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Inhibition of Tumor Endothelial ERK Activation, Angiogenesis, and Tumor Growth by Sorafenib (BAY43-9006)

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Activation of the Raf-MEK-ERK signal transduction pathway in endothelial cells is required for angiogenesis. Raf is the kinase most efficiently inhibited by the multikinase inhibitor sorafenib, which has shown activity against certain human cancers in clinical trials. To understand the mechanisms underlying this activity, we studied how it controlled growth of K1735 murine melanomas. Therapy caused massive regional tumor cell death accompanied by severe tumor hypoxia, decreased microvessel density, increased percentage of pericyte-covered vessels, and increased caliber and decreased arborization of vessels. These signs of K1735 angiogenesis inhibition, along with its ability to inhibit Matrigel neovascularization, showed that sorafenib is an effective anti-angiogenic agent. Extracellular signal-regulated kinase (ERK) activation in tumor endothelial cells, revealed by immunostaining for phospho-ERK and CD34, was inhibited, whereas AKT activation, revealed by phospho-AKT immunostaining, was not inhibited in K1735 and two other tumor types treated with sorafenib. Treatment decreased endothelial but not tumor cell proliferation and increased both endothelial cell and tumor cell apoptosis. These data indicate that sorafenib's anti-tumor efficacy may be primarily attributable to angiogenesis inhibition resulting from its inhibition of Raf-MEK-ERK signaling in endothelial cells. Assessing endothelial cell ERK activation in tumor biopsies may provide mechanistic insights into and allow monitoring of sorafenib's activity in patients in clinical trials. (*Am J Pathol* 2006, 169:1875-1885; DOI: 10.2353/ajpath.2006.050711)

Molecularly targeted cancer therapeutic agents are designed to inhibit signal transduction pathways important in cancer pathogenesis. Mutations in genes encoding proteins of the Ras-Raf-MEK-ERK signaling pathway are frequently found in human cancers, and certain cancers characteristically have Ras or Raf mutations. Pancreatic, colorectal, and lung adenocarcinomas often have K-ras mutations,¹⁻⁶ and melanomas, colorectal, ovarian, and papillary thyroid carcinomas often have B-Raf mutations.^{5,7-10} Activating mutations in RAS and RAF result in inappropriate activation of downstream kinases, mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK), and deregulated mitogenic and cell survival signaling.¹¹ The central importance and frequent derangement of Ras-Raf-MEK-ERK signaling in human cancers provide the rationale for developing small molecule inhibitors of Raf kinase for cancer therapy.¹² Sorafenib (BAY43-9006) is such a compound, binding Raf and inhibiting its kinase activity by maintaining it in an inactive configuration.¹³ It decreases ERK activation in human tumor cells, inhibits cell proliferation *in vitro*, and controls growth of human tumor xenografts in mice.^{12,14} Sorafenib has been Food and Drug Administration approved for treatment of renal cell carcinoma and has showed promise in the treatment of melanoma in clinical trials.^{15,16}

Additional characterization has shown that sorafenib inhibits other kinases, such as vascular endothelial growth factor (VEGF) receptors (VEGFR)-2 and -3 and platelet-derived growth factor receptor β , albeit with higher IC₅₀.¹⁴ VEGFR2-mediated VEGF activation of endothelial cells (ECs) is critical for angiogenesis. This is attributed to its ability to induce EC signaling pathways that regulate EC proliferation, migration, and survival. The

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two major pathways that regulate these processes are the PI3K-AKT and Raf-MEK-ERK pathways.^{17–22} Activation of these pathways in ECs is necessary for angiogenesis.^{23–27} Given this and the fact that a dominant-negative mutant Raf was shown to inhibit angiogenesis,²⁸ the VEGFR2 and Raf inhibitory activities of sorafenib have led to expectations that this drug is an inhibitor of angiogenesis. Evidence supporting this idea can be found in the observation that microvessel density (MVD) was reduced in tumors treated with sorafenib.¹⁴ However, it remains unclear whether the anti-angiogenic effect of sorafenib was a major mechanism for control of tumor growth and if tumor ECs are targeted by this drug *in vivo*.

