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BAY 43-9006 Exhibits Broad Spectrum Oral Antitumor Activity and Targets the RAF/MEK/ERK Pathway and Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis

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ABSTRACT

The RAS/RAF signaling pathway is an important mediator of tumor cell proliferation and angiogenesis. The novel bi-aryl urea BAY 43-9006 is a potent inhibitor of Raf-1, a member of the RAF/MEK/ERK signaling pathway. Additional characterization showed that BAY 43-9006 suppresses both wild-type and V599E mutant BRAF activity *in vitro*. In addition, BAY 43-9006 demonstrated significant activity against several receptor tyrosine kinases involved in neovascularization and tumor progression, including vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor β, Flt-3, and c-KIT. In cellular mechanistic assays, BAY 43-9006 demonstrated inhibition of the mitogen-activated protein kinase pathway in colon, pancreatic, and breast tumor cell lines expressing mutant KRAS or wild-type or mutant BRAF, whereas non–small-cell lung cancer cell lines expressing mutant KRAS were insensitive to inhibition of the mitogen-activated protein kinase pathway by BAY 43-9006. Potent inhibition of VEGFR-2, platelet-derived growth factor receptor β, and VEGFR-3 cellular receptor autophosphorylation was also observed for BAY 43-9006. Once daily oral dosing of BAY 43-9006 demonstrated broad-spectrum antitumor activity in colon, breast, and non–small-cell lung cancer xenograft models. Immunohistochemistry demonstrated a close association between inhibition of tumor growth and inhibition of the extracellular signal-regulated kinases (ERKs) 1/2 phosphorylation in two of three xenograft models examined, consistent with inhibition of the RAF/MEK/ERK pathway in some but not all models. Additional analyses of microvessel density and microvessel area in the same tumor sections using antimurine CD31 antibodies demonstrated significant inhibition of neovascularization in all three of the xenograft models. These data demonstrate that BAY 43-9006 is a novel dual action RAF kinase and VEGFR inhibitor that targets tumor cell proliferation and tumor angiogenesis.

INTRODUCTION

Many of the processes involved in tumor growth, progression, and metastasis are mediated by signaling pathways initiated by activated receptor tyrosine kinases (RTKs; ref. 1). RAS functions downstream of several RTKs, and activation of RAS signaling pathways is an important mechanism by which human cancer develops (2). Constitutive activation of the RAS pathways occurs through mutational activation of the RAS oncogene or of downstream effectors of RAS (3). RAS activation can also be exploited by overexpression of a variety of RTKs, including those for the epidermal (EGFR), platelet-derived (PDGFR), or vascular-endothelial (VEGFR) growth factors (4–9). In this way, the majority of human tumors, not just those with RAS mutations, depend on activation of the RAS signal transduction pathways to achieve cellular proliferation and survival (4).

RAS regulates several pathways that synergistically induce cellular transformation, including the well-characterized RAF/MEK/ERK cascade. RAF kinases are serine/threonine protein kinases that function in this pathway as downstream effector molecules of RAS. RAS localizes RAF to the plasma membrane, where RAF initiates a mitogenic kinase cascade that ultimately modulates gene expression via the phosphorylation of transcription factors (3), which can have profound effects on cellular proliferation and tumorigenesis.

The RAF kinase family is composed of three members: ARAF, BRAF, and Raf-1 (also termed c-Raf). BRAF is reportedly mutated in 70% of malignant melanomas (10), in 33% of papillary thyroid carcinomas (11), and in lower frequencies in other cancers (12). The V599E mutant form of BRAF activates the RAF/MEK/ERK pathway in human melanoma cells *in vitro*, and small interfering RNA silencing of V599E BRAF, but not Raf-1, inhibits soft agar growth of these cells (13). In addition, transformation of a melanocyte cell line with V599E BRAF activates the mitogen-activated protein kinase (MAPK) pathway. BAY 43-9006, a RAF kinase and VEGFR-2 inhibitor, and U0126, a MAP kinase kinase (MEK) inhibitor, block MAPK activation and inhibit cell proliferation in mutant BRAF- and KRAS-transformed melanocytes (14).

Recent evidence suggests that Raf-1 and BRAF participate in the regulation of endothelial apoptosis and, therefore, angiogenesis, a process essential for tumor development and metastasis (15, 16). Selective delivery of mutant Raf-1 to tumor blood vessels induces endothelial cell apoptosis, which inhibits angiogenesis and results in regression of established tumors (16). Mice deficient in BRAF or Raf-1 die during embryogenesis because of severe vascular defects and increased apoptosis that could be due, in part, to effects on endothelial cell survival (17, 18).

Angiogenesis is a tightly regulated multistep process that involves the interaction of multiple growth factors expressed as multiple isoforms, including VEGFs, basic fibroblast growth factor, and PDGFs. VEGF also regulates vascular permeability. Vessel stabilization through pericyte recruitment and maturation is primarily driven by PDGF (19). Several antiangiogenic agents are currently being investigated in clinical trials (20–24); however, because of the complex interactions between tumor cells, the invading stroma, and new blood vessels, a therapeutic agent targeting a single molecular entity might have limited efficacy across a spectrum of tumor types (25, 26).

BAY 43-9006 is a novel bi-aryl urea that has been previously shown to inhibit Raf-1 and tumor cell line proliferation and tumor growth in several human tumor xenograft models (27, 28). Here, we demonstrate that BAY 43-9006 inhibits another member of the RAF family, wild-type (wt) BRAF and V599E BRAF. In addition, BAY 43-9006 demonstrates potent inhibition of certain proangiogenic RTKs, including VEGFR-2, PDGFR-β, and VEGFR-3. BAY 43-9006 also substantially inhibits tumor growth of several human tumor

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xenograft models, even in the absence of MAPK pathway inhibition. Taken together, these data suggest that BAY 43-9006 functions as a novel dual action RAF kinase and VEGFR inhibitor targeting both the RAF/MEK/ERK pathway and RTKs that promote tumor angiogenesis.

MATERIALS AND METHODS

Preparation of BAY 43-9006

The chemical name of BAY 43-9006 is *N*-(3-trifluoromethyl-4-chlorophenyl)-*N'*-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea, and the structural formula is shown in Table 1. For *in vitro* experiments, BAY 43-9006 was dissolved in DMSO. For *in vivo* experiments, BAY 43-9006 was dissolved in Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethyl alcohol) at 4-fold (4×) of the highest dose, foil wrapped, and stored at room temperature. This 4× stock solution was prepared fresh every 3 days. Final dosing solutions were prepared on the day of use by dilution of the stock solution to 1× with water. Lower doses were prepared by dilution of the 1× solution with Cremophor EL/ethanol/water (12.5:12.5:75).

Biochemical Assays

***In vitro* Assays with Recombinant Raf-1 (Residues 305–648), BRAF (Residues 409–765), V599E BRAF (Residues 409–765), MEK-1, and Extracellular Signal-Regulated Kinase (ERK)-1.** COOH-terminal kinase domains of Raf-1 (residues 305–648) and BRAF (residues 409–765) were generated by PCR. The BRAF (residues 409–765) V599E mutation was introduced using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Recombinant baculoviruses expressing Raf-1 (residues 305–648), BRAF (residues 409–765), and V599E BRAF (residues 409–765) were purified as fusion proteins as described previously (29). Full-length human MEK-1 was generated by PCR and purified as a fusion protein from *Escherichia coli* lysates (30).

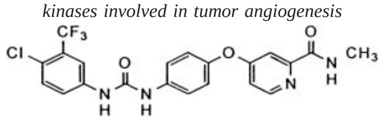
To test compound inhibition against various RAF kinase isoforms, BAY 43-9006 was added to a mixture of Raf-1 (80 ng), wt BRAF, or V599E BRAF (80 ng) with MEK-1 (1 μg) in assay buffer [20 mmol/L Tris (pH 8.2), 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, and 0.15% β-mercaptoethanol] at a final concentration of 1% DMSO. The RAF kinase assay (final volume of 50 μL) was initiated by adding 25 μL of 10 μmol/L γ-[^{33}P]ATP (400 Ci/mol) and incubated at 32°C for 25 minutes. Phosphorylated MEK-1 was harvested by filtration onto a phosphocellulose mat, and 1% phosphoric acid was used to wash away unbound radioactivity. After drying by microwave heating, a β-plate counter was used to quantify filter-bound radioactivity. Activated MEK-1 and ERK-1 were purchased from Upstate Biotechnology (UBI, Waltham, MA) and assayed according to manufacturer's instructions.

***In vitro* Assays for Murine (m)VEGFR-2 (flk-1), mVEGFR-3, mPDGFR-β, Flt-3, c-KIT, EGFR, HER2, c-MET, c-yes, FGFR-1, and IGFR-1.** mVEGFR-2 (flk-1; residues 785-1367), human VEGFR-2 (KDR) kinase domain, mPDGFR-β (residues 560-1098), mVEGFR-3 (residues 818-1363), EGFR (residues 669-1210), HER2/neu (residues 691-1255), and FGFR-1 (residues 398–882) were expressed and purified from Sf9 lysates as described previously (29, 31). Flt-3, c-KIT, insulin growth factor receptor (IGFR)-1, VEGFR-2, c-MET, and cdk-1/cyclin B were purchased from Proqinase (Freiburg, Germany). Activated protein kinase (PK)B, PKA, LCK, and c-yes were purchased from Calbiochem, Inc. (San Diego, CA) and assayed according to the manufacturer's instructions. BAY 43-9006 was assayed against recombinant pim-1 at Proqinase and PKCα and PKCγ at Pan Labs (Bothell, WA).

