Mutant V599EB-Raf Regulates Growth and Vascular Development of Malignant Melanoma Tumors

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Abstract

Activating mutations of the B-RAF gene are observed in >60% of human melanomas. Approximately 90% of these mutations occur in the activation segment of the kinase domain as a single-base substitution that converts a valine to glutamic acid at codon 599 (V599E) in exon 15. This mutation causes activation of the kinase as well as downstream effectors of the mitogen-activated protein kinase–signaling cascade, leading to melanoma tumor development by an as yet unknown mechanism. In this study, we have identified the role of V599EB-Raf in melanoma tumor development by characterizing the mechanism by which this mutant protein promotes melanoma tumorigenesis. Small interfering RNA targeted against B-Raf or a Raf kinase inhibitor (BAY 43-9006) was used to reduce expression and/or activity of V599EB-Raf in melanoma tumors. This inhibition led to reduced activity of the mitogen-activated protein kinase–signaling cascade and inhibited tumor development in animals. Targeted reduction of mutant V599EB-Raf expression (activity) in melanoma cells before tumor formation inhibited tumorigenesis by reducing the growth potential of melanoma cells. In contrast, reduction of mutant V599EB-Raf activity in preexisting tumors prevented further vascular development mediated through decreased vascular endothelial growth factor secretion, subsequently increasing apoptosis in tumors. These effects in combination with reduced proliferative capacity halted growth, but did not shrink the size of preexisting melanoma tumors. Thus, these studies identify the mechanistic underpinnings by which mutant V599EB-RAF promotes melanoma development and show the effectiveness of targeting this protein to inhibit melanoma tumor growth. (Cancer Res 2005; 65(6): 2412–21)

Introduction

Despite many clinical trials testing everything from surgery to immunotherapy, radiotherapy, and chemotherapy, there remains no effective long-term treatment for advanced-stage melanoma patients (1–4). The lack of effective therapeutic regimes reflects the need to identify the key proteins causing melanoma and therapies designed to correct these defects (1–4). Alterations activating members of the mitogen-activated protein kinase (MAPK) signaling pathway have been implicated in the development of 60% to 90% of melanomas with activating B-RAF mutations playing the most prominent role in this process (5–9). Activation of this pathway has been linked to cancer development by enhancing several key oncogenic features of the cell including increased cell proliferation, survival, invasion, and tumor angiogenesis (10–12). Therefore, therapies that target the activity of mutated B-Raf or other components of the MAPK pathway have potential to halt progression of advanced stage melanomas by slowing tumor growth, preventing angiogenesis, inhibiting invasion and metastasis, inducing tumor cell death, or promoting tumor differentiation (13–15).

B-Raf is one of three serine/threonine kinases in the Raf family, which includes A-Raf, B-Raf, and C-Raf (or Raf-1; ref. 10). Raf family members are intermediate molecules in the MAPK [Ras/Raf/MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK)] pathway, which is a signal transduction pathway that relays extracellular signals from cell membrane to nucleus via an ordered series of consecutive phosphorylation events (10, 12). Typically, an extracellular ligand binds to its tyrosine kinase receptor, leading to Ras activation and initiation of a cascade of phosphorylation events (10, 12). Activated Ras causes phosphorylation and activation of Raf, which in turn phosphorylates and activates MEK1 and MEK2. MEK kinases in turn phosphorylate and activate ERK1 and ERK2 (16), which phosphorylate several cytoplasmic and nuclear targets that ultimately lead to expression of proteins playing important roles in cell growth and survival (17).

Mutations that lead to activation of B-Raf have been found in the majority of sporadic melanomas, making B-RAF the most mutated gene in melanomas with a mutation rate ranging from 60% to 90% (5, 7, 18, 19). The majority of B-RAF mutations occur as a result of a single-base missense substitution that converts T to A at nucleotide 1796, which substitutes a valine for a glutamic acid at codon 599 (V599E) in exon 15 (5). This mutation increases basal kinase activity of B-Raf, resulting in hyperactivity of the MAPK pathway evidenced by constitutively elevated levels of downstream kinases MEK and ERK (5). B-RAF mutations are acquired, somatic, post-zygotic events that have not been identified in familial melanomas (20–22). The high frequency of mutation makes B-RAF an attractive therapeutic target to improve the effectiveness of treatments for advanced-stage melanoma patients (13). However, it is uncertain whether targeting B-RAF is sufficient to inhibit melanoma tumorigenesis, and the mechanism by which this could occur is unknown.

In this study, we evaluated the role that mutant V599EB-Raf plays in melanoma tumorigenesis by inhibiting expression and/or activity of V599EB-Raf in melanoma tumors. Small interfering RNA (siRNA) targeted against B-Raf or a Raf kinase inhibitor (BAY 43-9006) reduced activity of the MAPK-signaling cascade and inhibited tumor development in animals. Mechanistically, inhibition of mutant V599EB-Raf delayed melanoma tumor development by slowing cell growth and/or reducing vascular development, mediated through...
reduced vascular endothelial growth factor (VEGF) secretion from the tumor cells. Thus, these studies provide a mechanistic basis for therapeutically targeting V<sup>599E</sup>B-Raf in order to inhibit melanoma tumor development.

