AMG 706, an Oral, Multikinase Inhibitor that Selectively Targets Vascular Endothelial Growth Factor, Platelet-Derived Growth Factor, and Kit Receptors, Potently Inhibits Angiogenesis and Induces Regression in Tumor Xenografts

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Abstract

The growth of solid tumors is dependent on the continued stimulation of endothelial cell proliferation and migration resulting in angiogenesis. The angiogenic process is controlled by a variety of factors of which the vascular endothelial growth factor (VEGF) pathway and its receptors play a pivotal role. Small-molecule inhibitors of VEGF receptors (VEGFR) have been shown to inhibit angiogenesis and tumor growth in preclinical models and in clinical trials. A novel nicotinamide, AMG 706, was identified as a potent, orally bioavailable inhibitor of the VEGFR1/Flt1, VEGFR2/kinase domain receptor/Flk-1, VEGFR3/Flt4, platelet-derived growth factor receptor, and Kit receptors in preclinical models. AMG 706 inhibited human endothelial cell proliferation induced by VEGF, but not by basic fibroblast growth factor in vitro, as well as vascular permeability induced by VEGF in mice. Oral administration of AMG 706 potently inhibited VEGF-induced angiogenesis in the rat corneal model and induced regression of established A431 xenografts. AMG 706 was well tolerated and had no significant effects on body weight or on the general health of the animals. Histologic analysis of tumor xenografts from AMG 706–treated animals revealed an increase in endothelial apoptosis and a reduction in blood vessel area that preceded an increase in tumor cell apoptosis. In summary, AMG 706 is an orally bioavailable, well-tolerated multikinase inhibitor that is presently under clinical investigation for the treatment of human malignancies. (Cancer Res 2006; 66(17): 8715-21)

Introduction

Cancer and angiogenesis. Cancer is a group of diseases characterized by dysregulated cell growth. Growth of cancer cells is dependent on the continued supply of oxygen and nutrients that is delivered by vascular networks. Unless neovascularization occurs, solid tumor growth is limited by the diffusion limit for oxygen and does not progress beyond 1 to 2 mm in size (1). In experimental models of cancer, blocking angiogenesis prevents tumor growth and/or progression (2).

Some of the most potent proangiogenic substances are the vascular endothelial growth factor (VEGF) cytokines. These growth factors bind and activate VEGF receptor (VEGFR) 2 [also referred to as kinase domain receptor (KDR) or, in mice, fetal liver kinase-1 (Flk-1)] and other closely related receptor tyrosine kinases (RTK), including VEGFR1 (also referred to as Flt1), to elicit a stimulatory response in endothelial cells and promote neovascularization (3). Other receptors, such as platelet-derived growth factor (PDGF) receptor (PDGFR), are expressed on various cells, including pericytes, and have been shown to regulate pericyte differentiation and vascular survival (4). In addition, stem cell factor (SCF) receptor (Kit) is expressed on mast cells, melanocytes, and some hematopoietic stem cells and has been implicated in the pathogenesis of several tumor types, including gastrointestinal stromal tumors, small cell lung cancer, melanomas, ovarian carcinoma, and breast carcinoma (5). Furthermore, VEGFR3 is expressed exclusively on lymphatic endothelium and, in response to stimulation by its ligand VEGF-C, is thought to drive lymphangiogenesis and regulate metastatic spread (6). Inhibitors of VEGFRs, PDGFR, and/or Kit can potentially inhibit the growth of tumors by either blocking angiogenesis or perturbing dysregulated signaling pathways that cancer cells depend on for survival and growth.

Some small-molecule multikinase inhibitors targeting VEGFRs have shown promising clinical activity against various solid tumors (7–10), including gastrointestinal stromal (11), melanoma (12), and renal cell (13) tumors. Bevacizumab, a monoclonal antibody that selectively targets one isoform of VEGF, VEGF-A, was approved as first-line therapy in patients with metastatic colorectal cancer (10) and has shown activity against non–small cell lung cancer (14) and breast cancer (15).