Time-resolved fluorescence energy transfer assays for mVEGFR-2 (flk-1), mVEGFR-3, mPDGFR-β, Flt-3, c-KIT, EGFR, HER2, c-MET, c-yes, LCK, and IGFR-1 were performed in 96-well opaque plates in the time-resolved fluorescence energy transfer format. Final reaction conditions were as follows: 1 to 10 μmol/L ATP, 25 nmol/L poly GT-biotin, 2 nmol/L Europium-labeled phospho (p)-Tyr antibody (PY20; Perkin-Elmer, Wellesley, MA), 10 nmol/L APC (Perkin-Elmer), 1 to 7 nmol/L cytoplasmic kinase domain in final concentrations of 1% DMSO, 50 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 0.015% Brij-35, 0.1 mg/mL BSA, and 0.1% β-mercapto-ethanol. Reactions volumes were 100 μL and were initiated by addition of enzyme. Plates were read at both 615 and 665 nmol/L on a Perkin-Elmer VictorV Multilabel counter at ~1.5 to 2.0 hours after reaction initiation. Signal was calculated as a ratio: (665 nm/615 nmol/L) × 10,000 for each well. Signal to noise was generally 4 to 8-fold in each assay.

For IC_{50} generation, compounds were added before the enzyme initiation. A 50-fold stock plate was made with compounds serially diluted 1:3 in a 50% DMSO/50% distilled water solution. Final compound concentrations ranged from 10 μmol/L to 4.56 nmol/L in 1% DMSO. The data were expressed as percent inhibition = 100 − [(signal with inhibitor − background)/(signal without inhibitor − background)] × 100.

Table 1 *BAY 43-9006 inhibits the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor angiogenesis*



	IC_{50} (nmol/L) ± SD (*n*)*
Biochemical assay†	
Raf-1‡	6 ± 3 (7)
BRAF wild-type§	22 ± 6 (7)
V599E BRAF mutant¶	38 ± 9 (4)
VEGFR-2	90 ± 15 (4)
mVEGFR-2 (flk-1)	15 ± 6 (4)
mVEGR-3	20 ± 6 (3)
mPDGFR-β	57 ± 20 (5)
Flt-3	58 ± 20 (3)
c-KIT	68 ± 21 (3)
FGFR-1	580 ± 100 (3)
ERK-1, MEK-1, EGFR, HER-2, IGFR-1, c-met, PKB, PKA, cdk1/cyclinB, PKCα, PKCγ, pim-1	>10,000
Cellular mechanism‖	
MDA MB 231 MEK phosphorylation (human breast)	40 ± 20 (2)
MDA MB 231 ERK 1/2 phosphorylation (human breast)	90 ± 26 (7)
BxPC-3 ERK 1/2 phosphorylation (human pancreatic)	1,200** ± 165 (2)
LOX ERK 1/2 phosphorylation (human melanoma)	880** ± 90 (2)
VEGFR-2 phosphorylation (human, NIH 3T3 cells)	30 ± 21 (3)
VEGF–ERK 1/2 phosphorylation (human, HUVEC)	60** ± 26 (2)
PDGFR-β phosphorylation (human HAoSMC)	80 ± 40 (3)
mVEGFR-3 phosphorylation (mouse, HEK-293 cells)	100 ± 80 (2)
Flt-3 phosphorylation (human ITD, HEK-293 cells)	20 ± 10 (2)
Cellular proliferation	
MDA MB 231 (10% FCS)	2,600 ± 810 (3)
PDGF-BB HAoSMC (0.1% BSA)	280 ± 140 (5)

* IC_{50} mean ± SD; (*n* = number of trials).

† Kinase assay were carried out as described in Materials and Methods at ATP concentrations at or below K_m (1 to 10 μmol/L).

‡ Lck activated NH_2-terminal–truncated Raf-1.

§ NH_2-terminal–truncated BRAF (wild-type).

¶ NH_2-terminal V599E-truncated BRAF (mutant).

‖ Cellular mechanism assays (RTK autophosphorylation and RAF/MEK/ERK pathway) were performed in 0.1% BSA using phospho-specific antibodies or 4G10 for VEGFR-3 as described in Materials and Methods.

** Activated phospho-ERK 1/2 was quantitated with phospho-ERK 1/2 immunoassay (Bio-Plex; Bio-Rad, Inc.).

Cellular Mechanistic Assays

Tumor Cell Lines and Reagents. The MDA-MB-231 human mammary adenocarcinoma cell line was obtained from the National Cancer Institute. These cells were maintained in DMEM (Invitrogen, Inc., Carlsbad, CA), supplemented with 1% L-glutamine (Invitrogen, Inc.), 1% HEPES buffer (Invitrogen, Inc.), and 10% heat-inactivated fetal bovine serum. The Colo-205, HT-29, and DLD-1 human colon carcinomas and the NCI-H460 and A549 human non–small-cell lung cancer (NSCLC) carcinoma lines were obtained from and propogated as recommended by the American Type Tissue Culture Collection Repository (Manassas, VA).

Cellular MEK 1/2, ERK 1/2, and PKB Activation and Bio-Plex pERK Immunoassay. Tumor cell lines were plated at 2 × 10^5 cells per well in 12-well tissue culture plates in DMEM growth media (10% heat-inactivated FCS) overnight. Cells were washed once with serum-free media and incubated in DMEM supplemented with 0.1% fatty acid-free BSA (Sigma, St. Louis, MO) containing various concentrations of BAY 43-9006 in 0.1% DMSO for 120 minutes to measure changes in basal pMEK 1/2, pERK 1/2, or pPKB.

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Cells were washed with cold PBS (PBS containing 0.1 mmol/L vanadate) and lysed in a 1% (v/v) Triton X-100 solution containing protease inhibitors. Lysates were clarified by centrifugation, subjected to SDS-PAGE, transferred to nitrocellulose membranes, blocked in TBS-BSA, and probed with anti-pMEK 1/2 (Ser^{217}/Ser^{221}; 1:1000), anti-MEK 1/2, anti-pERK 1/2 (Thr^{202}/Tyr^{204}; 1:1000), anti-ERK 1/2, anti-pPKB (Ser^{473}; 1:1000), or anti-PKB primary antibodies (Cell Signaling Technology, Beverly, MA). Blots were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed with Amersham ECL reagent on Amersham Hyperfilm.

A 96-well pERK immunoassay, using the laser flow cytometry (Bio-Rad, Hercules, CA) platform, was developed to measure BAY 43-9006–mediated inhibition of basal pERK 1/2 in tumor cell lines. MDA-MB-231, LOX, and BxPC-3 cells were plated at 50,000 cells per well. One day after plating, tumor cells in DMEM with 0.1% fatty acid-free BSA were incubated for 2 hours with BAY compounds diluted to a final concentration of 3 μmol/L to 12 nmol/L in 0.1% DMSO. Cells were incubated washed, lysed, and directly transferred to assay plate or frozen at −80°C until processed. Tumor cell lysates were incubated with ~2000 of 5-μm Bio-Plex beads conjugated with an anti-ERK 1/2 antibody. The next day, biotinylated pERK 1/2 sandwich immunoassay was performed, beads were washed three times during each incubation, and phycoerythrin-streptavidin was used as a develop reagent. The relative fluorescence units of pERK 1/2 were detected by counting 25 beads with Bio-Plex flow cell (probe) at high sensitivity. The IC_{50} was calculated by taking untreated cells as maximum and no cells (beads only) as background using an Excel spreadsheet-based program.

VEGFR-2 Autophosphorylation and MAPK Phosphorylation in Human Umbilical Vascular Endothelial Cells (HUVECs) and NIH 3T3 VEGFR-2–Transfected Cells. Subconfluent HUVECs (ATCC or Cambrex) were cultured in growth factor deprived culture medium for 24 hours. Cells were serum starved by replacing media with basal media (EBM-2) containing 0.2% BSA for 1 hour. BAY 43-9006 was added to the cells with serum-free media for 1 hour followed by $VEGF_{165}$ treatment (final concentration of 30 ng/mL) for 10 minutes.

NIH 3T3 cells transfected with VEGFR-2 were obtained from Dr. Masabumi Shibuya (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and plated at 1×10^6 cells/well in 6-well tissue culture plates in DMEM, 10% fetal bovine serum, and 1.5 mg/mL G418. After 6 hours, culture medium was changed to 2 mL per well of 0.2% BSA/DMEM and incubated for 14 hours. Cells were preincubated with compound added in 0.1% BSA/PBS for 30 minutes followed by stimulation with 30 ng/mL $VEGF_{165}$ for 10 minutes. Cells were washed and lysed with buffer containing 0.3% Triton X-100 and protease inhibitors (Complete Protease Inhibitor Tablets). Twelve μg of protein from control and treated cell lysates were electrophoresed under reducing conditions and transferred onto nitrocellulose membranes. Blots were probed with anti-pVEGFR-2 (pTyr-1054 and pY1059) antibodies (PC460; Biosource, Inc., Camarillo, CA) or anti-VEGFR-2 antibody (sc-315; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed with HRP-conjugated secondary antibodies and developed with Amersham ECL reagent on Amersham Hyperfilm.

To monitor the effects of BAY 43-9006 on VEGF and basic fibroblast growth factor-dependent MAPK activation, exponentially growing human endothelial cells (HUVECs; Cambrex, East Rutherford, NJ) were seeded at 25,000 cells per well in 96-well plates in growth medium with (EBM-2 MV; Cambrex) and incubated at 37°C in 5% CO_2. Sixteen hours after plating, the cells were changed to serum-free RPMI 1640 containing 0.1% fatty acid-free BSA, and cells were preincubated at different concentrations of BAY43-9006. Cells were stimulated for 10 minutes with 50 ng/mL of either VEGF or basic fibroblast growth factor and processed for as described above for Bio-Plex pERK 1/2 immunoassay from tumor cell lysates.

