116 (1 μg/pellet), and corneal vascular response was measured 7 days postimplant as described in “Materials and Methods.” Fig. 3D shows that the angiogenesis index, which takes into account the length and width of the vessel area as described in “Materials and Methods,” was 8.23 ± 0.5 (n = 15) in the absence of GFA-116 and only 0.63 ± 0.3 (n = 8) in the presence of GFA-116 (P < 0.0001).
either vehicle or GFA-116. Daily i.p. injections of 50 mg/kg resulted in 64% tumor growth inhibition (Fig. 4C). In the second approach, we injected B16-F10 cells into the tail vein, and 14 days later, the lungs from both control and GFA-116-treated groups were harvested and analyzed grossly and microscopically as described in “Materials and Methods.” An excess of cold VEGF and PDGF was used to obtain nonspecific binding levels. Data are representative of four (PC3 cells), two (Flk-1 NIH 3T3 cells), and two (NIH 3T3 cells) independent experiments. In B, GFA-116 inhibits VEGF stimulation of Flk-1 tyrosine phosphorylation with an IC50 of 1.5 μM. NIH 3T3 cells stably overexpressing the VEGF receptor Flk-1 were treated with increasing concentrations of GFA-116 for 5 min before stimulation with VEGF (50 ng/ml) for 10 min. The cells were then lysed and processed for SDS-PAGE Western blotting with an antibody specific for phosphorytrosine-Flk-1. In C, GFA-116 inhibits VEGF-stimulated Erk1 and Erk2 phosphorylation selectively. NIH 3T3 or NIH 3T3 cells stably overexpressing either Flk-1 or EGFR were treated with GFA-116 (10 μM) before stimulation with VEGF (Flk-1/3T3), epidermal growth factor [EGF; EGFR receptor (EGFR)/3T3], PDGF (NIH 3T3), or basic fibroblast growth factor [bFGF; NIH 3T3] as described for B. The cells were then harvested and processed for SDS-PAGE Western blotting with an antibody specific for phospho-Erk1/2.

DISCUSSION

Protein–protein interactions such as those that occur between growth factor ligands and their cell surface receptors or between intracellular components of the signal transduction circuitry play pivotal roles in normal physiology as well as disease states. These interactions usually involve the formation of large surface area contacts (often >2000 Å2) between the protein partners. One of the unsolved problems in modern drug discovery is the identification of synthetic molecules that can mimic regions of these surface areas and hence disrupt protein–protein interactions. In this study, we have identified a potent disrupter of VEGF interaction with its receptor Flk-1. GFA-116 is a calix[4]arene with a strongly positively charged surface area formed by four peptide loops containing the glycine-lysine-glycine-lysine sequence. GFA-116 inhibited VEGF binding to its receptor with IC50 so of 500–750 nM and at 10 μM did not inhibit the binding of the closely related PDGF to its receptor. In contrast, GFA-116 selectively inhibited VEGF-stimulated Erk1 and Erk2 phosphorylation in NIH 3T3 cells stably overexpressing Flk-1, whereas EGFR-stimulated Erk1/2 phosphorylation in NIH 3T3 cells stably overexpressing EGFR was unaffected. These results suggest that GFA-116 may be a useful tool for studying the roles of VEGF signaling in normal physiology and disease states.
Fig. 3. In A, GFA-116 inhibits capillary network formation. Human middle cerebral artery endothelial cells (5 × 10⁴) were seeded onto Matrigel, and the cells were incubated with vascular endothelial growth factor (VEGF) in the presence or absence of GFA-116 or GFB-113 as described in “Materials and Methods.” Capillary network formation in A is quantified in B. For each culture, three randomly chosen fields were used. Student’s t test for an independent sample revealed a statistically significant difference between control and GFA-116 (P < 0.05). In B, GFA-116 potently inhibits human brain endothelial cell migration. Migration of adult human brain endothelial cells was evaluated using a modified Boyden chamber assay as described in “Materials and Methods.” Vehicle control or GFA-116 was added to 4% FBS-containing medium in the outer chamber, and the number of migrated cells was determined after an 18-h incubation. Endothelial cell migration in A is quantified in B. Student’s t test for independent samples revealed a statistically significant difference between control and GFA-116 (P < 0.05). In C, GFA-116 inhibits microvessel outgrowths in rat aortic rings. Rat aortic rings were cultured with vehicle or GFA-116. Pictures of the rings taken on day 4 (a) were quantified (b) as described in “Materials and Methods.” A significant difference between GFA-116-treated groups and control (ANOVA analysis; P < 0.005) was documented. In D, GFA-116 inhibits rat cornea angiogenesis in vivo. Results of rat corneal assay shown in a are quantified in b as described in “Materials and Methods.” A significant inhibition of VEGF-stimulated angiogenesis (P < 0.0001) was documented.
contrast, an analogue of GFA-116, GFB-111, which contains a partially anionic and hydrophobic surface area from four loops with the glycine-glutamic acid-glycine-tyrosine sequence, disrupted PDGF binding to its receptor, with an IC$_{50}$ of 250 nM, and inhibited VEGF/Flk-1 binding, with an IC$_{50}$ of 10 nM (29). These compounds are a new class of biologically active synthetic agents that disrupt protein–protein interactions which are involved in angiogenesis. Because VEGF is believed to be critical for initiation of angiogenesis, whereas PDGF is thought to play a major role in the maintenance of angiogenesis, it would be of great interest to determine whether a combination of GFA-116 and GFB-111 is synergistic. Similarly, designing a GFB that is able to inhibit both VEGF and PDGF signaling would also be of value. Recently, we have made GFB-204, which inhibits both PDGF and VEGF binding to PDGFR and FIK-1, respectively, and blocks angiogenesis.