This article describes a new, orally bioavailable, small-molecule multikinase inhibitor, AMG 706. AMG 706 is an ATP-competitive inhibitor of VEGFR1, VEGFR2, and VEGFR3. In addition, AMG 706 inhibits Kit and PDGFR, two RTKs related to VEGFR and implicated in the pathogenesis of several human cancers. AMG 706 inhibits VEGF-induced cellular proliferation and vascular permeability and induces tumor regression in vivo by selectively targeting neovascularization in tumor cells. AMG 706 is currently being studied in clinical trials for the treatment of cancer and has shown antiangiogenic and antitumor activity in a phase I clinical trial in patients with advanced solid malignancies (16, 17).

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Materials and Methods

Chemical synthesis. The chemical structure and physical-chemical properties of N-(2,3-dihydro-3,3-dimethyl-1H-indol-6-yl)-2-[(4-pyridinylmethythylamino)-3-pyridinecarboxamide (AMG 706) are shown in Fig. 1. It can be prepared according to procedures described in U.S. patent 6,878,714 (18). For in vitro studies, AMG 706 was dissolved in DMSO and diluted in growth medium before immediate use. For in vivo studies, AMG 706 was formulated as a suspension in Ora-Plus (Paddock Laboratories, Minneapolis, MN) vehicle adjusted to pH 2.0 and given orally.

Reagents and cells. A431, human epidermoid carcinoma cells, and MO7e, human megakaryocytic leukemia cells, were obtained and propagated as recommended by the American Type Culture Collection (Manassas, VA). The cells were determined to be free of mycoplasma and 17 different murine viral pathogens before use. Human umbilical vascular endothelial cells (HUVEC) and normal human dermal fibroblasts (NHDF) were obtained from Clonetics (East Rutherford, NJ) and grown according to the manufacturer’s instructions. DMEM, RPMI 1640, Dulbecco’s PBS (DPBS), fetal bovine serum (FBS), penicillin/streptomycin, penicillin/streptomycin/glutamine, Versene, and trypsin were obtained from Invitrogen Corp. (Carlsbad, CA). PDGF and granulocyte macrophage colony-stimulating factor (GM-CSF) were obtained from R&D Systems (Minneapolis, MN). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). PDGF and granulocyte macrophage colony-stimulating factor (GM-CSF) were obtained from R&D Systems (Minneapolis, MN). EPD growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). Epidermal growth factor (EGF) was from Upstate Systems (Chattanooga, VA). VEGF, basic fibroblast growth factor (bFGF), and SCF were produced (Carlsbad, CA). PDGF and granulocyte macrophage colony-stimulating factor (GM-CSF) were obtained from R&D Systems (Minneapolis, MN). Sodium orthovanadate (Na3VO4), ATP, Tris-HCl, MgCl2, NaCl, EGTA, DTT, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Corp. (St. Louis, MO).

Animals for in vivo studies. All in vivo experiments were conducted in accordance with the guidelines of the Amgen Animal Care and Use Committee. Female CD-1 nu/nu mice ages 4 to 6 weeks and female Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed in stainless caging. Harlan Teklad (Madison, WI) Sterilizable Rodent Diet 8656 and reverse osmosis water from the Amgen water supply system were provided ad libitum. Harlan Teklad (Madison, WI) Sterilizable Rodent Diet 8656 and reverse osmosis water from the Amgen water supply system were provided ad libitum. Harlan Teklad (Madison, WI) Sterilizable Rodent Diet 8656 and reverse osmosis water from the Amgen water supply system were provided ad libitum. Harlan Teklad (Madison, WI) Sterilizable Rodent Diet 8656 and reverse osmosis water from the Amgen water supply system were provided ad libitum.

In vitro kinase assays. In vitro kinase assays were done to establish IC50 values against a variety of recombinant receptor and nonreceptor kinases. Optimal enzyme, ATP, and substrate (gastrin peptide) concentrations were established for each enzyme using homogeneous time-resolved fluorescence (HTRF) assays. AMG 706 was tested in a 10-point dose-response curve for each enzyme using an ATP concentration of two-thirds Km for each. Most assays consisted of enzyme mixed with kinase reaction buffer [20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 5 mmol/L MnCl2, 100 mmol/L NaCl, 1.5 mmol/L EGTA]. A final concentration of 1 mmol/L DTT, 0.2 mmol/L NaVO3, and 20 μg/mL BSA was added before each assay. For Src, a modified kinase reaction buffer was used that included 20 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L MnCl2, 100 mmol/L NaCl, and 1.5 mmol/L EGTA. A final concentration of 1 mmol/L DTT, 0.2 mmol/L NaVO3, and 20 μg/mL BSA was added before each assay. For all assays, 5.75 mg/mL streptavidin-allophycocyanin (ProZyme, San Leandro, CA) and 0.1125 mmol/L Eu-PIT66 (Perkin-Elmer Corp., Boston, MA) were added immediately before the HTRF reaction. Plates were incubated for 30 minutes at room temperature and read on a Discovery instrument (Packard Instrument Co., Downers Grove, IL).