PDGFR-β Autophosphorylation and Cell Proliferation in Human Aortic Smooth Muscle Cells (HAoSMCs). A total of 1×10^5 HAoSMCs (P3-P6; Clonetics) were plated in 12-well cluster plates in 1 mL volume per well of SGM-2 (Clonetics). Cells were rinsed the next day with D-PBS (Life Technologies, Inc.), then serum starved in 500 μL of smooth muscle cell basal media (Clonetics) with 0.1% BSA (Sigma) overnight. Diluted compounds ranged from 10 μmol/L to 1 nmol/L in 0.1% DMSO. Media was removed, and 100 μL of each dilution were added to cells for 1 hour at 37°C. Cells were then stimulated with 10 ng/mL PDGF BB ligand (Leinco) for 7 minutes at 37°C. The media was decanted and 150 μL of isotonic 25 mmol/L bicine pH 7.6 lysis buffer (M-PER from Pierce) with protease inhibitor tablet (Complete; EDTA-free), and 0.2 mmol/L Na vanadate were added. Cells were lysed, and 15 μL of agarose-conjugated anti-PDGFR-β antibody (sc-339; Santa Cruz Biotechnology) were added. Next day, beads were rinsed, boiled in 1× LDS sample buffer, run on 3 to 8% gradient Tris-Acetate gels (Invitrogen), and transferred onto nitrocellulose. Membranes were probed with anti-pPDGFR-β (Tyr^{857}) antibody (sc-12907; Santa Cruz Biotechnology) and then the secondary goat antirabbit HRP IgG (Amersham). Positive bands were visualized using enhanced chemiluminescence. Subsequently, membranes were stripped and reprobed with SC-339 (Santa Cruz Biotechnology) for total PDGFR-β.

The PDGF-dependent bromodeoxyuridine incorporation assay measures the ability of compounds to inhibit the induction of DNA synthesis by PDGF in serum-starved HAoSMCs. For the assay, HAoSMCs (4×10^3/well) were plated in complete SMBM media in 96-well tissue culture plates, incubated overnight, and then serum starved for 16 hours in SMBM containing 0.1% BSA (SF-SMBM). Fresh SF-SMBM was then added to the cells. Dilutions of BAY 43-9006 in SF-SMBM were added in a dose range from 10 μmol/L to 4.57 nmol/L 1 hour before the addition of 10 ng/mL PDGF-BB. Cells were incubated for 24 hours and processed using the BrdUrd ELISA kit from Amersham.

mVEGFR-3 and Human Flt-3 (ITD) Receptor Autophosphorylation Assays. The human embryonic kidney (HEK-293)-Flt-3 (ITD) cells (CRL-1573; American Type Cell Culture) were plated at 2.5 to 5×10^5 cells/well in 6-well plates (RPMI +10% FBS). The following day, cells were treated with inhibitors (3 μmol/L to 10 nmol/L) for 2 hours in serum-free RPMI media. Cells were lysed, and 10 μg of proteins per lane were loaded on 3 to 8% Tris-Acetate NuPAGE gels (Invitrogen). Gels were transferred to nitrocellulose membranes, blocked, probed with anti-pFlt-3 monoclonal antibody (3466; Cell Signaling), washed, and probed with secondary HRP-conjugated antimouse IgG (Amersham). Membranes were washed, developed with ECL Western blot detection reagent (Amersham), and exposed on Hyperfilm ECL film (Amersham). For total Flt-3 quantification, membranes were stripped with RESTORE buffer (Pierce) and blotted as described above using anti-Flt-3 polyclonal antibodies (sc-479; Santa Cruz Biotechnology) and HRP-conjugated antirabbit IgG (Amersham).

For mVEGFR-3 studies, HEK-293 cells were transiently transfected with pcDNA3.1 vector (Invitrogen) containing a full-length cDNA of murine VEGFR3. Two days after transfection, cells were exposed to test compounds (3 μmol/L to 10 nmol/L in 0.1% DMSO) for 30 minutes at 37°C. Cells were washed, lysed in Triton-lysis buffer, pelleted, and 10 μg of total protein were loaded on 3 to 8% Tris-Acetate NuPAGE gels (Invitrogen). Gels were transferred to nitrocellulose membranes, which were probed with anti-mVEGFR-3 (ADI) or anti-p-mVEGFR-3 (4G10; Upstate Biotechnology) antibodies and secondary HRP-conjugated antimouse or antirabbit IgG, respectively (Amersham).

Tumor Cell Proliferation

Tumor cells were trypsinized and plated in 96-well plates at 3000 cells per well in complete media with 10% FCS. Cells were incubated overnight at 37°C, and the next day, compounds were added in complete growth media over a final concentration range of 10 μmol/L to 10 nmol/L in 0.1% DMSO. Cells were incubated with test compounds for 72 hours at 37°C in complete growth media, and cell number was quantitated using the Cell TiterGlo ATP Luminescent assay kit (Promega). This assay measures the number of viable cells per well by measurement of luminescent signal based on amount of cellular ATP.

Tumor Xenograft Experiments

Female NCr-*nu/nu* mice (Taconic Farms, Germantown, NY) were used for all studies. The mice were housed and maintained in accordance with Bayer Institutional Animal Care and Use Committee and state and federal guidelines for the humane treatment and care of laboratory animals and received food and water *ad libitum*.

Tumors for all but the DLD-1 model were generated by harvesting cells from mid-log phase cultures using Trypsin-EDTA (Invitrogen, Inc.). Three to five million cells were injected s.c. into the right flank of each mouse. DLD-1 tumors were established and maintained as a serial *in vivo* passage of s.c. fragments (3 × 3 mm) implanted in the flank using a 12-gauge trocar. A new

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generation of the passage was initiated every three weeks, and studies were conducted between generations 3 and 12 of this line.

Treatment was initiated when tumors in all mice in each experiment ranged in size from 75 to 144 mg for antitumor efficacy studies and from 100 to 250 mg for studies of microvessel density and ERK phosphorylation. All treatment was administered orally once daily for the duration indicated in each experiment. Tumor weight was calculated using the equation length $\times$ (width)^2)/2. Treatments producing >20% lethality and/or 20% net body weight loss were considered toxic.

Detection of Tumor Microvessels and Activated ERK 1/2

Immunostaining of paraffin sections of tumors with murine anti-CD31 antibodies was performed on the Dako Autostainer, model LV (DakoCytomation). Sections were deparaffinized and hydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2. Antigen retrieval was performed using Dako Target Retrieval Solution (S1699, DakoCytomation). Sections were blocked with an avidin/biotin block (Vector Laboratories) and rabbit serum. Immunostaining was performed using a goat Vectastain ABC elite kit (Vector Laboratories) and 3,3′-diaminobenzidine as the chromagen (DakoCytomation), according to the manufacturer's protocol with the following modifications: (*a*) 2 $\times$ 30-minute incubations were performed with primary antibody; and (*b*) after incubation with the secondary antibody, the slides were rinsed in buffer, distilled water, and then buffer again. Sections were incubated with anti-CD31 antibodies [PECAM-1 (M-20) SC-1506 goat polyclonal; Santa Cruz Biotechnology] diluted 1:750 in Dako Antibody Diluent (DakoCytomation, Carpinteria, CA) or goat IgG (1:750; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as a negative control. Sections were counterstained with Mayer's hematoxylin for 1 minute and washed with water.

Immunohisotchemical localization of activated ERK 1/2 was determined from paraffin sections. Sections were then placed in heated (95°C) 0.1M citrate buffer (pH 6.0) for 35 min, brought to RT for 30 min, and then blocked with 1.5% H_2O_2. Sections were stained using primary pERK 1/2 antibody (phospho-p44/42 Cell Signaling) diluted 1:100 with Dako Antibody Diluent. Staining was performed using the Envision Plus HRP (3,3′-diaminobenzidine) System from Dako (4011) according to the manufacturer's protocol. The slides were counterstained with Mayer's hematoxylin for 1 min and washed with water.

Quantification of Microvessels

Histologic slides were blind-coded during the assessment. The tissue sections were viewed at $\times$100 magnification ($\times$10 objective lens and $\times$10 ocular lens; 0.644 mm^2 per field). Tissue image was captured with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Four fields per section were randomly analyzed, excluding peripheral surrounding connective tissues and central necrotic tissues. Total tissue area analyzed in each section was 2.576 mm^2.