We undertook the current study to understand the mechanism of tumor growth control by sorafenib. Our results show that in K1735 murine melanomas treated with this agent, inhibition of tumor angiogenesis contributes significantly to control of tumor growth and, in fact, may be the predominant anti-tumor mechanism. We then examined the effect of therapy on the activity of EC signal transduction pathways in tumors *in vivo*. Marked inhibition of ERK activation and unaltered AKT activation in tumor vascular ECs was accompanied by inhibition of EC proliferation and induction of EC apoptosis. Together with similar findings in two other tumor models treated with this agent, these data show that sorafenib is a potent inhibitor of ERK activation in tumor ECs and tumor angiogenesis. Finally, our studies suggest methods for assessing the activity of this agent at relevant target sites, which may be useful both for monitoring its effect and understanding its activity against human cancers in clinical trials.

Materials and Methods

Murine Tumor Therapy Studies

Murine K1735 melanoma and RENCA renal carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin.²⁹ Female C3H/HeN and BALB/c mice, 6 to 8 weeks old, were purchased from Charles River (Wilmington, MA) and Taconic Farms (Germantown, NY), respectively, and maintained in microisolate cages. K1735 and RENCA tumors were generated by injecting 2×10^6 viable tumor cells subcutaneously into the lower right flank of C3H/HeN and BALB/c mice, respectively. Tumors were measured by calipers at regular intervals, and volume was calculated using the formula for approximating the volume of a spheroid [$0.5 \times (\text{diameter})^3$]. Mice were treated with either vehicle (5% Cremaphore EL/5% ETOH/90% ddH₂O) or sorafenib (30 mg/kg) by gavage daily. Colo-205 human colon carcinoma tumors were generated in nude mice and treated as previously described.¹⁴ Plasma was collected from a subset of animals 3 hours after gavage and analyzed for drug concentration. For histological studies, all animals were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) by intracardiac injection. Tumors were excised and either frozen or processed for paraffin embedding. Size-matched untreated and vehicle-treated tumors

were generated for histological analysis. Tumor samples were stained with hematoxylin and eosin (H&E), and the area of necrosis was calculated by Image J software (National Institutes of Health, Bethesda, MD).

Matrigel Assay for Angiogenesis

C3H/HeN mice were injected subcutaneously with 0.5 ml of Matrigel (BD Biosciences, Bedford, MA) mixed with 15 U of heparin (Sigma, St. Louis, MO) with or without 100 ng of basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN). In treated mice, vehicle or 30 mg/kg of sorafenib was administered daily by gavage for 6 days. Matrigel pellets were harvested on day 6, fixed in 4% paraformaldehyde overnight, and processed for paraffin embedding. Neovascularization was determined as previously described.³⁰ Briefly, sections were stained with Masson-Trichrome and examined for erythrocytes in nuclei-lined luminal structures.

Tumor Immunostaining

Detection of tumor cell hypoxia by EF5 was performed as previously described.²⁹ In brief, EF5 (provided by Dr. Cameron Koch, University of Pennsylvania, Philadelphia, PA) was injected into tumor-bearing mice via tail vein 3 hours before euthanasia. Sections of frozen tumors were stained with Cy3-conjugated anti-EF5 monoclonal antibody (provided by Dr. Cameron Koch). Vasculature in frozen tumor sections was studied by epifluorescence and confocal microscopy as previously described³¹ using anti-CD31 (PECAM) antibody (Pharmingen, San Jose, CA) to identify ECs followed by Alexa Fluor 488 goat anti-rat IgG (Molecular Probes, Eugene, OR). Pericytes were identified using anti-smooth muscle actin (SMA) (DAKO, Carpinteria, CA) antibody in M.O.M. blocking buffer (Vector Laboratories, Burlingame, CA) followed by Texas Red-conjugated goat anti-mouse IgG antibody (Molecular Probes). MVD measurements were calculated as previously described.³²

Cell Signaling

Five- μ m paraffin-embedded tumor sections were stained for p-ERK with rabbit anti-phospho-ERK antibody (Cell Signaling, Beverly, MA), for p-AKT with rabbit anti-phospho-AKT antibody (Cell Signaling, MA), and for proliferation using mouse anti-Ki-67 antibody (Vector Laboratories). These were incubated with biotinylated secondary antibodies (Vector Laboratories) and ABC reagent (Vector Laboratories). Immune complexes were detected with 3,3'-diaminobenzidine (Vector Laboratories). The same sections were subsequently stained for vessels using CD34 (Abcam, Cambridge, MA) by immunofluorescence. A vessel was counted as positive for p-ERK, p-AKT, or Ki-67 when at least one EC within a vessel stained positively for these antigens. Apoptotic cells were identified by terminal dUTP nick-end labeling (TUNEL) staining using the Apoptag indirect fluorescein isothiocyanate detection kit (Chemicon, Temecula, CA) followed