HUVEC proliferation assay. HUVECs were cultured in endothelial growth medium-2 (Cambrex, Walkersville, MD). Cells were seeded into flat-bottomed, 96-well plates (BD Falcon, San Jose, CA) at 3,000 per well in DMEM (high glucose) with 10% FBS and penicillin/streptomycin. After culture for 22 hours, medium was removed and cells were preincubated for 2 hours with serial dilutions (1:400) of AMG 706 in DMEM with 10% FBS plus penicillin/streptomycin. HUVECs were then challenged with 50 ng/mL VEGF or 20 ng/mL bFGF and incubated for 72 hours at 37°C, 5% CO2. Cells were washed twice with DPBS, and plates were frozen at −70°C for 24 hours. Proliferation was assessed by the addition of CyQuant dye (Molecular Probes, Eugene, OR), and plates were read on a Victor 1420 workstation (Perkin-Elmer Corp., Wellesley, MA). IC50 data were calculated using the Levenberg-Marquardt algorithm into a four-parameter logistic equation (ID Business Solutions Ltd., Alameda, CA).

PDGF proliferation assay. NIH3T3s were cultured in fibroblast growth medium-2 (Clonetics). Cells were washed with DPBS, trypsinized, resuspended in DMEM (low glucose) plus 10% FBS plus penicillin/streptomycin, and incubated at 37°C for 22 hours. Cells were serum starved for 72 hours in DMEM plus 0.5% FCS plus penicillin/streptomycin before the addition of AMG 706. After 2 hours, 25 ng/mL PDGF was added and the cells were incubated for an additional 24 hours before the addition of 0.05 μCi/well [3H]thymidine (Amersham, Piscataway, NJ). The amount of radioactivity incorporated into cells was determined following a 24-hour incubation period using a MicroBeta counter (Perkin-Elmer). IC50 data were calculated using the Levenberg-Marquardt algorithm into a four-parameter logistic equation.

EGF receptor phosphorylation assay. A431 cells were cultured in DMEM (high glucose) plus 10% FBS plus penicillin/streptomycin. Cells were washed with DPBS, trypsinized, seeded into 96-well plates, and incubated at 37°C for 5 hours. Cells were serum starved for 24 hours in DMEM plus penicillin/streptomycin before the addition of AMG 706. After a 2-hour incubation, 50 ng/mL EGF was added and the cells were incubated for an additional 10 minutes. Medium was removed, and the cells were washed twice with ice-cold PBS plus 300 μmol/L NaVO3, before incubation with radioimmunoprecipitation assay buffer plus 300 μmol/L NaVO3 plus protease inhibitors for 30 minutes at 4°C. Cell lysate was diluted 1:1 with PBS containing 1% BSA plus 0.1% Tween 20 plus 0.2 μg/mL biotinylated anti-EGF receptor (EGFR) antibody (R&D Systems) and 0.2 μg/mL abzyme anti-phosphotyrosine antibody (IGEN International, Gaithersburg, MD). After incubation for 1 hour at room temperature, 2.5 mL of Dynabeads M-280 streptavidin beads (IGEN International) were added and incubated for 30 minutes at room temperature. Samples were read on an IGEN M-Series M8 Analyzer (IGEN International).