Area and number of CD31 positive objects were quantified using the software ImagePro Plus version 3.0 (Media Cybernetics, Silver Spring, MD). Percentage of microvessel area (MVA, %) in each field was calculated as [(area of CD31-positive objects/measured tissue area) $\times$ 100%]. Microvessel density (MVD, number/mm^2) in each field was calculated as (number of CD31-positive objects/ 0.644 mm^2). Mean values of MVA and MVD in each group were calculated from

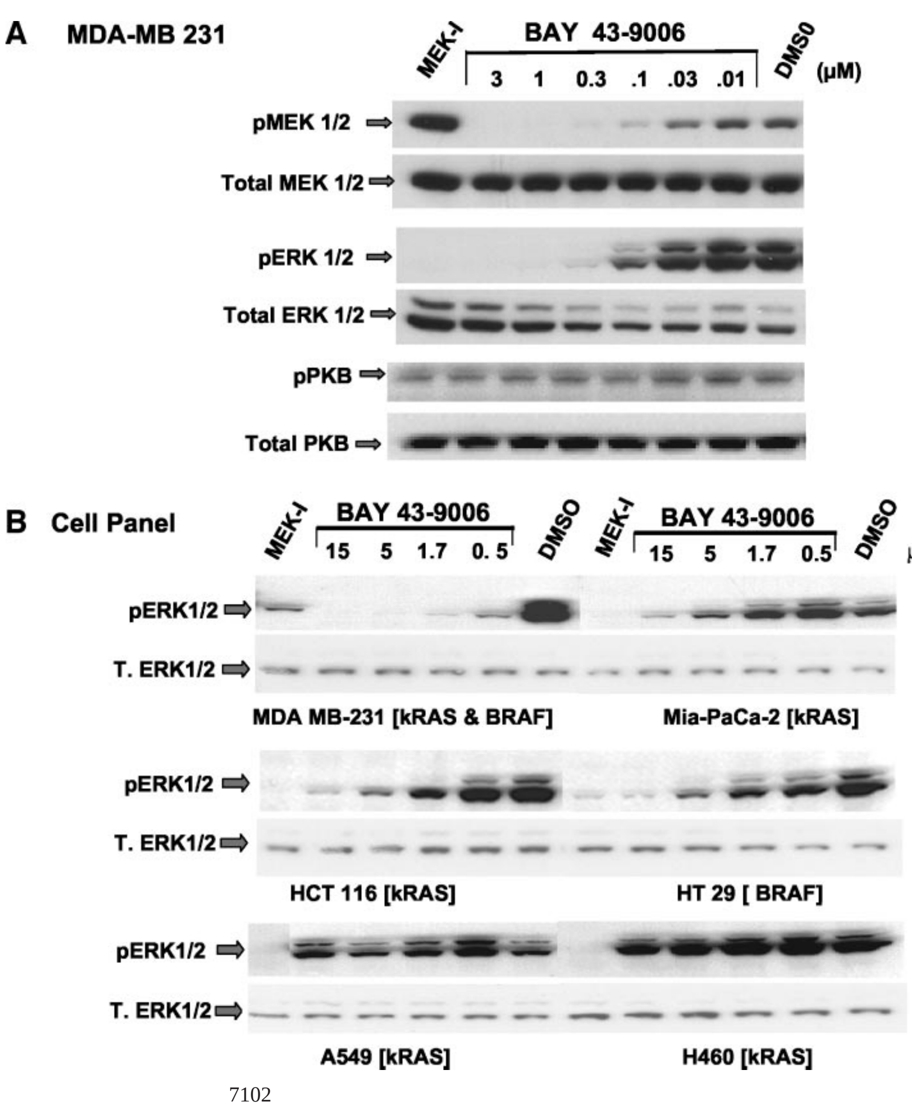

Fig. 1. BAY 43-9006 inhibits activation of the RAF/MEK/ERK pathway in most but not all human tumor cell lines. *A.* MDA-MB-231 cells were incubated with various concentrations of BAY 43-9006 or DMSO. Cell lysates were subjected to Western blot analysis for phosphorylated (p) and total (T) MEK 1/2 (*top box*), ERK 1/2 (*middle box*), and PKB (*bottom box*). Changes in total MEK 1/2, ERK 1/2 levels, or PKB were not observed. The MEK inhibitor U0126 (10 μmol/L) was used in all Western blot experiments as a control for detecting RAF/MEK/ERK pathway inhibition (*Lane 1:* MEK-1). *B.* Subconfluent human tumor cells were incubated with BAY 43-9006 for 2 hours, lysed, and processed for Western blotting. Activated ERK 1/2 was detected with anti-pERK antibodies. Changes in total ERK 1/2 levels were not observed. U0126 was used at 10 μmol/L (*Lane 1:* MEK-1).

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four tumor samples. Data were analyzed statistically with one-way ANOVA followed by Fisher's PLSD (StatView, version 4.5; Abacus Concepts, Inc., Berkeley, CA). $P < 0.05$ was considered significant.

RESULTS

BAY 43-9006 Inhibition of the MAPK Pathway. BAY 43-9006 is a synthetic molecule that can be broadly defined as a bi-aryl urea (Table 1), which was originally identified through inhibition of Raf-1 kinase biochemical and cellular mechanistic assays (27, 32). BAY 43-9006 was additionally profiled against wt BRAF and V599E mutant BRAF (Table 1). Biochemical assays were performed in which varying concentrations of BAY 43-9006 were tested for the capacity to inhibit MEK-1 phosphorylation by the catalytic domains of Raf-1, BRAF, and V599E BRAF. As shown in Table 1, BAY 43-9006 potently inhibited Raf-1 (IC_{50}, 6 nmol/L), wt BRAF (IC_{50}, 22 nmol/L), and V599E mutant BRAF (IC_{50}, 38 nmol/L) but did not significantly inhibit MEK-1 or ERK-1 activity (IC_{50}, >10,000 nmol/L).

The ability of BAY 43-9006 to block activation of the MAPK pathway was examined by measuring ERK 1/2 phosphorylation in several tumor cell lines by Western blot analysis or Bio-Plex pERK immunoassay. Genotyping of each cell line revealed mutations in KRAS (Mia PaCa 2, HCT 116, A549, and NCI-H460), V599E BRAF (LOX melanoma, HT-29), or both KRAS and G463V BRAF (MDA-MB-231), suggesting that transformation of these cells is driven, in part, by disruption of the MAPK pathway. Results show that BAY 43-9006 inhibits ERK phosphorylation in most of these cell lines, independent of which mutation caused aberrant activation of the RAS/RAF pathway (Fig. 1).

In MDA-MB-231 breast cancer cells, BAY 43-9006 completely blocked activation of the MAPK pathway (Fig. 1*A*). Cells were preincubated with BAY 43-9006 at concentrations ranging from 0.01 to 3 μmol/L, and dose-dependent inhibition of basal MEK 1/2 and ERK 1/2 phosphorylation (IC_{50}, 40 and 100 nmol/L, respectively) was observed (Fig. 1*A*). BAY 43-9006 had no effect on the PKB pathway in MDA-MB-231 cells, demonstrating selectivity for inhibition of the MAPK, but not the PKB, pathway in these cells (Fig. 1*A*).

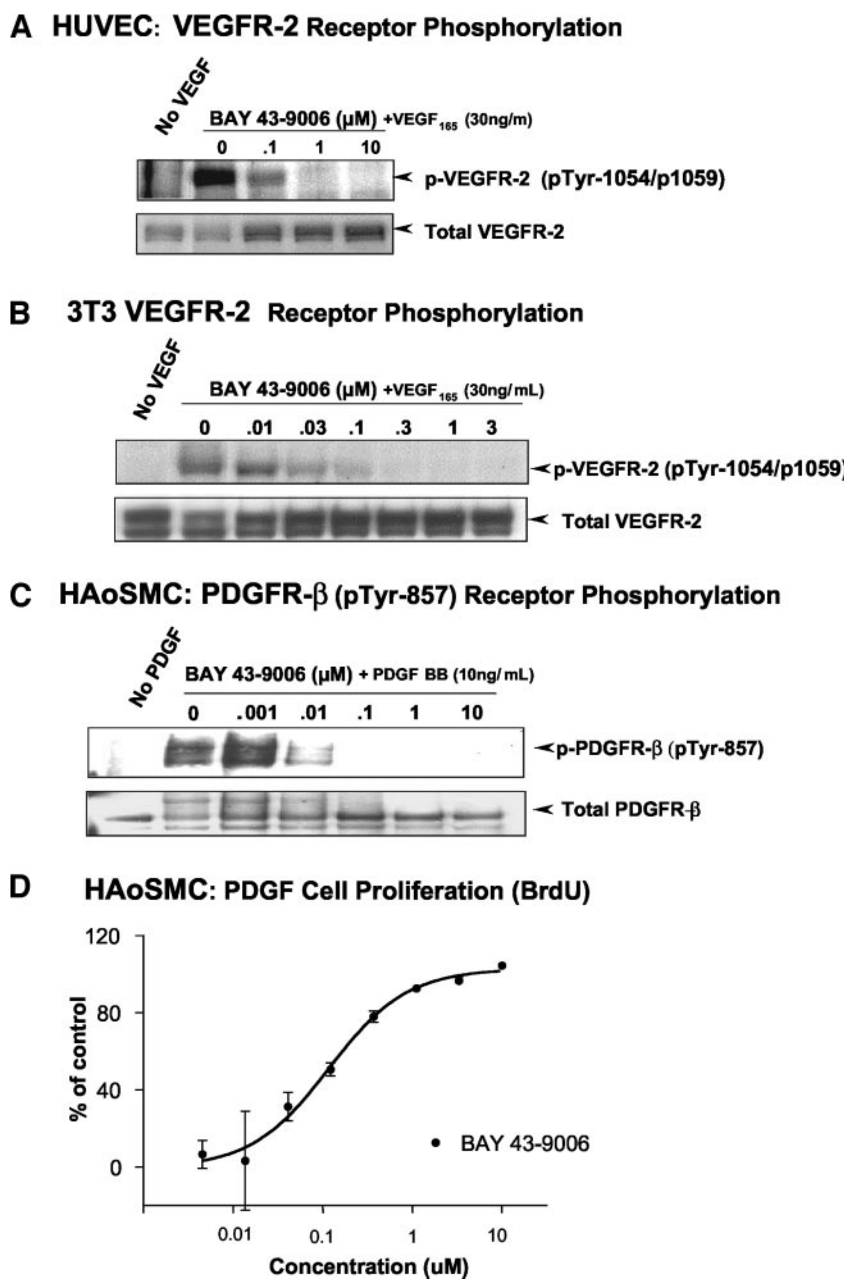

Fig. 2. BAY 43-9006 targets receptor tyrosine kinases (VEGFR-2 and PDGFR-β) involved in tumor angiogenesis. *A*, VEGF-stimulated VEGFR-2 autophosphorylation in HUVECs. *B*, VEGF-stimulated VEGFR-2 autophosphorylation in NIH 3T3 VEGFR-2 cells. *C*, PDGF-BB–stimulated PDGFR-β phosphorylation in HAoSMCs. *D*, PDGF-BB–stimulated bromodeoxyuridine (BrdUrd) proliferation assay in HAoSMCs.