Figure 1. K1735 tumor growth is inhibited by sorafenib treatment. Female C3H/HeN mice bearing 1- to 2-mm diameter K1735 tumors were started on treatment with sorafenib (30 mg/kg) or vehicle by gavage daily. Tumor size was measured with calipers, and volumes were calculated using the formula $0.5 \times (\text{width})^2 \times (\text{length})$. Plotted is time taken by tumors to reach 1.2-cm³ volume at which time mice were euthanized (x axis, days after initiation of treatment; y axis, percentage of tumors in each treatment group < 1.2 cm³). **A:** Untreated tumors are represented by black lines ($n = 4$), vehicle-treated tumors are represented by dashed lines ($n = 9$), and sorafenib-treated tumors are represented by dotted lines ($n = 10$). **B:** Sections from sorafenib or vehicle-treated K1735 tumors were stained with H&E. Pink regions are primarily necrotic; purple regions are primarily viable.

by vessel staining by CD31 immunofluorescence (Pharmingen, La Jolla, CA) on 10- μ m frozen tumor sections. IP Lab Software (Scanalytics, Fairfax, VA) was used to quantify tumor cell staining of Ki-67 and TUNEL.

In Vitro Studies

Cultured mouse brain capillary endothelial cells (MBECs, gift from Dr. Dounan Yu, University of Pennsylvania)³³ and human microvascular dermal endothelial cells (HMVEC-d; Clonetics, San Diego, CA) were treated with different concentrations of sorafenib for 2 hours and lysed. Thirty to 60 μ g of protein were run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and probed for p-ERK or p-AKT (Cell Signaling). Blots were subsequently stripped and reprobed for ERK or AKT (Cell Signaling). K1735 tumors were homogenized in lysis buffer. Tumor lysate was loaded at increasing concentrations of protein (50, 100, and 300 μ g) along with K1735 tumor cell lysate and run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The gel was probed for p-ERK, stripped, and reprobed for ERK. Relative p-ERK and p-AKT expressions were quantitated by calculating (optical density of phosphorylated proteins/optical density of unphosphorylated proteins) and normalized to control levels (Image J).

Statistical Analysis

Assessment of statistical significance for survival analysis was performed by log rank test (R Statistical Software, <http://www.r-project.org>). All other statistical analyses were performed by Student's *t*-test (Excel; Microsoft, Redmond, WA).

Results

Sorafenib Inhibits K1735 Tumor Growth

C3H/HeN mice bearing subcutaneous K1735 melanoma tumors were treated daily with sorafenib (30 mg/kg) or vehicle by gavage when their tumors reached 2 mm in diameter. Mice were treated for 4 weeks or until their

tumors reached euthanizable size (1.2 cm³). After 4 weeks of treatment, all control tumors and eight of nine vehicle-treated tumors were greater than 1.2 cm³, whereas only 3 of 10 sorafenib-treated tumors had reached this size (Figure 1A). This difference in growth between vehicle and sorafenib-treated tumors was significant ($P < 0.01$, log rank test). Similar results were obtained in another experiment in which we tested treatment with sorafenib at 30 and 100 mg/kg/day (data not shown).

Tumors were removed and processed for histological analysis at the end of therapy. H&E-stained sections from size-matched tumors revealed large regions of necrosis in the sorafenib-treated group that were primarily absent in the vehicle-treated group (Figure 1B; necrotic areas accounted for $44 \pm 28\%$ of the cut surface of sorafenib-treated tumors versus $1 \pm 1\%$ of the cut surface of vehicle-treated tumors, $P < 0.05$). These results show that sorafenib treatment controls K1735 tumor growth, causing massive regional tumor cell death consistent with ischemic necrosis.

Sorafenib Inhibits bFGF-Induced Matrigel Neovascularization

To demonstrate that sorafenib can inhibit angiogenesis *in vivo*, Matrigel pellets containing 100 ng/ml bFGF were implanted subcutaneously in C3H/HeN mice and the hosts subjected to daily treatment with either 30 mg/kg sorafenib or vehicle. After 6 days, plugs were removed and analyzed by Masson-Trichrome staining.³⁰ Untreated and vehicle-treated Matrigel showed large numbers of vascular structures that reached the center of the pellet (Figure 2, B and C). In contrast, Matrigel treated with sorafenib had fewer vascular structures, and these were located predominantly at the periphery of the pellets (Figure 2D). Sorafenib treatment, however, did not reduce the number of vessels to levels seen in Matrigel without bFGF (Figure 2A). These results show that sorafenib treatment inhibits but does not completely abrogate bFGF-induced angiogenesis *in vivo*.