The inhibition by GFA-116 of the binding of VEGF to Flk-1 resulted in selective suppression of VEGF- but not PDGF-, EGF-, or fibroblast growth factor-dependent signaling, as measured by Erk1/2 mitogen-activated protein kinase activation. This disruption of VEGF signaling resulted in major biological consequences. VEGF is known to be one of the major regulators of angiogenesis and tumor growth. Inhibiting angiogenesis and tumor growth by blocking the binding of both VEGF and PDGF to their receptors could be a promising strategy for cancer treatment.

Table 1. GFA-116 anti-angiogenic activity in vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>Microvessel Count (Mean ± SE, 400X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.9 ± 1.3</td>
</tr>
<tr>
<td>GFA-116</td>
<td>6.1 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2. GFA116 inhibits B16-F10 melanoma lung metastasis in vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor nodule count in lungs (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.8 ± 4.1</td>
</tr>
<tr>
<td>GFA-116</td>
<td>9.6 ± 1.8</td>
</tr>
</tbody>
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Fig. 4. In A, GFA-116 inhibits tumor growth of the human lung adenocarcinoma A-549 in nude mice. A-549 cells were implanted into the flanks of nude mice, and when the tumors reached an average size of ~100 mm$^3$, the mice were randomized and treated either with vehicle (●) or GFA-116 (▲); tumor sizes were measured as described in “Materials and Methods.” Daily injections of GFA-116 (50 mpk, i.p.) resulted in 53% tumor growth inhibition. In B, GFA-116 inhibits angiogenesis. Tumors were processed 2 h after the last i.p. injection for CD31 staining as described in “Materials and Methods.” In C, GFA-116 inhibits tumor growth of B16-F10 melanoma in an immune competent mouse model. B16-F10 mouse melanoma cells were implanted s.c. into C57BL/6 mice. When the tumors reached an average size of ~200 mm$^3$, the mice were randomized and treated with either vehicle (●) or GFA-116 (▲; 50 mpk/day), and tumor sizes were measured as described in “Materials and Methods.” In D and Table 2, GFA-116 inhibits B16-F10 melanoma metastasis to the lungs of C57BL/6 mice. B16-F10 mouse melanoma cells were injected i.v. into C57BL/6 mice and treated with vehicle or GFA-116 as described in “Materials and Methods.” Lungs of vehicle or GFA-116-treated mice were harvested, and lung tumor nodules were counted as described in “Materials and Methods.” Whole lung (top) and H&E staining of lung sections (bottom) from control and GFA-116-treated mice are shown. Quantification of B16-F10 tumor nodule count in lungs is shown in Table 2.
the most critical growth factors responsible for stimulating endothelial cell proliferation, migration, and new blood vessel formation (1, 35–38).

Here, we have demonstrated that GFA-116 inhibits VEGF-dependent stimulation of the proliferation of human brain endothelial cells and rat aortic endothelial cells, as well as suppresses VEGF-induced blood vessel formation in rat corneas. Disruption of VEGF binding to its receptor has also been accomplished by using Avastin, a monoclonal antibody to VEGF. Avastin binds VEGF with a Kd of about 1 nM and inhibits tumor growth in animal models with doses of 5 mg/kg (i.p. twice a week; Ref. 39). One drawback of this approach is the labor intensive and costly generation of the antibody. Furthermore, this approach to inhibit angiogenesis and tumorigenesis was tested clinically with success in colorectal but not breast cancer (27). In addition to targeting the interaction of VEGF with its receptor, inhibitors of the tyrosine kinase activity of Flk-1 have also been used to inhibit angiogenesis and tumorigenesis. Several of these, including SU5416, SU6668, DZ4190, and PTK787/ZK2284, inhibit potently Flk-1 tyrosine kinase in vitro and in whole cells (IC50 ranging from 50 to 3 μM) and suppress tumor growth in animal models at doses ranging from 25 to 200 mpk (22–25).

The fact that a synthetic molecule that disrupts binding of VEGF to its receptor and subsequent downstream signaling resulted in inhibition of angiogenesis suggested that such a molecule might have antitumor activity. In this study, we have demonstrated that GFA-116 inhibits angiogenesis in vivo in a nude mouse xenograft model where human lung cancer cells were grown s.c. Furthermore, the abilities of the tumors to grow in mice was significantly inhibited. More importantly, in an immune competent mouse model, GFA-116 also inhibited the growth of the highly aggressive mouse melanoma B16-F10 cells implanted s.c.; although GFA-116 was efficacious, we do not know if prolonged treatment would result in resistance development.

In a yet third model where B16-F10 cells injected i.v. metastasize to the lung, GFA-116 effectively inhibited metastasis. This is consistent with the fact that for metastatic tumors to grow at distant sites from primary tumors, they require angiogenesis. Indeed, it was recently reported that an anti-Flk-1-neutralizing antibody inhibits metastasis in orthotopic prostate cancer xenografts (40). Similarly, VEGF antisense treatment was also shown to be antitumorigenic (41).

Taken together, these results demonstrate that a synthetic molecule capable of disrupting the binding of VEGF to its receptor selectively inhibits VEGF-dependent signaling and suppresses angiogenesis and tumorigenesis.

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Blocking Angiogenesis and Tumorigenesis with GFA-116, a Synthetic Molecule that Inhibits Binding of Vascular Endothelial Growth Factor to its Receptor

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