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BAY 43-9006 inhibition of pERK was also observed for the human pancreatic (Mia PaCa 2) and colon (HCT 116 and HT-29) tumor cell lines by Western blot analysis (Fig. 1*B*) and in the human LOX melanoma and pancreatic BxPC-3 cell lines using the Bio-Plex pERK immunoassay (Table 1). However, BAY 43-9006, at concentrations as high as 10 μmol/L, had no effect on inhibition of ERK 1/2 phosphorylation in the two NSCLC cell lines (Fig. 1*B*).

BAY 43-9006 Targets Receptor Tyrosine Kinases Involved in Tumor Progression and Angiogenesis. Additional characterization of BAY 43-9006 in biochemical assays demonstrated potent inhibition of several RTKs, including human and murine VEGFR-2 (IC_{50}, 90 and 15 nmol/L, respectively), mVEGFR-3 (IC_{50}, 20 nmol/L), mPDGFR-β (IC_{50}, 57 nmol/L), Flt-3 (IC_{50}, 58 nmol/L), c-KIT (IC_{50}, 68 nmol/L), and FGFR-1 (IC_{50}, 580 nmol/L; Table 1). By contrast, EGFR, IGFR-1, c-met, and HER-2 RTKs were not inhibited by BAY 43-9006 (IC_{50}, >10,000 nmol/L). Other kinases tested included PKB, PKA, cdk1/cyclinB, PKCα, PKCγ, and pim-1, which were all insensitive to inhibition by BAY 43-9006 (Table 1).

Inhibition of VEGFR-2 autophosphorylation by BAY 43-9006 was examined in two cell culture systems, HUVECs, and NIH 3T3–VEGFR-2 cells (28). Inhibition of VEGFR-2 autophosphorylation by BAY 43-9006 is shown in Fig. 2, *A* and *B*. Consistent with previous reports (29), VEGFR-2 expression was detected as a doublet at Mr ~200,000 in HUVEC cell lysates. $VEGF_{165}$ dramatically induced VEGFR-2 autophosphorylation, which was blocked by BAY 43-9006 (Fig. 2). At 100 nmol/L BAY 43-9006, >50% inhibition of VEGFR-2 phosphorylation was observed (Fig. 2*A*). As shown in Table 1, ERK 1/2 phosphorylation, determined by the Bio-Plex pERK immunoassay, was also substantially inhibited by BAY 43-9006 in HUVECs stimulated with either VEGF (IC_{50}, 60 nmol/L). Concentration-dependent inhibition of VEGFR-2 phosphorylation in NIH 3T3–VEGFR-2 cells was also detected (IC_{50}, ~30 nmol/L; Fig. 2*B*). Consistent with the biochemical data, these findings indicate that BAY 43-9006 is a potent inhibitor of VEGFR-2 signaling in cells.

To determine whether BAY 43-9006 can block activation of other proangiogenic RTKs in cell culture, we induced PDGFR-β autophosphorylation in primary HAoSMCs (Fig. 2*C*). Addition of varying concentrations of BAY 43-9006 inhibited PDGFR-β autophosphorylation (Fig. 3*C*) and PDGF-stimulated HAoSMC proliferation (IC_{50}, 280 nmol/L; Fig. 3*D*). Two other RTKs, mVEGFR-3 and Flt-3 (human ITD), were also sensitive to inhibition of receptor phosphorylation by BAY 43-9006 in cell-based assays (Table 1).

Antitumor Activity of BAY 43-9006 in Human Xenografts. Fig. 3 illustrates the spectrum of antitumor efficacy of orally administered BAY 43-9006 in a representative panel of tumor xenograft models. Mice bearing 75 to 150 mg tumors were treated orally with BAY 43-9006 at dose levels of 7.5 to 60 mg/kg, administered daily for 9 days. In each model, BAY 43-9006 produced dose-dependent tumor growth inhibition with no evidence of toxicity, as measured by increased weight loss relative to control animals or drug-related lethality.

During treatment with 30 to 60 mg/kg BAY 43-9006, complete tumor stasis in the HT-29, Colo-205, and DLD-1 human colon tumor models and the A549 NSCLC model was observed (Fig. 3). Each of these tumor lines expresses a mutation in either *KRAS* or *BRAF* that would constitutively activate signaling through the RAF/MEK/ERK pathway. In another NSCLC model (NCI-H460) tumor growth was inhibited, but complete stasis during treatment was not achieved at doses up to 60 mg/kg. The NCI-H460 model also contains an activating mutation of the *KRAS* gene, suggesting that the presence of a genetic alteration that stimulates signaling through the RAF/MEK/

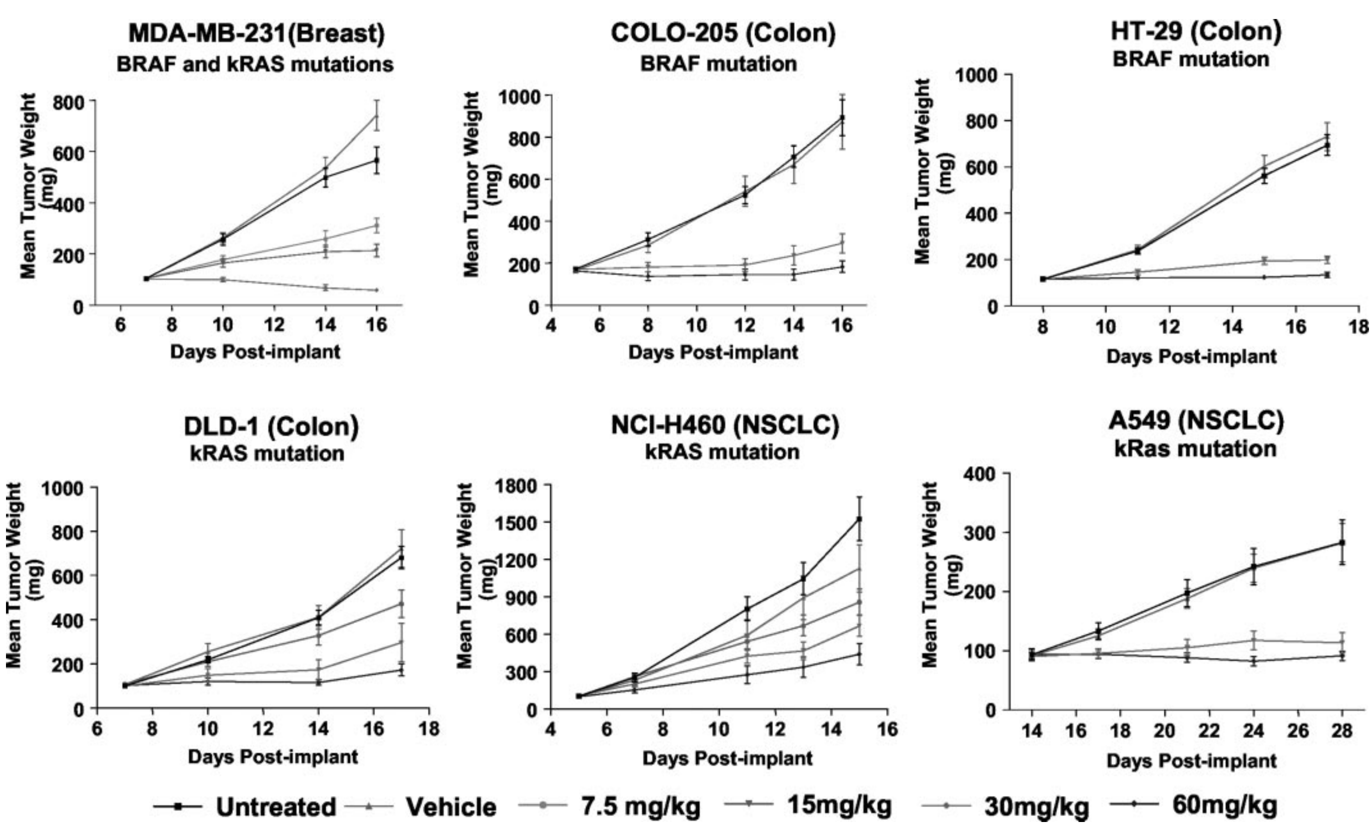

Fig. 3. BAY 43-9006 demonstrates broad oral antitumor efficacy in panel of human tumor xenograft models. MDA-MB-231, Colo-205, HT-29, DLD-1, NCI-H460, and A549 tumor cells were implanted sc in the flank of athymic mice as described in Materials and Methods. Treatment in each experiment was initiated on the day shown when all mice had tumors ranging in size from 75 to 150 mg. BAY 43-9006 was given orally at 7.5 to 60 mg/kg, qd×9. There was no lethality and no increase in weight loss in any treated group relative to the corresponding control group. Daily oral administration of BAY 43-9006 at 30 to 60 mg/kg produced complete tumor stasis during treatment in five of the six models.