Materials and Methods

Cell Lines, Culture Conditions, and B-Raf Mutational Status. The human melanoma cell lines UACC 903, 1205 Lu, and C8161, as well as the HEK 293T cell line were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). The HEK 293T cell line were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). The presence or absence of the T1796A mutation in the UACC 903 and C8161 cell lines was undertaken as described previously (23). Furthermore, the presence of this mutation in UACC 903 and 1205 Lu cells has been reported previously (23–25).

In vitro siRNA Studies. siRNA (100 pmol) was introduced into 1 × 10<sup>6</sup> UACC 903, 1205 Lu, or C8161 cells via nucleofection with an Amamax Nucleofector (Koeln, Germany) using Solution R/program K-17 as described in ref. (26). The resultant transfection efficiency was >90%. Following nucleofection, cells were replated for 24 to 48 hours after which protein lysates were harvested for Western blot analysis. To measure the duration of siRNA knockdown, cells were harvested at 0, 2, 4, 6, and 8 days following nucleofection with siRNA to B-Raf or C-Raf and subjected to Western blot analysis. Duplexed Stealth siRNA (Invitrogen) were used for these studies with the B-Raf sequences modified from ref. (27). The siRNA sequences used were as follows: wild-type B-Raf (COM4 or 4)-GGACAAAGAUGUGGAGAU-CUGGAUCAU; MUT B-Raf (MuA or A)-GGUCUAGCUACAGAGAAAUCUC-GAU; C-Raf-GGUCAAUGGCGAAAUGGAAUGAGC; LAMIN A/C-GAGGAAUCUUGCUAGAAGAACG; and VEGF-GACACATGGAGATGAGCCTCTTA.

Western Blot Analysis. For Western blot analysis, cell lysates were harvested in Petri dishes by the addition of lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mmol/L sodium molybdate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 5 μg/mL leupeptin. Whole cell lysates were centrifuged (≥10,000 × g) for 10 minutes at 4°C to remove cell debris. Proteins were quantitated using the bicinchoninic acid assay from Pierce (Rockford, IL), and 30 μg of lysate per lane were loaded onto a NuPage Gel Life Technologies, Inc. (Carlsbad, CA). Following electrophoresis, samples were transferred to polyvinylidene difluoride membrane (Pall Co., Pensacola, FL). The blots were probed with antibodies according to each supplier's recommendations: anti-pERK and anti-pMEK (Millipore, Billerica, MA); antibodies to B-Raf, C-Raf, and MEK2, and c-enamele from Santa Cruz Biotechnology (Santa Cruz, CA); and an antibody to Lamin A/C from Biomeda Co. (Foster City, CA). Secondary antibodies were conjugated with horseradish peroxidase and obtained from Santa Cruz Biotechnology. The immunoblots were developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

In vivo siRNA Studies. Animal experimentation was undertaken according to protocols approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine. Tumor kinetics were measured by s.c. injection of 1 × 10<sup>6</sup> UACC 903 or 1205 Lu cells into nude mice. After 6 days when a small tumor (50-100 mm<sup>3</sup>) had developed, the mice received an i.p. injection of adenoviruses consisting of 50 μL of vehicle (DMSO), the drug BAY 43-9006 at concentrations of 10, 50, or 100 μg/kg body weight for UACC 903 cells and 50 μg/kg body weight for 1205 Lu cells. For studies involving pretreatment with BAY 43-9006, 50 μg/kg body weight of drug was i.p. injected twice (–4 and –2 days) before s.c. injection of UACC 903 or 1205 Lu cells. The mechanism by which pharmacologic inhibition of mutant V<sup>599E</sup>B-Raf delays tumor development was identified by comparing tumors of the same size developing in parallel. This was achieved by s.c. injection of 5 × 10<sup>6</sup> UACC 903 cells followed at day 6 by i.p. injection every 2 days with 50 mg/kg of BAY 43-9006. For temporal and spatial matching of control DMSO with drug treated tumors, either 1 × 10<sup>6</sup>, 2.5 × 10<sup>6</sup> or 5 × 10<sup>6</sup> UACC 903 cells were s.c. injected and from day 6 treated i.p. with DMSO every 2 days. Drug or vehicle treated tumors of the same size developing in parallel were harvested at days 9, 11, 13, and 15 for comparison. At each time point, tumors from mice treated with vehicle or drug were harvested for analysis of cell proliferation, apoptosis, and vascular density as described previously (26, 28).