Figure 2. Effect of sorafenib on Matrigel neovascularization and tumor ischemia. Matrigel pellets containing PBS (**A**) or bFGF (100 ng/ml) (**B–D**) were implanted in mice that went untreated (**A, B**) or were treated with vehicle (**C**) or sorafenib (30 mg/kg) (**D**) for 7 days. Sections of the pellets were stained with Masson-Trichrome to reveal vascular structures containing erythrocytes (red). Mice with K1735 tumors were treated with vehicle or sorafenib (30 mg/kg) for 7 days. These mice were injected with EF5 to label hypoxic cells and fluorescein isothiocyanate-tomato lectin to illuminate perfused vessels before tumor excision. Shown are representative sections of vehicle-treated (**E, F**) and sorafenib-treated (**G, H**) tumors stained with Cy3-conjugated anti-EF5 monoclonal antibody. Red indicates severely hypoxic tumor regions labeled by EF5, and green indicates perfused tumor vessels. Original magnifications, $\times 100$.

Figure 3. Inhibition of tumor angiogenesis by sorafenib. K1735 tumor vessels were revealed by CD31 staining and quantitated as previously described.³¹ **A:** Histograms of MVDs in untreated ($n = 5$, gray), vehicle-treated ($n = 6$, clear bar), and sorafenib-treated ($n = 9$, black bar) tumors are shown (*significant difference at $P < 0.05$, Student's t -test). Tumor vessels were characterized for coverage by pericytes by staining for smooth muscle actin (SMA) in addition to CD31. **B:** The percentage of pericyte-covered vessels in individual untreated, vehicle-treated, and sorafenib-treated K1735 tumors are shown ($n = 3$). The mean of each group is indicated by a horizontal bar (*significant difference at $P < 0.05$, Student's t -test). Thick sections of untreated (**C**), vehicle-treated (**D**), and sorafenib-treated (**E**) tumors were stained for CD31 and viewed by confocal microscopy. Original magnifications, $\times 200$.

Sorafenib Inhibits Development of K1735 Tumor Vasculature

To determine whether sorafenib treatment inhibits angiogenesis and vascular development in tumors, we examined development of hypoxia in treated tumors. Because necrosis interferes with detection of hypoxia, and extensive necrosis was seen in K1735 tumors treated with sorafenib for 3 to 4 weeks, we studied K1735 tumors treated with sorafenib for only 7 days. We started treatment after tumors reached 5 mm in diameter to ensure that they would be sufficiently large for analysis at the end of the week of therapy. Hypoxic tumor cells were labeled *in vivo* with EF5, which was subsequently detected by immunostaining with Cy3-conjugated anti-EF5 antibody.³⁴ As previously seen,²⁹ untreated K1735 tumors had few, if any, regions of EF5 labeling (data not shown). Vehicle-treated tumors had limited areas of EF5 staining, which were not intense (Figure 2, E and F). In contrast, tumors treated with sorafenib had widespread areas of intense EF5 staining (Figure 2, G and H). As in K1735 tumors treated successfully with other anti-angiogenic agents,²⁹ EF5 staining in sorafenib-treated tumors tended to be in tumor regions most distant from perfused vessels.

To investigate the effect of treatment on MVD, tumors treated for 7 days with vehicle or sorafenib were immunostained with anti-CD31 (PECAM) antibody. MVD was significantly reduced in sorafenib-treated tumors compared to vehicle-treated tumors ($P < 0.01$) (Figure 3A). To assess vessel pericyte coverage, tumor sections were immunostained with anti-smooth muscle actin (SMA) and anti-CD31 antibodies. Pericyte coverage of tumor vessels increased significantly after sorafenib treatment but not after vehicle treatment (Figure 3B). Thick (50 μ m) tumor sections were stained with anti-CD31 and examined by confocal microscopy to assess vessel morphology. Vessels in vehicle-treated tumors appeared only slightly larger in caliber and resembled those in untreated tumors in arborization (Figure 3, C and D). In contrast, vessels in sorafenib-treated tumors were obviously larger in caliber and were much less arborized (Figure 3E). The marked increase in tumor cell hypoxia, the decrease in tumor MVD, the increase in the percentage of pericyte-covered vessels, and the changes in vessel morphology are hallmarks of angiogenesis inhibition in K1735 tumors.^{29,32,35,36} These led us to conclude that sorafenib is a potent inhibitor of angiogenesis and that this activity contributes significantly to its control of tumor growth.