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ERK pathway is not sufficient to predict sensitivity to therapy with BAY 43-9006. As illustrated in Fig. 2, the level of ERK 1/2 phosphorylation was not reduced after exposure to BAY 43-9006 in either the A549 or NCI-H460 NSCLC tumor lines. The antitumor efficacy of BAY 43-9006 against these two xenograft models may be due to inhibition of RTKs involved in angiogenesis (see below). Of the tumor models examined, the MDA-MB-231 breast tumor model was the most sensitive to BAY 43-9006. A dose level of 30 mg/kg produced a 42% reduction in the mean size of these tumors after only 9 days of treatment (Fig. 3).

In all, BAY 43-9006 demonstrated robust antitumor efficacy in xenograft tumor models of human colon, lung, breast, ovarian, pancreatic, and melanoma origin (Fig. 3 and data not shown). Thus, BAY 43-9006 exhibits broad-spectrum efficacy against histologic types of human cancers that encompass mutations in certain genes (*KRAS* and *BRAF*) controlling signaling in the pathways inhibited by BAY 43-9006.

Correlation between Antitumor Activity and Inhibition of the MAPK Pathway. The association between antitumor activity and inhibition of the RAF/MEK/ERK pathway was determined in HT-29, Colo-205, and MDA-MB-231 tumor xenografts. In parallel to the antitumor efficacy studies described above, additional groups of four mice bearing 100 to 200 mg tumors were treated orally with vehicle or 30 to 60 mg/kg BAY 43-9006, administered daily for 5 days, which is the shortest treatment duration producing complete tumor stasis in the treated groups. In mechanism of action studies, tumors were collected on day 5 3 hours after administration of either BAY 43-9006 or vehicle (Figs. 4–6). As shown in Fig. 4 by Western blot analysis (Fig. 4*B*) and immunohistochemistry (Fig. 4*C*), pERK levels were reduced in HT-29 tumors obtained from animals treated with 30 or 60 mg/kg BAY 43-9006 compared with untreated or vehicle-treated mice. No differences were observed for total ERK levels (Fig. 4*B*). Similar inhibition of MEK 1/2 phosphorylation was observed in HT-29 tumor lysates by Western blotting (data not shown).

Similar results were observed with MDA-MB-231 xenografts (Fig. 5), which also showed significant tumor necrosis in BAY 43-9006-treated mice as visualized by hematoxylin staining at day 5 after initiation of drug treatment (Fig. 5C). Parallel immunohistochemistry experiments revealed that pERK 1/2 levels were substantially reduced in MDA-MB-231 tumors with BAY 43-9005 treatment (Fig. 5*D*). In addition, Ki-67 staining, a marker of cell proliferation, was significantly reduced in MDA-MB-231 tumors obtained from BAY 43-9006–treated animals (data not shown).

Interestingly, BAY 43-9006 inhibition of MAPK activation could be dissociated from inhibition of tumor growth in the Colo-205 colon xenograft model. Although tumor growth was delayed in Colo-205 colon tumor xenografts (Figs. 3 and 6), no significant effect on inhibition of ERK 1/2 phosphorylation was detected in treated Colo-205 tumors either by Western blotting (Fig. 6*B*) or immunohistochemistry (Fig. 6*C*), indicating that the MAPK pathway was not blocked by BAY 43-9006 administration. BAY 43-9006 did block activation of the MAPK pathway in Colo-205 (data not shown) and HT-29 (Fig. 1*B*) cell lines, which contain the V599E mutation; it is therefore possible that an alternative pathway other than the RAF/MEK/ERK cascade activates ERK 1/2 in Colo-205 tumors.

Antiangiogenic Activity of BAY 43-9006 in Human Tumor Xenografts. The experiments above demonstrated that BAY 43-9006 inhibits ERK activation in most xenograft tumors and that BAY 43-9006 inhibits growth of human breast, lung, and colon tumor xenografts. As shown in Table 1 and Fig. 2, BAY 43-9006 also exhibits significant activity against RTKs known to promote angiogenesis. For this reason, we evaluated tumor MVA and MVD in the

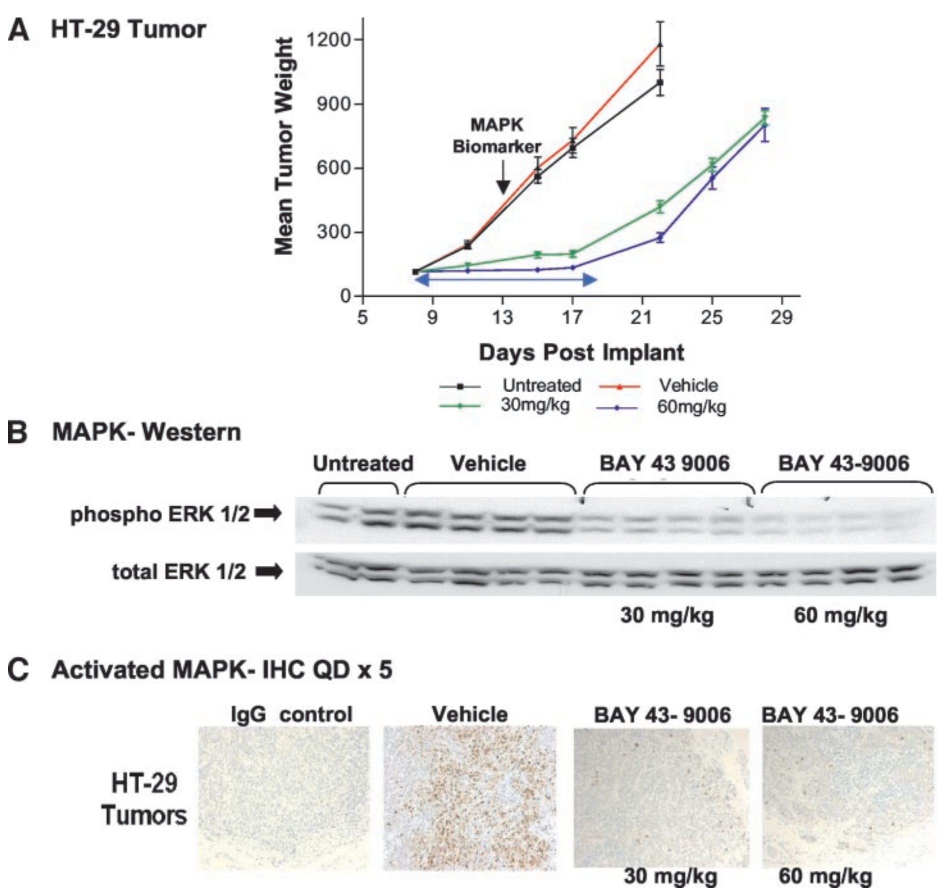

Fig. 4. Association between inhibition of HT-29 tumor growth and the MAPK pathway by BAY 43–9006. *A*, inhibition of HT-29 tumor growth by BAY 43-9006. Female NCr mice were implanted sc with 5×10^6 HT-29 cells. BAY 43-9006 was administered orally at 30 or 60 mg/kg, qd×9, starting on day 8 when all mice had 75 to 150-mg tumors. *B* and *C*. Additional groups of four mice bearing 100 to 200-mg tumors were treated with BAY 43-9006 orally qd×5 at 30 or 60 mg/kg. *B*. Western blot analysis using anti-pERK and anti-ERK antibodies demonstrate that BAY 43-9006 inhibits RAF/MEK/ERK pathway activation in HT-29 human tumor xenografts. *C*. Immunohistochemistry with anti-pERK 1/2 antibodies was performed on paraffin sections of HT-29 human tumor xenografts from vehicle or BAY 43-9006–treated mice and showed reduced staining in tumors obtained from BAY 43-9006 animals.

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same tumor samples described above (Figs. 4 and 6). As shown in Fig. 7, *A* and *B*, daily oral administration of 30 or 60 mg/kg BAY 43-9006 produced 50 to 80% inhibition of MVA and MVD in the drug treated relative to vehicle-treated HT-29 tumors, which was significantly greater than MVA and MVD inhibition in vehicle-treated animals. Interestingly, a reduction in both MVA and MVD was observed for Colo-205 tumors, despite a lack of MAPK inhibition in the same tumor samples (Figs. 6 and 7). BAY 43-9006 treatment was also associated with MVA and MVD inhibition in MDA-MB-231 tumors, demonstrating that angiogenesis was significantly inhibited in this tumor type (Fig. 5*A*).

DISCUSSION

There is a significant unmet medical need for the development of effective therapies that can stabilize or slow the progression of solid tumors. In this article, we identify BAY 43-9006 as a novel, orally active, dual action RAF kinase and VEGFR inhibitor that targets tumor cell proliferation and tumor angiogenesis. Biochemical assays described here demonstrated that BAY 43-9006 inhibits Raf-1 (IC_{50}, 6 nmol/L), wt BRAF (IC_{50}, 22 nmol/L), and V599E mutant BRAF (IC_{50}, 38 nmol/L), as well as the split kinase family: VEGFR-2 (IC_{50}, 90 nmol/L), VEGFR-3 (IC_{50}, 20 nmol/L), PDGFR-β (IC_{50}, 57 nmol/L), c-KIT (IC_{50}, 68 nmol/L), and Flt3 (IC_{50}, 58 nmol/L). Furthermore, BAY 43-9006 is not active against ERK-1, MEK-1, EGFR, HER-2, IGFR-1, c-met, c-yes, PKB, PKA, cdk1/cyclinB, PKCα, PKCγ, and pim-1. The bi-aryl urea BAY 43-9006 was recently co-crystallized in complex with both wt and oncogenic V599E BRAF kinase (33). The structure revealed that the distal pyridyl ring of BAY 43-9006 directly interacts with three amino acids within the ATP adenine binding pocket and that the urea moiety forms several hydrogen bonds with the enzyme. BAY 43-9006 appears to promote formation of the inactive conformation of BRAF similar to that reported for the structure of c-Abl STI-571 complex (33).