Apoptosis, Cell Proliferation, and Vessel Density Measurements in Tumors. Apoptosis measurements on formalin-fixed, paraaffin-embedded tumor sections were undertaken using the TUNEL and anti-cleaved caspase 3 (caspase 3) that uses the monoclonal antibody (PharMingen, San Diego, CA) has been described previously (26, 28). The proportional area of the tumors occupied by the vessels over the total area was calculated using the IP Lab imaging software program. For all tumor analyses, a minimum of 6 different tumors with four to six fields per tumor was analyzed and results represented as the average ± SE. In vivo pERK Measurements. To quantitate changes in pERK levels in formalin-fixed, paraaffin-embedded tumor sections, antibody retrieval was undertaken with 0.01 mol/L citrate buffer at pH 6.0 for 20 minutes in a 95°C water bath. Slides were cooled for 20 minutes, rinsed in PBS, and incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes to quench endogenous peroxidase activity. Next, sections were blocked with 1% bovine serum albumin for 30 minutes and incubated with anti-pERK antibody at a 1:100 dilution (Cell Signaling Technologies) overnight at 4°C. Following rinsing in PBS, sections were incubated with biotinylated anti-rabbit IgG for 1 hour, rinsed again in PBS, and incubated with peroxidase labeled streptavidine for 30 minutes. Visualization was accomplished using the aminothiol carbazole substrate kit for 5 to 10 minutes (Zymed Laboratories, Inc., South San Francisco, CA) and nuclei counterstained with hematoxylin before coverslip mounting using an aqueous mounting solution. The average percentage of cells ± SE that stained positive for pERK was counted from a minimum of six different tumors with four to six fields counted per tumor.

In vitro Doublet Times and In vivo Tumor Latency Periods. The in vitro doubling time of UACC 903 cells nucleofected with siRNA was estimated by plating 5 × 10<sup>3</sup> cells per well in 200 μL of DMEM supplemented with 10% FBS in multiple rows of wells in five 96-well plates. Growth was measured every 24 hours over a period of 5 days by performing a colorimetric assay on one plate each day using the Sulforhodamine B binding assay (Sigma Chemical Co., St Louis, MO) and

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the doubling time calculated, as described previously (28). The in vivo tumor latency period was measured by estimating number of days required for mean tumor size to reach 10 mm³.

**BAY 43-9006 Growth Inhibition/IC₅₀ of UACC 903 Melanoma Cells.** To measure the growth inhibitory effects or IC₅₀ of BAY 43-9006 on UACC 903 cells, 5 × 10⁴ cells per well were plated into 96-well plates. Following 24 hours, varying concentrations of BAY 43-9006 (0, 0.02, 0.1, 0.4, 1.6, 6.3, 25, or 100 μmol/L) was added to duplicate 8-strip wells in the plate. After 72 hours of growth at 37°C in a 5% CO₂ humidified atmosphere, media was discarded and cells were fixed in 10% trichloroacetic acid. Surviving cells at each concentration of the drug were calculated using the Sulforhodamine B binding assay (28). Western blot analysis was used to show the effects of increasing concentrations of BAY 43-9006 (5, 10, 15, or 20 μmol/L) on phosphorylation levels of MEK1/MEK2 and ERK1/ERK2 in UACC 903 cells following 2 hours of drug exposure.

**VEGF Expression Analysis.** To determine the amount of VEGF secreted by cells following siRNA-mediated knockdown of B-Raf protein or after treatment with BAY 43-9006, the human VEGF Quantikine kit (DVE00) was used (R&D Systems, Inc., Minneapolis, MN). UACC 903 or 1205 Lu cells (5 × 10⁴) nucleofected with the various siRNA were plated in 60-mm Petri dishes and 24 hours later media replaced with DMEM containing 2% FBS. Following an additional 24 hours, media was again replaced and conditioned media for ELISA analysis was collected 24 and 48 hours later. For BAY 43-9006 studies, 3 × 10⁵ UACC 903 or 1205 Lu cells were plated into 60-mm Petri dishes and 24 hours later media was changed to DMEM containing 2% FBS. After an additional 24 hours, media was replaced with DMEM supplemented with 2% FBS alone or in combination with BAY 43-9006 (5, 10, and 15 μmol/L) or DMSO vehicle. After 12 or 24 hours, conditioned media was collected for ELISA analysis. The media was cleared by centrifugation at 14,000 rpm (4°C) for 5 minutes and stored at −80°C. VEGF ELISA analysis was performed in triplicate on duplicate experiments according to the manufacturer’s instructions.

**Statistics.** For statistical analysis, the Student’s t test was used for pairwise comparisons and the one-way ANOVA or the Kruskal-Wallis test was used for groupwise comparisons, followed by the appropriate post hoc tests (Dunnett’s, Tukey’s or Dunn’s). Results were considered significant at \( P < 0.05 \).