Figure 4. Inhibition of ERK but not AKT activation in tumor endothelial cells by sorafenib. K1735 tumors treated with either vehicle or sorafenib were stained with anti-p-ERK or anti-p-AKT antibody (brown immunohistochemistry) followed by vessel staining with anti-CD34 antibody (green immunofluorescence) and a light hematoxylin counterstain. **A:** The p-ERK/p-AKT images and the CD34 images of the same representative fields are superimposed; vascular ECs staining for p-ERK or p-AKT are indicated by **arrows**. Histograms of the percentage of tumor vessels staining for p-ERK (**B**) or p-AKT (**C**) are shown for tumors treated for 7 or 28 days with sorafenib (black bars; $n = 3$ for p-ERK, $n = 6$ for p-AKT) or vehicle (white bars; $n = 3$ for p-ERK, $n = 4$ for p-AKT) and for size-matched untreated tumors (gray bars, $n = 3$). *Indicates significant difference at $P < 0.05$; ** indicates significant difference at $P < 0.01$. **D:** Colo-205 and RENCA tumors were stained for p-ERK, p-AKT, and CD34 as described above. Original magnifications, $\times 400$.

Sorafenib Inhibits ERK Phosphorylation in Tumor Vasculature

Activation of the Raf-MEK-ERK kinase pathway in ECs has been shown to be necessary for neovascularization,^{25–27} so we asked whether sorafenib treatment inhibited ERK phosphorylation in K1735 tumor vasculature. Tumors treated for 1 and 4 weeks were stained for phospho-ERK (p-ERK) using anti-p-ERK antibody by immunohistochemistry and for vessels using anti-CD34 antibody by immunofluorescence. In untreated and vehicle-treated K1735 tumors, p-ERK staining was frequently seen in ECs and was usually nuclear (Figure 4A). The specificity of this stain was confirmed by immunostaining these tumors with a different (monoclonal) anti-p-ERK antibody and through use of a blocking peptide for the polyclonal anti-p-ERK antibody (data not shown). In addition, p-MEK staining co-localized with p-ERK staining in tumor ECs, indicating that p-ERK activation resulted from Raf-MEK-ERK signaling in these EC (Supplemental Figure S1 at <http://ajp.amjpathol.org>). Interestingly, p-ERK staining was rarely seen in the tumor cells themselves in most

K1735 tumors. The dearth of detectable p-ERK in tumor cells was confirmed by Western blot analysis of K1735 tumor lysates. This was a surprising finding, because K1735 cells cultured *in vitro* contain abundant p-ERK (Figure 5A).

Quantitation of staining revealed that $\sim 22\%$ of vessels in untreated K1735 tumors stained for p-ERK and that 28 and 18% of vessels stained in tumors treated with vehicle for 1 and 4 weeks, respectively (Figure 4, A and B; $P > 0.05$). In contrast, vascular p-ERK expression was significantly reduced in sorafenib-treated tumors with 14% of vessels stained after 1 week of treatment ($P < 0.05$) (Figure 4, A and B). p-ERK expression further decreased with continued treatment, such that only 3% of tumor vessels stained with p-ERK after 4 weeks of treatment ($P < 0.01$). In treated tumors with reduced vascular p-ERK staining, staining for total ERK was not decreased (data not shown). Another EC signaling pathway known to be activated during angiogenesis is the PI3K-AKT pathway. We examined activation of this pathway and the effect of sorafenib treatment by staining tumor sections for phospho-AKT (p-AKT). In contrast to p-ERK staining,

Figure 5. ERK and AKT activation in K1735 cells and tumors and the effect of sorafenib treatment on ERK and AKT activation in ECs. **A:** Western blots of lysates (50, 100, and 300 µg) of cultured K1735 tumor cells and K1735 tumors were probed for p-ERK and subsequently reprobed for ERK. Cultured HMVEC-d and MBECs were treated with vehicle or different concentrations of sorafenib for 2 hours. Western blots of cell lysates were probed for p-ERK and p-AKT. **B:** After stripping, these blots were reprobed for ERK and AKT, respectively. HMVEC-d expression of these antigens is shown. After densitometry of the relevant bands, p-ERK content was normalized for ERK content, and p-AKT content was normalized for AKT content. Shown are histograms of normalized p-ERK and p-AKT content for HMVEC-d (**C**) and MBECs (**D**) under different treatment conditions relative to normalized p-ERK and p-AKT content in untreated HMVEC-d and MBECs (set at 1.0).

anti-p-AKT antibody stained tumor cells as well as ECs in K1735 tumors (Figure 4A). Treatment with sorafenib for 1 or 4 weeks did not significantly change the percentage of vessels staining for p-AKT (Figure 4, A and C).