As a potent inhibitor of RAF kinase, BAY 43-9006 inhibited ERK 1/2 phosphorylation, an indicator of MAPK pathway blockade, in most, but not all, tumor cell lines examined, while having no effect on inhibition of the PKB pathway. Sensitivity to BAY 43-9006 treatment varied depending on the cell line tested. MDA-MB-231 human breast carcinoma cells were the most sensitive cell line identified for inhibition of the MAPK pathway by BAY 43-9006 (IC_{50}, 90 nmol/L). Several other cell lines responded to BAY 43-9006 treatment, including the LOX human melanoma (IC_{50}, 880 nmol/L), BxPC3 human pancreatic (IC_{50}, 1200 nmol/L), and the HCT 116, DLD-1, and Colo-205 human colon carcinoma cells (IC_{50}s ranging between 2000 and 4000 nmol/L). However, NCI-H460 and A549 (expressing mutant KRAS) NSCLC cells were insensitive to inhibition of ERK 1/2 phosphorylation by BAY 43-9006. The reason for this observation is not clear but could be due to RAF-independent MEK activation

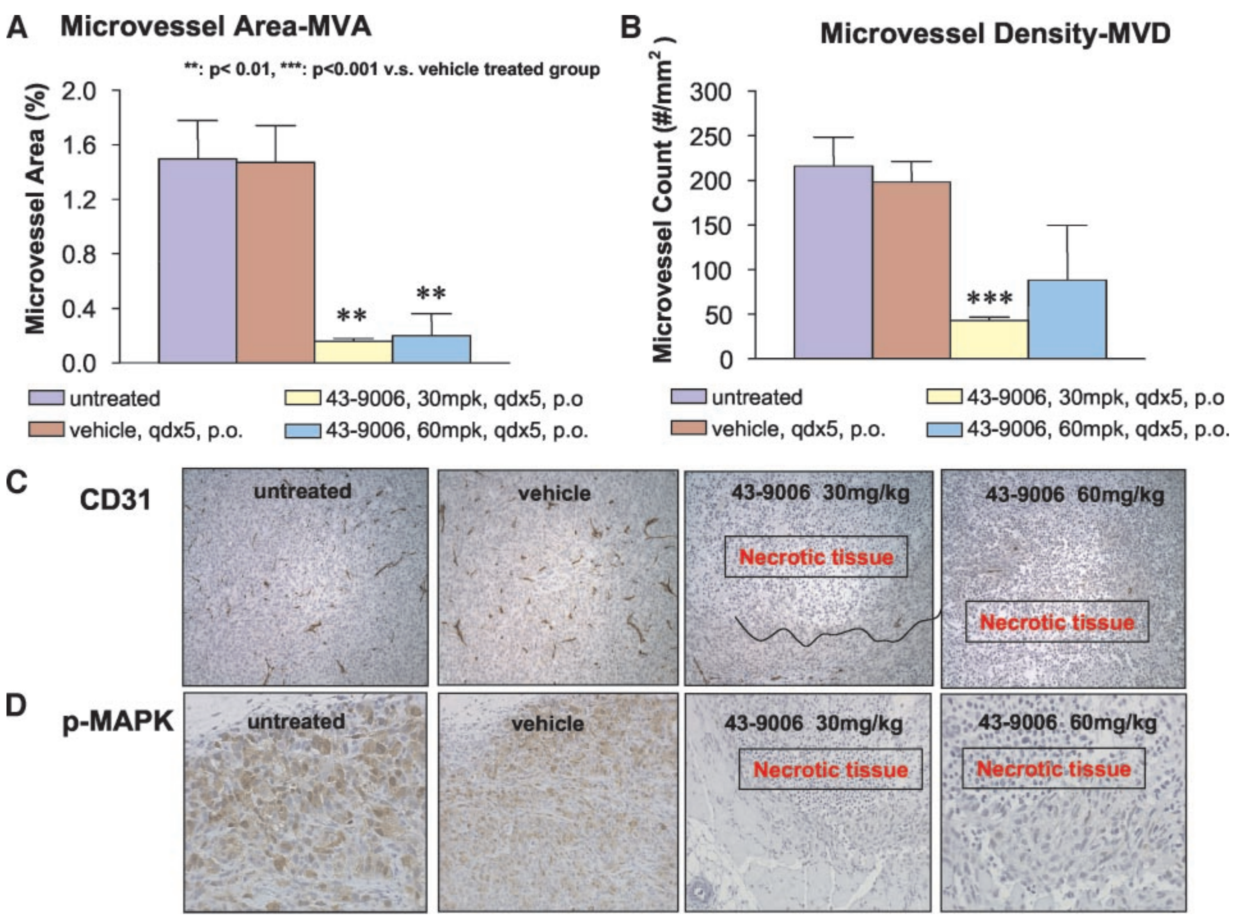

Fig. 5. BAY 43-9006 significantly inhibits tumor MVD and MVA and MAPK activation in MDA MB-231 tumor xenografts. Mice with 100 to 200 mg of MDA-MB-231 tumors were treated for 5 days with either vehicle or oral BAY 43-9006 at 30 or 60 mg/kg. Tumors were collected 3 hours after the last dose of BAY 43-9006. Sections of tumors were stained with anti-CD31 antibodies. *A* and *B*. The number and area of microvessels were measured, and percent area and density of microvessel were calculated in each group. Data were presented as mean ± SE (four samples per group). *C*. CD31-positive objects were showed in the tumor tissue (*brown-color objects*). Necrotic areas are highlighted in drug treated samples and were visualized by hematoxylin staining at day 5 after initiation of drug treatment. *D*. pERK 1/2 was reduced in MDA-MB-231 tumors obtained from animals treated with BAY 43-9006.

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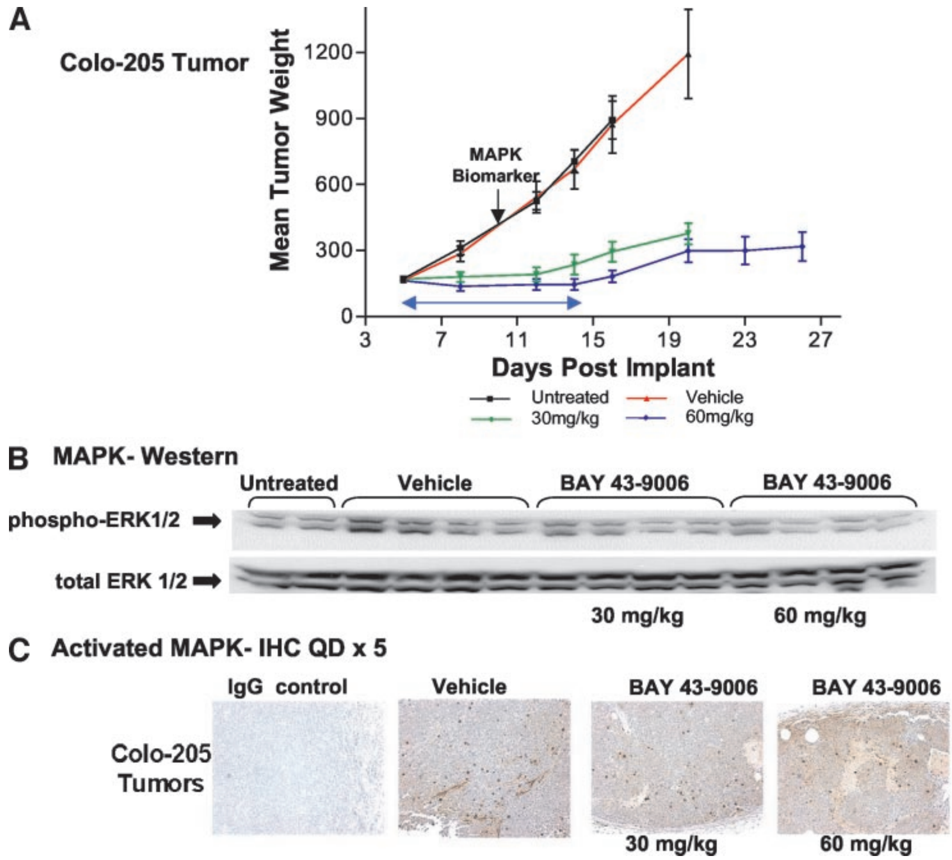

Fig. 6. Dissociation between inhibition of Colo-205 tumor growth and the MAPK pathway by BAY 43-9006. *A*, inhibition of Colo-205 tumor growth by BAY 43-9006. Female NCr mice were implanted sc with 3×10^6 Colo-205 cells. BAY 43-9006 was administered orally at 30 or 60 mg/kg, qd×9 starting on day 5 when all mice had 100 to 150-mg tumors. *Blue line* indicates BAY 43-9006 treatment. *B* and *C*. Additional groups of four mice bearing 150 to 250-mg tumors were treated with oral BAY 43-9006 qd×5 at 30 or 60 mg/kg. Western blots of tumor lysates (*B*) and paraffin sections (*C*) were stained with polyclonal anti-pERK antibodies. Treatment with BAY 43-9006 inhibited tumor growth without substantially reducing MAPK activation.

because a reference MEK inhibitor (U0126) blocked MAPK activation in these cell lines (Fig. 1*B*).