**Results**

**siRNA-Mediated Targeting of Mutant V₅⁹⁹⁹B-Raf Inhibits Melanoma Tumor Development.** The role of mutant V₅⁹⁹⁹B-Raf in melanoma tumorigenesis is currently unknown. To address this issue, we reasoned that inhibition of expression or activity of mutant V₅⁹⁹⁹B-Raf protein could be used to identify the role this protein plays in melanoma tumorigenesis. An siRNA-mediated approach was used to knockdown expression of mutant V₅⁹⁹⁹B-Raf in UACC 903 and 1205 Lu cell lines containing mutant protein or B-Raf in the C8161 cell line lacking the T1796A mutation. The MuA or A siRNA was designed to reduce expression of wild-type and mutant protein whereas the Com4 or 4 siRNA only lowered expression of mutant protein as described previously (27). siRNA for these studies was introduced into the cell lines via nucleofection resulting in transfection efficiencies of >90% (data not shown; ref. 26). Effectiveness of siRNA for reducing the expression of B-Raf and C-Raf protein in UACC 903 (Fig. 1A), 1205 Lu (Fig. 1B), and C8161 (Fig. 1C) cells after nucleofection was measured by Western blot analysis. At 24 and 48 hours after nucleofection, each siRNA reduced only expression of the protein against which it was made, thereby demonstrating the specificity and effectiveness of the siRNA knockdown in each of these cell lines. In UACC 903 and 1205 Lu cells, only siRNA to B-Raf reduced phosphorylation (activity) levels of the downstream targets MEK and ERK, whereas scrambled siRNA or siRNA to C-Raf had no effect on these proteins (Fig. 1A and B). Maximal decrease in phosphorylation (activity) levels of MEK and ERK in UACC 903 and 1205 Lu cells were observed 48 hours after nucleofection. In contrast, reduced expression of B-Raf or C-Raf in C8161 cells had a negligible insignificant effect on levels of phosphorylated MEK and ERK (Fig. 1C). Thus, inhibition of V₅⁹⁹⁹B-Raf expression in melanoma cell lines containing mutant protein leads to reduced
activity of MEK and ERK, whereas lowering expression of B-Raf protein in melanoma cells lacking the T1796A mutation does not seem to affect activity of downstream targets.

To measure the effect of reduced V599E-B-Raf expression (activity) on melanoma tumor development, V599E-B-Raf expression in UACC 903 and 1205 Lu cell lines was inhibited using siRNA followed by s.c. injection into mice using a transient knockdown approach that we have reported previously (26). siRNA-mediated knockdown of protein expression persisted for a minimum of 8 days in UACC 903 (Fig. 2A) and 1205 Lu (Fig. 2B) cells. Furthermore, a corresponding decrease in pERK levels was also observed for the same period (Fig. 2B). The size of the developing tumor was measured on alternate days up to 17.5 days after nucleofection to determine the effect of B-Raf knockdown on melanoma tumorigenesis. A reduction in tumor development was observed in both UACC 903 (Fig. 2C) and 1205 Lu (Fig. 2D) cells in which mutant V599E-B-Raf expression had been knocked down. In contrast, siRNA-mediated inhibition of C-Raf, a scrambled siRNA or buffer controls did not alter tumor development. Lack of an effect following knockdown of C-Raf, suggested that signaling through V599E-B-Raf was specifically necessary for tumor development. Thus, siRNA-mediated reduction of V599E-B-Raf expression (activity) in melanoma cells before injection into mice inhibited tumorigenesis.

A similar experiment was undertaken using a Raf kinase inhibitor, called BAY 43-9006 to inhibit the activity of B-Raf protein in UACC 903, 1205 Lu, or C8161 cells. This compound, originally identified in a screen for Raf kinase inhibitors, has been shown to effectively inhibit the activity of wild-type B-Raf protein (31, 32). Initially, we determined the concentration of BAY 43-9006 that reduced UACC 903 cell survival by half, also called the IC50, and found it to be 5 to 6 μmol/L (data not shown). Therefore, a concentration of 5 μmol/L was chosen for subsequent in vitro studies. Next, we showed that BAY 43-9006 inhibited activity of both mutant and wild-type B-Raf protein to a similar extent by expressing either HA-tagged wild-type or mutant V599E-B-Raf constructs in HEK 293T cells (Fig. 3A). As reported previously, we observed levels of phosphorylated (active) ERK or MEK in cells expressing V599E-B-Raf to be 5- to 7-fold higher than in cells transfected with only wild-type B-RAF (5). HEK 293T cells expressing either wild-type or mutant V599E-B-Raf protein were then exposed to 5 μmol/L BAY 43-9006 for 2 hours to examine the effect on the activity of the signaling pathway. Exposure to BAY 43-9006 reduced levels of phosphorylated MEK and ERK in cells expressing either wild-type or mutant V599E-B-Raf protein by 5- to 6-fold and 3- to 4-fold, respectively (Fig. 3A). Thus, BAY 43-9006 inhibits the activity of both wild-type and mutant B-Raf.