Kit phosphorylation assay. MO7e cells were cultured in RPMI 1640 plus 10% FBS plus penicillin/streptomycin/glutamine plus 5 μg/mL GM-CSF. Cells were washed and starved overnight in RPMI 1640 plus 0.1% FBS plus penicillin/streptomycin/glutamine. Cells were washed and seeded into 12-well plates at 2.5 × 104 per well in starvation medium. AMG 706 was added to wells, and after a 2-hour incubation, 100 ng/mL SFC was added and the cells were incubated for an additional 15 minutes. Cells were pelleted and lysed in 150 μmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1% IGEPEAL, 0.5% deoxycholate, 0.1% SDS, protease cocktail (Sigma-Aldrich), and 300 μmol/L activated Na3VO4. Kit was immediately immunoprecipitated from supernatants using 5 μg Kit polyclonal antibody (R&D Systems). Samples were loaded onto an 8% Tris-glycine gel and transferred to nitrocellulose membrane (Invitrogen). Membranes were probed with 4G10 anti-phosphotyrosine antibody (Upstate Systems) and then developed using

![Figure 1. Chemical structure and physical properties of AMG 706.](image-url)
Relative Evans blue units refer to the percentage Evans blue as determined with 10% FBS and penicillin/streptomycin/glutamine. Cells were incubated in PBS containing either 0.1% BSA or 0.1% BSA with 10 μmol/L VEGF (R&D Systems) for 1 hour at 4°C. A single disc was inserted into a pocket in the cornal stroma of anesthetized rats. Animals were given vehicle (Ora-Plus) or AMG 706 as indicated via oral gavage. After 7 days of treatment, the corneas were photographed, and for each corneal image, the number of vessels intersecting the midpoint between the disc and the limbus was measured. Image analysis was done in a blinded fashion. Values represent the group mean ± SE. Statistical significance was assessed by ANOVA followed by Fisher’s protected least significant difference post hoc test.

**Rat corneal angiogenesis model.** Corneal angiogenesis was evaluated in adult female Sprague-Dawley rats as described (20). Briefly, 0.6-mm diameter circular discs of Nylaflo filter paper (Pall Corp., Ann Arbor, MI) were incubated in PBS containing either 0.1% BSA or 0.1% BSA with 10 μmol/L VEGF (R&D Systems) for 1 hour at 4°C. A single disc was inserted into a pocket in the cornal stroma of anesthetized rats. Animals were given vehicle (Ora-Plus) or AMG 706 as indicated via oral gavage. After 7 days of treatment, the corneas were photographed, and for each corneal image, the number of vessels intersecting the midpoint between the disc and the limbus was measured. Image analysis was done in a blinded fashion. Values represent the group mean ± SE. Statistical significance was assessed by ANOVA followed by Fisher’s protected least significant difference post hoc test.

**Tumor xenograft models.** A431 cells were cultured in DMEM (low glucose) with 10% FBS and penicillin/streptomycin/glutamine. Cells were harvested by trypsinization, washed, and adjusted to a concentration of 5 × 10^6/mL in serum-free medium. Animals were challenged s.c. with 1 × 10^7 cells in 0.2 mL over the left flank. Approximately 10 days thereafter, mice were randomized based on initial tumor volume measurements and treated with either vehicle (Ora-Plus) or AMG 706. Tumor volumes and body weights were recorded twice weekly and/or on the day of sacrifice. Tumor volume was measured with a Pro-Max electronic digital caliper (Sylvac, Crissier, Switzerland) and calculated using the formula length (mm) × width (mm) × height (mm) and expressed in mm^3. Data are expressed as mean ± SE. Repeated measures ANOVA followed by Scheffe post hoc testing for multiple comparisons was used to evaluate the statistical significance of observed differences.