Previous reports have shown that BAY 43-9006 inhibits cellular proliferation of HCT 116 and Mia PaCA 2 cells (27). Here, we show that BAY 43-9006 blocks ERK 1/2 phosphorylation in MDA-MB-231 cells and inhibits MDA-MB-231 cellular proliferation (IC_{50}, 2600 nmol/L). However, this proliferation IC_{50} was >10-fold higher than that observed for inhibition of ERK 1/2 phosphorylation in these cells (see Table 1). One possibility for this difference might be due to BAY 43-9006 binding to serum proteins in the cell proliferation assays because the cellular ERK 1/2 phosphorylation assays were performed in low protein containing media (0.1% BSA). Indeed, addition of serum to MDA-MB-231 cells assayed for ERK 1/2 phosphorylation resulted in a 7-fold increase in the IC_{50} for ERK 1/2 inhibition (IC_{50}, 630 nmol/L). The inhibition of ERK1/2 phosphorylation and tumor shrinkage in MDA-MB-231 tumors indicates clearly that this cell type responds to BAY 43-9006 via inhibition of MAPK signaling.

In addition to inhibiting members of the RAF kinase family, we show here that BAY 43-9006 exhibits potent inhibition of RTKs that play a role in angiogenesis. In cell-based assays, BAY 43-9006 blocked autophosphorylation of VEGFR-2, VEGFR-3, PDGFR-β, Flt-3, and c-KIT. In HUVEC endothelial cells, BAY 43-9006 inhibited both VEGFR-2 autophosphorylation and ERK 1/2 phosphorylation. Interestingly, because VEGF induction of angiogenesis requires RAS activation (9), BAY 43-9006 inhibition of VEGF signaling can occur both at the level of the VEGFR-2 receptor and later in the signaling cascade through inhibition of the RAF/MEK/ERK pathway. In addition, blockade of PDGFR-β activation with BAY 43-9006 treatment resulted in inhibition of PDGFR-stimulated HAoSMC proliferation.

In general, inhibition of cellular autophosphorylation of the split kinase receptor tyrosine kinases, VEGFR-2, VEGFR-3, PDGFR-β, and Flt-3 (see Table 1) occurred at significantly lower drug concentrations (20 to 100 nmol/L) than observed for inhibition of the RAF/MEK/ERK pathway (90 to 4000 nmol/L) in tumor cells. The reason for this difference is not clear. One possibility is that inhibition of multiple RAF isoforms is necessary for inhibition of the RAF/MEK/ERK pathway. This is supported by the report that knocking out both Raf-1 and BRAF expression is required to completely inhibit MAPK activation upon B cell receptor activation (34) and the observation that there is no inhibition of growth factor-mediated MAPK activation in mouse embryonic fibroblasts from Raf-$1^{-/-}$ embryos (18).

BAY 43-9006 demonstrated broad spectrum, dose-dependent antitumor activity against preclinical xenograft models representing human colon, lung, breast, ovarian, pancreatic, and melanoma cancers (27, 35). BAY 43-9006 acts predominantly to prevent the growth of tumors evaluated in preclinical studies. Only the MDA-MB-231 model showed evidence of tumor regressions after 9 days of oral dosing of BAY 43-9006 ≤ the 30 mg/kg dose level. Because treatment with BAY 43-9006 is well tolerated, it is possible that increasing the duration of therapy would have produced increased numbers of tumor regressions in this model.

Aberrant proliferation is likely driven by expression of mutant KRAS or BRAF in certain cell lines. MDA-MB-231 cells, which were the most sensitive to BAY 43-9006 treatment in cell culture and in the xenograft experiments, contain two activating mutations of the MAPK pathway, one each in the *KRAS* and *BRAF* genes. The presence of these activating mutations might provide a selective proliferative advantage to these cells associated with a greater dependence on signaling through the RAF/MEK/ERK pathway for survival. Because BAY 43-9006 blocks activation of this pathway, it is possible that the greater sensitivity of this tumor type to BAY 43-9006 treatment is because BAY 43-9006 inhibits both of these mutations. Indeed, BAY 43-9006 inhibited MDA-MB-231 tumor cell proliferation and also

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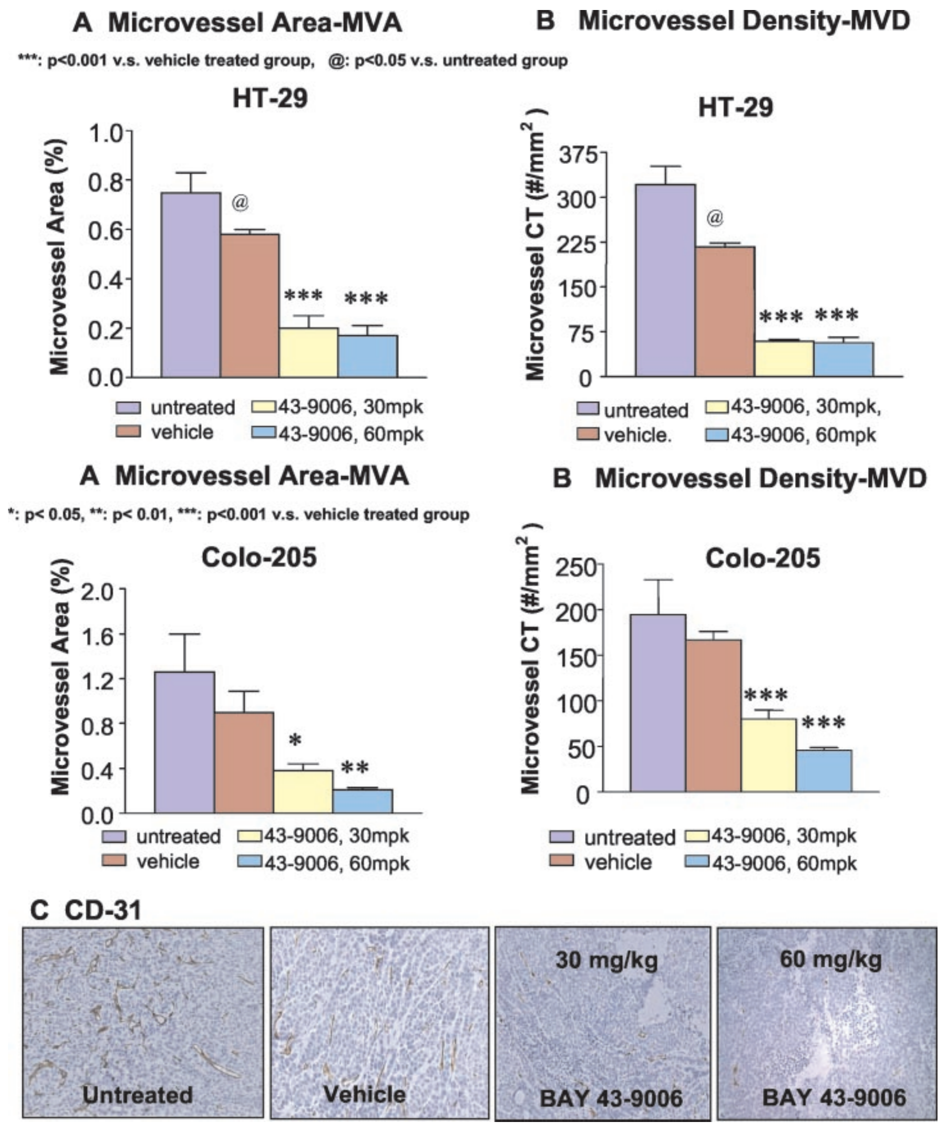

Fig. 7. BAY 43-9006 significantly inhibits tumor MVD and MVA in HT-29 and Colo-205 tumor xenografts. Mice with 150–250 mg HT-29 or Colo-205 tumors were treated for 5 days with either vehicle or oral BAY 43-9006 at 30 or 60 mg/kg. Tumors were collected, sectioned, and stained with anti-CD31 antibodies. *A* and *B*. The number and area of microvessels, indicated by CD31-positive objects in either HT-29 or Colo-205 tumors, were quantified, and percent area and density of microvessel were calculated in each group. Data are presented as mean ± SE (four samples per group). *C*. Detection of CD31-positive objects in Colo-205 tumor tissue was apparent in tumors obtained from untreated and vehicle-treated animals (left two pictures) but was substantially reduced in BAY 43-9006 treated tumors (right two pictures).

promoted cell death, as evidenced by extensive tumor cell necrosis (Fig. 5) seen as early as day 5 after initiation of drug treatment.

One important goal of this investigation was to define the mechanism of action of BAY 43-9006 *in vivo*. In the HT-29 colon tumor, BAY 43-9006 induced tumor growth inhibition that was correlated with inhibition of ERK phosphorylation (Fig. 4). BAY 43-9006 treatment was also associated with significant inhibition (50 to 80%) of HT-29 tumor neovascularization (Fig. 7), indicating that inhibition of HT-29 tumor growth may have been mediated by both inhibition of the MAPK pathway and inhibition of tumor angiogenesis. In contrast, ERK phosphorylation was not affected by BAY 43-9006 treatment in the Colo-205 colon tumor model. However, BAY 43-9006 treatment did inhibit Colo-205 tumor growth *in vivo*, which suggests that tumor growth inhibition in this model is the result of decreased tumor angiogenesis as demonstrated by the reduction of CD31 staining. Taken together, these results support the likelihood that BAY 43-9006 affects tumor growth by blocking two cellular processes essential for tumor development: tumor cell proliferation and tumor angiogenesis.

These data support the development of BAY 43-9006 as a novel, dual action RAF kinase and VEGFR inhibitor targeting both the RAF/MEK/ERK pathway and receptor tyrosine kinases that promote angiogenesis. Currently, BAY 43-9006 is being tested in phase III human clinical trials for renal cell carcinoma and in phase II clinical trials across multiple tumor types.

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