To show that BAY 43-9006 inhibited mutant V599E-B-Raf protein signaling in UACC 903 cells, in vitro cultures were exposed for 2 hours to increasing concentrations of BAY 43-9006. BAY 43-9006

![Figure 2. Melanoma tumor development was inhibited with siRNA to B-Raf but not with siRNA to C-Raf or scrambled siRNA. siRNA-mediated knockdown of B-Raf protein persists for 6 to 8 days after nucleofection into UACC 903 (A) and 1205 Lu (B) cell lines growing in culture. A corresponding decrease was observed in phosphorylated ERK1/ERK2 levels (B). ERK2 served as a loading control. siRNA-mediated reduction of B-Raf led to decreased tumorigenic potential of UACC 903 (C) and 1205 Lu (D) cells. siRNA against B-Raf, C-Raf and scrambled siRNA were introduced into UACC 903 or 1205 Lu cells (white arrow) and 36 hours later cells were injected into nude mice (black arrow). Size of tumors was measured at 2-day intervals. siRNA-mediated down-regulation of B-Raf reduced the tumorigenic potential of UACC 903 and 1205 Lu melanoma cells. Control cells were nucleofected with buffer only, a scrambled siRNA or siRNA against C-Raf. Values are means of a minimum of 12 injection sites in six mice from two separate experiments; bars, ±SE.](/images/Inhibition_of_Mutant_V599E_B-Raf_in_Malignant_Melanoma.png)
reduced the levels of phosphorylated (active) MEK and ERK in UACC 903 cells in a dose responsive manner (Fig. 3B). The inhibitory effects of BAY 43-9006 on MAPK signaling persisted for at least 2 to 3 days in UACC 903 and 1205 Lu cell lines (data not shown). We next evaluated the effect of pretreating animals with BAY 43-9006 before s.c. injection of UACC 903 or 1205 Lu cells. For these experiments, mice were exposed to 50 mg/kg BAY 43-9006 for 4 days before s.c. injection of 5 × 10⁶ cells, which was followed by i.p. injection of drug every 2 or 3 days (arrowheads). Tumor size is shown at 2-day intervals up to day 22. Bars, ±SE. D, decreased tumor cell proliferation accompanies siRNA-mediated inhibition of melanoma tumor development. Five- to 8-fold decrease in bromodeoxyuridine-positive cells occurs following siRNA-mediated inhibition of B-Raf but not C-Raf or scrambled siRNA. *, P < 0.05. Columns, means from six different tumors with four to six fields counted per tumor; bars, ±SE.

**Figure 3.** Pharmacologic inhibition of B-Raf activity using BAY 43-9006 inhibits melanoma tumor development. A, BAY 43-9006 inhibits both wild-type and mutant V599E-B-Raf activity. HA-tagged wild-type or mutant V599E-B-RAF were expressed in HEK 293T cells and exposed to 5 μmol/L BAY 43-9006 or DMSO vehicle. HA indicates ectopically expressed B-Raf protein. Activation or inhibition of the MAPK pathway was determined by comparing levels of pMEK and pERK. ERK2 served as a loading control. B, BAY 43-9006 decreases pMEK and pERK (activity) levels in UACC 903 melanoma cells containing mutant V599E-B-Raf in a dose responsive manner. Western blot analysis showing reduced pMEK and pERK levels in UACC 903 cells with increasing concentrations of BAY 43-9006. The loading control was ERK2. C, pretreatment of mice with BAY 43-9006 inhibits development of melanoma tumors. Four days before injection of 5 × 10⁶ UACC 903 cells, mice were pretreated twice i.p. with 50 mg/kg BAY 43-9006 or DMSO vehicle, which continued every 2 days (arrowheads). Tumor size is shown at 2-day intervals up to day 22. Bars, ±SE. D, decreased tumor cell proliferation accompanies siRNA-mediated inhibition of melanoma tumor development. Five- to 8-fold decrease in bromodeoxyuridine-positive cells occurs following siRNA-mediated inhibition of B-Raf but not C-Raf or scrambled siRNA. *, P < 0.05. Columns, means from six different tumors with four to six fields counted per tumor; bars, ±SE.

inhibition of mutant V599E-B-Raf activity by pretreatment of the host animal with BAY 43-9006 significantly reduced the tumorigenic potential of melanoma cells expressing mutant V599E-B-Raf.