**Tumor microvasculature analysis.** A431 cells (1 × 10^5) were injected s.c. into the left flank of female CD-1 nu/nu mice. Eleven days thereafter, mice were randomized based on initial tumor volume measurements and oral gavage with either vehicle (Ora-Plus) or AMG 706 was initiated. Two hours before harvest, each mouse received an i.p. injection of bromodeoxyuridine (BrdUrd) to enable subsequent labeling of S-phase nuclei. Tumors were isolated along with a section of ileum to serve as a positive control for endothelial cell apoptosis, blood vessel density, and tumor cell apoptosis. Endothelial cell apoptosis was assessed by double immunostaining for CD31 (BD Biosciences) using alkaline phosphatase and for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Roche Applied Sciences, Indianapolis, IN) using FITC and counterstained with Hoechst nuclear dye (Molecular Probes). Images from each field were captured thrice using different fluorescent filters to sandwich the images. The newly created sandwiched images allowed the precise identification of both normal and apoptotic endothelial cell nuclei due to the superimposition of the CD31, Hoechst, and TUNEL signals. Blood vessel area was expressed as a percentage of the viable tumor area that was occupied by blood vessels as visualized by CD31 staining. Endothelial cells were immunostained for CD31 using peroxidase localization with DAB substrate. Image analysis was done in a blinded fashion. Analyses were done by pair-wise comparison of AMG 706 versus vehicle control for each time point using JMP version 4.0.4 (SAS Institute, Cary, NC). For each dependent variable, Levene’s test was used to determine homogeneity of variance, with P < 0.05 indicating that variance was not homogeneous. If Levene’s test indicated that variances were homogeneous for each time point being analyzed, the Student’s t test was used to compare AMG 706 versus vehicle control groups for that dependent variable at each time point. If Levene’s test indicated that variances were not homogeneous for each time point being analyzed, the Wilcoxon rank sum test was used to compare AMG 706 versus vehicle control groups for that dependent variable at each time point.

**Results**

**Activity and selectivity of AMG 706.** The chemical structure and physical-chemical properties of N-(2,3-dihydro-3,3-dimethyl-1H-indol-6-yl)-2-[[4-pyridinylmethyl]amino]-3-pyridinecarboxamide (AMG 706) are shown in Fig. 1 (18). AMG 706 is a potent, orally bioavailable, ATP-competitive inhibitor of human VEGFR2 when tested using *in vitro* kinase assays (Table 1). AMG 706 has broad activity against the human VEGFR family, including VEGFR1, VEGFR2, and VEGFR3, and displays similar activity against human Kit (Table 1). The compound also displays activity against PDGFR and Ret, although the potency was reduced ~10-fold relative to the VEGFR family (Table 1). The compound is highly selective against a broad range of ~47 other kinases tested, including EGFR, Src, and p38 kinase (Table 1).

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<th>Table 1. Biochemical activity and selectivity of AMG 706</th>
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NOTE: AMG 706 was tested in a 10-point dose-response curve for each enzyme using an ATP concentration of two-thirds K_{d} for each. IC_{50} data were calculated using the Levenberg-Marquardt algorithm into a four-parameter logistic equation. Data are mean ± SD (n > 2).
The selectivity of AMG 706 was preserved in cellular assays. Potent inhibition of receptor phosphorylation and functional responses was observed in biologically relevant cells. AMG 706 potently inhibited VEGF-induced but not bFGF-induced proliferation of HUVECs with IC50 values of 10 nmol/L and >3,000 nmol/L, respectively. Similarly, AMG 706 potently inhibited both PDGF-induced proliferation and SCF-induced c-kit phosphorylation with IC50 values of 207 and 37 nmol/L, respectively. AMG 706 displayed no activity against unrelated kinases as evidenced by the lack of activity against EGF-induced EGFR phosphorylation in A431 cells (IC50 >25 μmol/L). Importantly, incubation of A431 tumor cells for 3 days with up to 25 μmol/L AMG 706 did not affect their viability as determined by Alamar Blue uptake according to the manufacturer's instructions.

**In vivo inhibition of vascular permeability.** The effects of AMG 706 were examined in an animal model of VEGF-induced vascular permeability to extend and confirm the results that were observed with enzymatic and cell-based assays. A single 100 mg/kg dose of AMG 706 given 24 hours after injection of VEGF-transfected or vector control cells inhibited VEGF-induced vascular permeability in a time-dependent manner. AMG 706 rapidly and significantly \( P = 0.0022 \) inhibited VEGF-induced vascular permeability (Fig. 2). Inhibition of vascular permeability was maintained, although not significantly, for up to 16 hours after administration of AMG 706, an effect that correlates with the maintenance of plasma levels above the calculated HUVEC IC50 value. Inhibition of vascular permeability was rapidly reversed once plasma levels declined below the estimated IC50 value.