The decrease in vascular p-ERK staining but not p-AKT staining in tumors treated with sorafenib was also seen in the two other tumor types examined, Colo-205 human colon carcinoma xenografts and RENCA renal

cell carcinoma tumors (Figure 4D, Table 1). Quantitation of p-ERK and p-AKT expression in tumor vasculature and changes induced by therapy for all tumors examined are summarized in Table 1.

These results show that sorafenib treatment inhibited ERK activation but not AKT activation in tumor ECs in three different tumor types. This pattern of signaling inhibition was confirmed by *in vitro* correlative studies. We

Table 1. Examination of Phospho-ERK, Phospho-AKT, and Proliferation in Endothelial Cells of Three Tumor Types Treated with Sorafenib

Expression ^{1,2} treatment ³	p-ERK vehicle	p-ERK sorafenib	p-AKT vehicle	p-AKT sorafenib	Proliferation vehicle	Proliferation sorafenib
K1735	29 ± 5% ^α	14 ± 5% ^α	54 ± 7% ⁴	47 ± 4%	14 ± 2% ^δ	2 ± 1% ^δ
Colo-205	16 ± 6% [*]	4 ± 2% [*]	45 ± 3%	45 ± 0.6%	12 ± 3% [#]	6 ± 1% [#]
RENCA	13 ± 4% ^β	7 ± 3% ^β	24 ± 6%	27 ± 3%	12 ± 3% ^δ	5 ± 1% ^δ

¹Tumors were stained by immunohistochemistry with either anti-p-ERK, p-AKT, or Ki67 (proliferation) followed by immunofluorescence with anti-CD34 to identify endothelial cells.

²Expressed as percentage of p-ERK+ CD34+ vessels/total CD34+ vessel, p-AKT and Ki67 percentages were obtained similarly.

³Tumors were either treated with vehicle or sorafenib (30 mg/kg) for 5 to 7 days before excision for analysis.

⁴For each mean ± standard deviation. 15 representative fields in each of three tumors (vehicle) and four tumors (sorafenib) were counted.

^{*},^δ,[#]Signify pairs of numbers that are statistically different, *P* < 0.01, Student's *t*-test.

^α,^βSignify pairs of numbers that are statistically different, *P* < 0.05, Student's *t*-test.

Table 2. Proliferation and Death of Cells in K1735 Tumors Treated with Sorafenib

Cells studied ¹ treatment ²	Tumor cells vehicle	Tumor cells sorafenib	Vessels vehicle	Vessels sorafenib
Proliferation ³	26 ± 5% ⁵	25 ± 3%	14 ± 2%*	2 ± 1%*
Apoptosis ⁴	0.8 ± 0.2% [#]	1.4 ± 0.5% [#]	3 ± 2% ^{&}	7 ± 3% ^{&}

¹Tumors were stained with anti-CD34 to identify endothelial cells; all other cells were considered tumor cells in this analysis.

²Tumors were either treated with vehicle or sorafenib (30 mg/kg) for 7 days before excision for analysis.

³Expressed as a percentage of all cells present in a field of view, proliferating cells were defined as those staining for Ki-67.

⁴Expressed as a percentage of all cells present in a field of view, apoptotic cells were determined as those staining by TUNEL.

⁵For each mean ± standard deviation, ≥4 representative fields in each of six tumors were counted.

*,&,#Signify pairs of numbers that are statistically different, $P < 0.01$, Student's *t*-test.

measured drug levels in mice treated with sorafenib and found plasma levels were 4 to 12 $\mu\text{mol/L}$ 3 hours after administration. The addition of sorafenib to HMVEC-d and MBECs *in vitro* showed that p-ERK but not p-AKT levels were decreased by treatment with drug in this concentration range and that p-AKT levels began to decrease slightly at higher drug concentrations (Figure 5, B–D).