To identify the mechanism leading to tumor inhibition in cells pretreated with siRNA to knockdown V599E-B-Raf activity, rates of tumor cell proliferation and apoptosis were measured in UACC 903 tumors 4 days after s.c. injection. No difference in the rate of apoptosis (1-2%) was detected using the TUNEL assay (data not shown). However, UACC 903 cells treated with siRNA to B-Raf had 5- to 6-fold fewer proliferating cells compared with control cells nucleofected with buffer only, scrambled siRNA or C-Raf siRNA (Fig. 3D). Next, in vitro, doubling times, in vivo proliferation rates and tumor latency periods of UACC 903 cells nucleofected with V599E-B-Raf siRNA were compared to controls to determine whether reduced growth could account for delayed tumor development (Table 1). UACC 903 cells nucleofected with siRNA to C-Raf or scrambled siRNA doubled in number in vitro every 1.2 days
Table 1. Growth properties of UACC 903 cells treated with siRNA against B-Raf, C-Raf, or scrambled siRNA

<table>
<thead>
<tr>
<th>siRNA treatment</th>
<th>Doubling time in vitro in days (h)</th>
<th>% Proliferating cells at day 4 in tumors ± SE</th>
<th>Latent period for tumor formation (d)*</th>
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<tr>
<td>Scrambled</td>
<td>1.25 (30)</td>
<td>10 ± 0.7</td>
<td>5</td>
</tr>
<tr>
<td>C-Raf</td>
<td>1.1 (26)</td>
<td>15 ± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>B-Raf (4)</td>
<td>1.6 (38.4)</td>
<td>2 ± 0.6</td>
<td>14</td>
</tr>
<tr>
<td>B-Raf (A)</td>
<td>1.7 (40.8)</td>
<td>2 ± 0.4</td>
<td>16</td>
</tr>
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*Latent period for tumor formation was defined as the number of days required for mean tumor size to reach 10 mm³.

(or ~29 hours), whereas cells nucleofected with siRNA against B-Raf doubled every 1.65 days (or ~40 hours), which was a delay of ~38%. In contrast, analysis of proliferating cells in tumors showed a significant difference between control tumors nucleofected with siRNA to C-Raf or scrambled siRNA (ANOVA; \( P < 0.05 \)), which had 10% to 15% proliferating cells, compared with tumors cells nucleofected with siRNA to B-Raf that had 2% to 3% proliferating cells. The ~82% reduction in proliferative capacity of cells nucleofected with B-RAF siRNA could account for the delayed latency period of tumor development. Hence, for tumors of the same size as controls at day 5, cells nucleofected with siRNA to B-Raf required an additional 10 days to form tumors of the same size (Table 1). Because tumor development was delayed >200%, the reduced growth rate observed in vitro and in vivo could account for the reduced tumorigenic potential of these cells. Therefore, inhibition of mutant V599E-B-Raf expression (activity) in melanoma cells before tumor formation significantly reduced the in vivo growth potential of cells, thereby delaying tumorigenesis.

Inhibition of Melanoma Tumor Development by Targeting Mutant V599EB-Raf in Preexisting Tumors. It is currently unknown whether targeting mutant V599EB-Raf in established preexisting melanoma tumors could retard tumor development, and if so, whether the mechanism is the same as that occurring when targeting V599E-B-Raf in cells before tumor formation. Therefore, we next examined whether pharmacologically targeting B-Raf in preexisting melanoma tumors would inhibit tumor development by a similar mechanism. Five million UACC 903 cells, or one million 1205 Lu or C8161 cells were s.c. injected into 4- to 6-week old female nude mice. On day 6, vehicle (DMSO) or BAY 43-9006 compound dissolved in vehicle (10, 50, or 100 mg/kg) was given to mice via i.p. injection every 48 hours. A 48-hour time period between drug administrations was chosen because inhibitory effects on the MAPK signaling

Figure 4. Inhibition of B-Raf activity using BAY 43-9006 inhibits melanoma tumor development. The effects of BAY 43-9006 treatment are shown on UACC 903 (A) and 1205 Lu (B) tumor development. UACC 903 and 1205 Lu cells were injected into nude mice and tumor development allowed to occur to day 6 at which point mice were injected i.p. every 2 days with BAY 43-9006 dissolved in DMSO (arrowheads). Control conditions were DMSO treatment only. C, decreased amounts of phosphorlated (active) ERK were observed for these cells following treatment with BAY 43-9006 but not with vehicle treatment. Immunohistochemical comparison of the number of pERK-positive cells in UACC 903 tumor sections treated with 50 mg/kg BAY 43-9006 (in DMSO) or in DMSO vehicle alone. A 3-fold difference was detected between control vehicle and BAY 43-9006 treated cells (D). *, \( P < 0.05 \). Columns, means from six different tumors with four to six fields counted per tumor; bars, ± SE.
pathway in UACC 903, 1205 Lu and C8161 cells persisted for at least that period (data not shown). Size of the developing tumors was measured using calipers on alternate days and the results are shown for UACC 903 cells in Fig. 4A and 1205 Lu cells in Fig. 4B. Whereas all concentrations of the BAY 43-9006 compound slowed UACC 903 tumor development, only concentrations $\geq$50 mg/kg caused tumor development to plateau 7 days following the start of treatment (Fig. 4A). Tumor development in mice treated with BAY 43-9006 at 10 mg/kg was delayed $\approx$1 week, but UACC 903 tumors steadily increased in size and mice had to be euthanized on day 27 when tumors reached sizes $>2,400 \text{mm}^3$. For UACC 903 cells, a small increase occurred in the size of the tumor up to day 13; however, after a week of drug treatment, tumor sizes stabilized and there was no statistically significant increase in tumor sizes from days 13 to 31 (Fig. 4A; ANOVA; $P > 0.05$). Treatment of 1205 Lu tumors with 50 mg/kg BAY 43-9006 also reduced tumor development in a similar manner causing a plateau in tumor size from days 17 to 31 (Fig. 4B; ANOVA; $P = 0.12$). In contrast, whereas BAY 43-9006 inhibited pMEK and pERK levels in C8161 cells, no difference was observed in the kinetics of tumor formation (data not shown). Thus, pharmacologic inhibition of mutant V599E-B-Raf activity retards tumor development in preexisting melanoma tumors but does not cease tumor regression. In contrast, inhibition of B-Raf in melanoma cells lacking the T1796A mutation did not seem to alter tumorigenic potential.