**Inhibition of VEGF-induced rat corneal angiogenesis.** To determine if AMG 706 had antiangiogenic activity in vivo, AMG 706 was evaluated in the rat corneal angiogenesis model (Fig. 3). AMG 706 inhibited VEGF-induced angiogenesis in a dose-dependent manner (Fig. 3). Twice daily (b.i.d.) oral dosing of AMG 706 significantly inhibited \( P < 0.004 \) angiogenesis, with an estimated ED50 of 2.1 mg/kg [area under the plasma concentration versus time curve (AUC)0-24 = 2.2 μg * h/mL; \( C_{\text{max}} = 0.28 \mu g/mL \); Fig. 3A]. In a second study, AMG 706 was given orally once daily (q.d.) for 7 days (Fig. 3B). AMG 706 significantly \( P < 0.018 \) inhibited angiogenesis, with an estimated ED50 of 4.9 mg/kg/d (AUC0-24 = 5.0 μg * h/mL; \( C_{\text{max}} = 2.1 \mu g/mL \)). Of note, with both q.d. and b.i.d.

![Figure 2](image-url)  
**Figure 2.** Inhibition of VEGF-induced vascular permeability in mice treated with AMG 706. HEK 293 cells were mixed with Matrigel and injected s.c. into CD-1 nu/nu mice. A single dose of AMG 706 was given by oral gavage (100 mg/kg) 24 hours after implantation of cells. At various time points after administration, plasma was obtained for measurement of pharmacokinetics, and vascular permeability in the skin overlying the Matrigel plug was measured by quantitation of extravasation of Evans blue dye. Columns, relative Evans blue units (n = 5 per group); bars, SE. *, \( P < 0.0022 \), significant difference from VEGF plus vehicle-injected control mice.

![Figure 3](image-url)  
**Figure 3.** AMG 706 inhibition of VEGF-induced angiogenesis in rats. Angiogenesis was induced by implanting a VEGF-soaked or BSA control-soaked nylon disc into the corneal stroma (n = 8 per group). For each corneal image, the number of vessels intersecting the midpoint between the disc and the limbus was measured. AMG 706 was given orally b.i.d. for 7 days (1, 3, 5, or 10 mg/kg/dose) or q.d. for 7 days (1, 3, 10, or 30 mg/kg/dose). Columns, mean; bars, SE. *, \( P < 0.004 \); **, \( P < 0.018 \), significant difference from VEGF plus vehicle-treated animals.
dosing, AMG 706 was able to inhibit angiogenesis below that observed in the 0.1% BSA control group. We attribute this background effect to a slight stimulation of angiogenesis induced by the surgical procedure of disc implantation.

**In vivo tumor xenograft growth inhibition.** The antitumor activity of AMG 706 was examined in several human xenograft models in athymic nude mice. In this study, the effects of AMG 706 on A431 human epidermoid xenografts to treatment with AMG 706 were evaluated. Female athymic nude mice aged 5 to 6 weeks were injected with 1 × 10⁷ A431 cells on day 0. Treatment with AMG 706 (10, 30, or 100 mg/kg/dose b.i.d.) was begun on day 10 when tumors were ∼125 mm³. Six of 10 mice initially treated with vehicle began treatment with AMG 706 at 100 mg/kg/dose on day 27. The remaining 4 animals were sacrificed for immunohistochemical analysis. Fractions, number of mice with no measurable tumor over the total number of animals in the group. Points, mean (n = 10 per group); bars, SE. *, P < 0.05, statistically significant difference from day 10 values. B, blanching and regression of A431 xenografts in mice treated with AMG 706. Female CD-1 nude mice with large, established (∼1,000 mm³) A431 tumor xenografts were dosed with AMG 706 at 75 mg/kg/dose b.i.d. A representative animal illustrates the time-dependent blanching and regression of disease associated with continued treatment.

**Mechanism of tumor xenograft growth inhibition.** To characterize the mechanism of AMG 706–induced regression of tumors, we examined the physiologic response of established tumors to treatment with AMG 706. Initially, the xenografts were highly vascularized with extensive vasculature networks visible throughout the tumor. AMG 706 induced rapid tumor regression as early as 3 days after AMG 706 administration, and loss of tumor vasculature could be observed as well as the appearance of a scab of necrotic tissue. By day 5, the tumor was completely blanched in appearance, reflecting almost complete loss of the tumor-associated vasculature. Further treatment resulted in tumor regression associated with additional disappearance of tumor-associated vessels as well as an increase in the area of necrotic tissue (Fig. 4B).