Sorafenib Inhibits Proliferation and Promotes Apoptosis in Tumor Vasculature

Angiogenesis requires EC proliferation, and anti-angiogenic agents inhibit EC proliferation and/or induce EC apoptosis. Inhibition of K1735 tumor angiogenesis by sorafenib suggests that one, the other, or both processes are affected by treatment. To examine proliferation in K1735 vasculature, tumor sections were stained for the proliferation-associated antigen, Ki-67, by immunohistochemistry followed by CD34 vessel staining by immunofluorescence. Quantification revealed that a similar percentage of vessels (~14%) in untreated and vehicle-treated tumors stained for Ki-67 (data not shown). In contrast, a much lower percentage of vessels (2%) stained for Ki-67 after 7 days of sorafenib treatment (Table 1, $P < 0.01$). A significant decrease in Ki-67 staining was also seen in the vascular ECs of treated Colo-205 and RENCA tumors (Table 1). Cell death in K1735 tumors was studied by TUNEL staining and CD31 immunostaining. There was significantly more EC death in sorafenib-treated tumor vasculature compared to untreated or vehicle-treated tumor vasculature (7 ± 3 versus $3 \pm 2\%$, $P < 0.01$; Table 2). These data show that both decreased formation of new tumor vessels and regression of existing tumor vessels contribute to sorafenib's inhibition of tumor vasculature development. Because a decrease in tumor vasculature could be attributed to primary effects on tumor cells, we investigated tumor cell proliferation and apoptosis in K1735 tumors after sorafenib treatment. There was no decrease in tumor cell staining for Ki-67 after sorafenib treatment, but treatment increased tumor cell TUNEL staining (1.4 ± 0.5 versus $0.8 \pm 0.2\%$, $P < 0.01$; Table 2).

Discussion

Sorafenib is a novel multikinase inhibitor that was recently approved by the Food and Drug Administration for treatment of renal cell carcinoma. In addition to Raf kinases, several other kinases, such as VEGFR2, VEGFR3, PDGFR- β , c-Kit, and FLT-3, are also inhibited at concentrations of sorafenib that may be achieved *in vivo*.¹⁴ This spectrum of inhibitory activity has led to the idea that sorafenib may exhibit two anti-tumor activities, inhibition of tumor vascularization (via inhibition of VEGFR and PDGFR) as well as inhibition of proliferation/survival of malignant cells (via inhibition of Raf, kit, FLT-3). We studied its effect in K1735 tumors to examine these issues and to delve into the drug's effects on EC signaling *in vivo*. Our studies showed that sorafenib markedly inhibited tumor growth and unequivocally inhibited tumor vascular development. Effects associated with angiogenesis inhibition in K1735 tumors—induction of hypoxia, reduction in vascularity, altered vessel characteristics and morphology—were all present in treated tumors, and actual inhibition was demonstrated when EC proliferation was shown to be decreased. Angiogenesis inhibition was almost certainly a primary effect of therapy and not secondary to tumor cell loss with reduced production of angiogenic factors. We base this conclusion on the fact that inhibition of EC proliferation and the decrease in tumor vascularity (MVD) occurred early in therapy, before marked loss of tumor cells was evident, and on the fact that adverse effects on EC cells were more prominent than adverse effects on K1735 cells, as indicated by Ki-67 and TUNEL staining. In addition, if tumor cell loss preceded and was responsible for reduced angiogenesis (ie, angiogenesis is a trailing event), tumor MVD, an expression of vessel to tumor cell ratio, would not be expected to decrease and should remain unchanged or possibly increase.

In response to growth factor stimulation, cells activate the conserved Raf-MEK-ERK pathway to transduce signals that regulate differentiation, growth, and proliferation.^{37–40} *In vitro*, ECs activate this pathway on stimulation of multiple proangiogenic EC receptors including, VEGFR2, FGFR2, Tie2, integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$), and EDG1,^{17,18,21,41–44} and EC activation of this pathway is necessary for angiogenesis.^{22,25–27} Inhibition of EC signaling through Raf-MEK-ERK was shown in tumors treated with sorafenib by decreased immunohistochemical staining for p-ERK. In control K1735 tumors, 18 to 28% of vessels stained for p-ERK (Figure 4B). Because quiescent vessels in most normal mouse organs do not stain (data not shown), this is a sign of activated vasculature in tumors. Sorafenib treatment decreased the number of tumor vessels staining for p-ERK by 85 to 90%. This was accompanied by a significant decrease in Ki-67 staining within treated tumor endothelium. We believe this decrease reflects sorafenib inhibition of the Raf-MEK-ERK pathway in ECs and is not merely a manifestation of angiogenesis inhibition, because EC p-ERK expression was not reduced in K1735 tumors treated with the angiogenesis inhibitor rIL-12 ($22 \pm 4\%$ untreated versus $19 \pm 6\%$ treated; $P > 0.05$). Residual staining for p-ERK in