To confirm that the BAY 43-9006 compound affected activity of the mutant V599E-B-Raf signaling pathway in tumors, the percentage of cells expressing elevated levels of phosphorylated ERK was scored in tumors from mice 9 days after start of treatment with vehicle (DMSO) or vehicle containing 50 mg/kg BAY 43-9006 (Fig. 4C). Quantification of the number of pERK-positive cells showed that BAY 43-9006 treated tumors had $\approx$3-fold fewer pERK-positive cells than control vehicle treated tumors (Fig. 4D; Student’s $t$-test; $P < 0.05$). The significantly greater number of phosphorylated ERK-positive cells in vehicle-treated tumors indicated that BAY 43-9006 was inhibiting the activity of the mutant V599E-B-Raf signaling pathway in vivo. Thus, these results show that pharmacologic inhibition of mutant V599E-B-Raf with BAY 43-9006 reduces MAPK pathway signaling in tumors, thereby mediating tumor inhibition.

**Mechanistically, BAY 43-9006 Inhibits Vascular Development of Preexisting Melanoma Tumors Leading to Increased Apoptosis.** The foregoing experiments showed a consistent relationship between inhibition of mutant V599E-B-Raf activity and reduced tumor development; therefore, subsequent studies focused on identifying the mechanism by which this occurred in existing melanoma tumors. For these studies, temporally and spatially matched UACC 903 tumors exposed to either vehicle or BAY 43-9006 were analyzed for vascular development as well as apoptosis and proliferation rates to identify the key event delaying growth of existing established tumors. Matched tumors were harvested every 2 days, starting at day 9 and ending at day 15; rates of apoptosis, growth, and vascular development were compared at each time point (Fig. 5). A statistically significant difference in vessel development at day 9 was observed between vehicle and BAY 43-9006 treated tumors (Fig. 5A; Student’s $t$-test; $P < 0.05$). In contrast, no statistically significant difference was detected in number of proliferating cells (Student’s $t$ test; $P = 0.61$) or apoptotic areas (Student’s $t$-test; $P = 0.15$) in tumor masses at day 9 between control and BAY 43-9006 treated tumors (Fig. 5B and C). However, for all analyses from day 11 onwards, a statistically significant difference was observed between control and drug treated tumors (Student’s $t$ test; $P < 0.05$). Collectively, these data suggest that significantly reduced vascular development observed at day 9 in BAY 43-9006 treated tumors was an initiating event leading to delayed tumor growth. Apoptosis became evident in the BAY 43-9006 treated tumors at day 11 and occupied up to 25% of the tumor area by day 15 (Fig. 5B). By day 20, $\approx$50% of the tumor area was undergoing apoptosis (data not shown). BAY 43-9006 also affected tumor cell proliferation of preexisting tumors leading to a 32% to 57% decrease in percentage of proliferating cells (Fig. 5C). Collectively, these data led to the conclusion that inhibition of vascular development is a key event leading to growth inhibition of preexisting melanoma tumors.

Because vascular development in tumors occurs via angiogenesis, or the growth of new vessels from the surrounding vascular beds, and is triggered by angiogenic factors secreted by tumor cells (33), we predicted that BAY 43-9006 and siRNA-mediated inhibition of V599E-B-Raf were reducing the activity of a key angiogenic factor, thereby decreasing vascular development (34, 35). To examine this possibility, an ELISA assay was used to determine whether secretion of VEGF decreased following inhibition of V599E-B-RAF. Initially, UACC 903 and 1205 Lu cells in which V599E-B-Raf expression was inhibited using siRNA were examined and revealed significant reduction in VEGF secretion compared with controls (Fig. 6A). Next,
the effects of BAY 43-9006 mediated inhibition of V599EB-Raf in UACC 903 and 1205 Lu cells was examined and also found to decrease VEGF secretion in a dose dependent manner (Fig. 6B). To determine whether siRNA-mediated reduction of VEGF resulted in tumor inhibition similar to that seen following V599EB-Raf inhibition, siRNA against VEGF was nucleofected into UACC 903 or 1205 Lu cells. Decreased VEGF expression was observed using VEGF specific siRNA (Fig. 6A), which reduced the tumorigenic potential of UACC 903 (Fig. 6C) and 1205 Lu (Fig. 6D) cells in a manner consistent with that occurring following decreased V599EB-Raf expression. Thus, reduced VEGF secretion mediated by decreased V599EB-Raf activity led to inhibition of vascular development, which consequently affected melanoma tumor development.