The specific effects of AMG 706 on tumor microvasculature from vehicle-treated and AMG 706–treated animals were evaluated using anti-CD31 immunohistochemistry (Fig. 5). After 7 days of
treatment with 75 mg/kg/dose b.i.d. of AMG 706, there was a distinct absence of blood vessels in tumor tissue as evidenced by the lack of CD31 antibody staining compared with tumor tissue from vehicle-treated mice (Fig. 5A). Only vessels located on the periphery of the tumor were still evident. In addition, as opposed to tissue from vehicle-treated mice, the presence of tumor cell necrosis was seen in the tissue isolated from the AMG 706–treated mice.

To further investigate the mechanism of action of AMG 706, A431 xenografts from mice treated with vehicle or AMG 706 (75 mg/kg/dose b.i.d.) were examined by histology. Xenografts were harvested at various treatment intervals and evaluated by quantitative histomorphometry for blood vessel area, endothelial cell apoptosis, and tumor cell apoptosis (Fig. 5). A significant increase ($P < 0.026$) in endothelial cell apoptosis and corresponding decrease ($P < 0.0005$) in blood vessel area were the first temporal events observed following administration of AMG 706 (Fig. 5B and C). This was followed by a significant increase ($P < 0.009$) in tumor cell apoptosis at 48 hours after AMG 706 administration (Fig. 5D). These observations are consistent with targeting of tumor-associated endothelial cells and blood vessels as a primary mechanism of antitumor activity of AMG 706 in this model. Additional studies using specific inhibitors of PDGFR and Kit are required to delineate the contribution of these targets to the antitumor activities of AMG 706.

**Discussion**

In this report, we describe the characterization of AMG 706, a novel, orally bioavailable, multikinase inhibitor. AMG 706 inhibits a subclass of RTKs, including VEGFR1, VEGFR2, VEGFR3, PDGFR, and Kit. This compound competitively inhibits VEGFRs, as shown by *in vitro* assays (data not shown), as well as the reversibility of vascular permeability in mice. The ability of AMG 706 to inhibit VEGF-induced permeability correlated well with its cellular inhibition of HUVEC proliferation, highlighting the pharmacokinetic-pharmacodynamic nature of this response. This result also suggests that maximal inhibition of the targets will require sustained plasma concentration of drug to maximize efficacy.

The *in vivo* activity of this compound is generally attributable to its broad activity against all VEGFRs tested, including VEGFR1,
VEGFR2, and VEGFR3, although we do not know what contribution inhibition of the other targets may play in this response. Although VEGFR2/KDR is presumed to be the dominant receptor mediating the angiogenic activity of VEGF, both VEGFR1 and VEGFR3 have also been implicated. VEGFR1 has been shown to mediate recruitment of endothelial precursor cells to areas of active angiogenesis (3), whereas VEGFR3 plays an important role in lymphangiogenesis. In addition, the stabilization of nascent vessels involves associations with pericytes, a process that involves the PDGFR (5). Modulation of these key mediators should provide more complete inhibition of angiogenesis than the targeting of any single component.

The high selectivity of this molecule is evidenced by the lack of activity against a variety of other kinases tested. Furthermore, no activity against bFGF-induced HUVEC proliferation was observed, implying that AMG 706 does not inhibit the receptor for bFGF nor any downstream kinases or other proteins that mediate the proliferative signals of bFGF. Examination of the physiologic response of established tumors to treatment with AMG 706 provided direct evidence that the mechanism of tumor regression in A431 xenografts is due to the antiangiogenic effects of AMG 706. Increased endothelial cell apoptosis in association with decreased blood vessel area were the first temporal events observed following administration of AMG 706. This was followed by significant increases in tumor cell apoptosis. The sequence of these observations is consistent with targeting of tumor-associated endothelial cells and blood vessels as a primary mechanism of the antitumor activity of AMG 706 in the model systems explored in this study.

In conclusion, we have described the identification of AMG 706 and its characterization as a potent, orally bioavailable, novel multikinase inhibitor. This compound potently inhibited angiogenesis in a variety of in vivo models and was able to induce regressions of large established tumor xenografts. AMG 706 is currently being studied in clinical trials for the treatment of cancer and has shown acceptable safety and promising activity in early clinical trials in patients with advanced solid malignancies (16, 17).

Acknowledgments

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References

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