Figure 6. ERK activation in human melanomas and renal cell carcinomas. Paraffin sections of human melanomas and renal cell carcinomas were stained with anti-p-ERK antibody (brown immunohistochemistry) followed by vessel staining with anti-CD34 antibody (green immunofluorescence) and a light hematoxylin counterstain. Tumor vascular endothelial cells expressing p-ERK are indicated by **arrows**. Original magnifications, $\times 400$.

tumor ECs could be attributable to incomplete kinase inhibition by sorafenib or attributable to the activity of alternative pathways of ERK activation.⁴⁵ Interestingly, ECs seemed to either stain or not stain for p-ERK, and gradations of p-ERK staining (and, presumably, of p-ERK levels) were not evident. In contrast to its consistent and marked inhibition of EC ERK activation, AKT activation in tumor ECs, revealed by p-AKT staining, is not significantly diminished by sorafenib treatment.

The virtually undetectable levels of p-ERK in K1735 tumor cells *in vivo* was unexpected. If p-ERK levels reflect activity of the Raf-MEK-ERK pathway, it implies that activity of this pathway is low. Perhaps this should not be so surprising, because K1735 tumor cells do not contain a mutant Ras or B-Raf⁴⁶ to constitutively activate this pathway. Relative inactivity of the targeted signaling pathway in tumor cells highlights the therapeutic significance of sorafenib inhibitory effects on ECs. From this, it may be argued that angiogenesis inhibition played a prominent role in sorafenib efficacy against K1735 tumors only because tumor cell cytotoxic/cytostatic effects were relatively weak in these tumors. Indeed, it is difficult to know whether deleterious effects seen among K1735 cells in treated tumors were a direct effect of sorafenib or a consequence of angiogenesis inhibition. Although effects on tumor cells may play a larger role when the malignant cells depend more on Raf-MEK-ERK activation, angiogenesis dependency on activation of this pathway in ECs will probably not vary, so that angiogenesis inhibition should be a consistently significant feature of sorafenib

therapy. This thesis is supported by the results of our Colo-205 tumor studies. These tumor cells have an activating B-Raf mutation¹⁴ and express p-ERK *in vivo* (Figure 4D). Sorafenib treatment inhibited p-ERK in EC (Table 1) much more than in tumor cells,¹⁴ indicating that, even when tumor cells have activated Raf-MEK-ERK signaling, sorafenib inhibits signaling in ECs and produces anti-angiogenic effects.

Candidate targets of sorafenib inhibition in EC include VEGFR2 and Raf. Inhibition of either VEGFR2 or Raf can produce angiogenesis inhibition,^{28,47,48} so both are reasonable therapeutic targets in sorafenib-treated tumors. Unfortunately, the pattern of EC signaling inhibition does not allow identification of the kinase targeted. Down-modulation of the p-ERK would be expected if either or both kinases were inhibited by sorafenib. Absence of p-AKT down-modulation does not argue for Raf or against VEGFR2 being the target, because receptors other than VEGFR2 (eg, Tie2) may be responsible for activation of the PI3K-AKT pathway in tumor ECs. Distinguishing whether Raf or VEGFR2 (or both) is the kinase targeted by sorafenib in ECs *in vivo* may be therapeutically significant, because many angiogenic factors besides VEGF signal through the Raf-MEK-ERK pathway, and inhibiting Raf may attenuate the action of all these factors.

The approaches used here to study EC dynamics and treatment response in mouse tumors can be adapted to study similar parameters and events in human tumors. Phospho-ERK in microvessels reflects EC stimulation and

activation and is present in a significant fraction of vascular ECs in different types of human cancers. Examples of human melanoma and renal cell carcinoma stained for CD34 and p-ERK are shown in Figure 6. Sorafenib is being tested in patients with these cancers in clinical trials,^{15,16} and the presence of p-ERK in vascular ECs in these cancers indicates that their vessels are likely targets of therapy. If tumor tissue obtained during treatment were also available for study, this type of histological analysis could also report the effects of targeted anti-angiogenic agents and provide a definitive assessment of therapeutic response.

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