**Discussion**

This study shows that use of siRNA or pharmacologic inhibition of mutant V599EB-Raf expression (activity) effectively reduces the tumorigenic potential of melanoma cells by lowering the proliferative and/or angiogenic capacity of the tumor cell. As such, melanoma cells having mutant V599EB-Raf are better suited for proliferation in the *in vivo* tumor environment. We have shown that targeted reduction of V599EB-Raf expression (activity) in melanoma cells before tumor development significantly reduced the growth potential of melanoma cells, thereby inhibiting tumor development. In contrast, apoptosis played no significant role in this process. Furthermore, inhibition of tumor development was only observed in cells in which mutant V599EB-Raf expression had been knocked down and not following knockdown of C-Raf or following knockdown of B-Raf in melanoma cells lacking the T1796A B-RAF mutation. Therefore, it is apparent that signaling through V599EB-Raf was specifically necessary for melanoma tumor development. These data are consistent with our previous study demonstrating that siRNA-mediated inhibition of V599EB-Raf in WM793 melanoma cells reduced the *in vitro* growth potential of these cells (27). Similar *in vitro* studies using UACC 903 cells in this report further confirm these earlier observations. Knockdown of mutant V599EB-Raf expression (activity) also specifically reduced constitutive ERK signaling leading to reduced growth, which did not occur following knockdown of C-Raf. Thus, mutant V599EB-Raf promotes growth of melanoma cells both *in vitro* and *in vivo*. Moreover, targeted inhibition before tumor development inhibits tumorigenesis mediated through reduced growth of tumor cells.

Targeting mutant V599EB-Raf in preexisting established tumors halted growth; however, growth inhibition played only a partial role in this process. More significantly, comparison of size- and time-matched tumors revealed that inhibition of vascular development played an initiating role in delaying tumor growth. As in all solid tumors, vascular development occurs through angiogenesis.
in which growth of new vessels from surrounding vascular beds is driven by angiogenic factors secreted by tumor cells (33). In this study, we found that inhibition of V999E-B-Raf reduced VEGF secretion by UACC 903 and 1205 Lu melanoma cells. B-Raf has been reported to exert an important role in embryonic vascular development because B-Raf knockout mice exhibit significant endothelial cell death leading to hemorrhage and embryonic lethality (36). However, we observed no significant endothelial cell death in preexisting tumor vessels following inhibition of V999E-B-Raf using BAY 43-9006. Rather, inhibition of V999E-B-Raf inhibited angiogenesis, halting growth of preexisting melanoma tumors. Our study also shows that BAY 43-9006 inhibits V999E-B-Raf activity in vitro and in vivo, leading to reduced phosphorylation of downstream targets MEK and ERK, which slowed melanoma tumor development. We observed that pretreatment of animals with BAY 43-9006 reduced melanoma tumor development in manner similar to siRNA-mediated inhibition. However, BAY 43-9006 treatment only retarded development of established tumors by disrupting their vascular development. Complete regression of tumors did not occur, rather tumor size became relatively static after treatment. This observation is in agreement with preliminary data from clinical trials in which BAY 43-9006 monotherapy was relatively ineffective for treatment of advanced stage melanoma patients (13, 39). However, in combination with traditional chemotherapy (paclitaxel and carboplatinum), a 50% response rate occurred in patients (13, 40). Therefore, whereas BAY 43-9006 slows tumor development, it is likely that the drug will need to be combined with other synergistic therapeutics to cause regression of established preexisting tumors (13, 14, 32). It is also possible that the route of drug administration could alter efficacy of BAY 43-9006 in melanoma patients. Whereas the clinical trial involved oral administration of the drug, our study gave the drug via i.p. injection every 2 to 3 days. An alternative route of administration might be more effective by increasing the drug’s local bioavailability (41–44). Therefore, therapeutically targeting V999E-B-Raf activity in combination with chemotherapeutic agents may offer an effective approach to shrink established melanoma tumors containing this mutant protein.

In conclusion, we identified mechanisms by which mutant V999E-B-Raf promotes melanoma tumor development and show how this mutation provides melanoma cells with selective growth and angiogenic advantages in the tumor environment.

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References

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