Molecular determinants of melanoma malignancy: selecting targets for improved efficacy of chemotherapy

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Abstract

The BRAFV600E mutation is common in human melanoma. This mutation enhances K:B kinase (IKK)/nuclear factor-κB (NF-κB) and extracellular signal-regulated kinase/activator protein signaling cascades. In this study, we evaluated the efficacy of targeting either B-Raf or IKKβ in combination with the DNA alkylating agent temozolomide for treatment of advanced metastatic melanoma. Xenografts of Hs294T human metastatic melanoma cells exhibiting the BRAFV600E mutation were treated with inhibitors of IKKβ (BMS-345541), B-Raf (BAY 54-9085), and/or temozolomide. Drug response was mechanistically analyzed in vitro and in vivo. In this study, we determined that the antitumor activity of all three drugs depends on inhibition of NF-κB. BMS-345541 inhibits IKKβ-mediated phosphorylation of IkBα and thus blocks the nuclear localization of NF-κB, whereas BAY 54-9085 inhibits activation of NF-κB through a mechanism that does not involve stabilization of IkBα. Moreover, BMS-345541, but not BAY 54-9085, activates the death pathways of p53 and c-Jun-NH2-kinase, contributing to the killing of melanoma cells. Temozolomide inhibits both NF-κB and extracellular signal-regulated kinase activity, conferring effective in vivo antitumor activity. Thus, temozolomide, but not BAY 54-9085, has a synergistic in vivo antitumor effect with BMS-345541. We conclude that the efficacy of antimelanoma therapy depends on inhibition of expression of antiapoptotic genes transcriptionally regulated by NF-κB. In contrast, drug targeting of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway alone in melanoma cells is ineffective for melanoma therapy in cases where NF-κB is not also targeted. [Mol Cancer Ther 2009;8(3):636–47]

Introduction

Malignant melanoma is the most deadly skin cancer and its incidence is growing more rapidly than other types of cancer (1). Moreover, metastatic melanoma is largely refractory to the current therapies and has a poor prognosis, with a mean 6-month survival rate and a <5% five-year survival rate. The effect of current systemic therapies on advanced melanoma remains dismal. For example, the most effective chemotherapeutic agent for melanoma has a <20% response rate and a <5% complete response rate (2). Combinations of chemotherapeutic agents or biochemotherapeutic regimens have not yet improved patient survival rate compared with single-agent chemotherapy (3). Thus, it is imperative to improve our understanding of melanoma biology and develop new therapeutic approaches (4).

Melanoma is a complex genetic disease. Mutations in critical regulatory genes disrupt intracellular signaling in melanocytes, allowing them to produce autocrine growth factors and lose growth inhibitory signals and cell cycle regulation (5). Consequently, melanocytes with constitutive proliferative signal activation and loss of tumor suppressor genes often develop into melanoma lesions and progress to metastatic melanoma. One of the major oncogenic events of malignant melanoma is activation of the Ras/Raf/Mek/extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK) pathway. More than 90% human melanoma cells exhibit hyperactivation of Erk, and this is often due to gain-of-function mutations in the signaling gene BRAF. Ninety-two percent of BRAF mutations involve substitution of a valine for a glutamic acid at codon 600 (V600E) in exon 15 (6). BRAF mutation is acquired during melanoma development and is not linked to familial melanoma (7). The BRAFV600E is responsible for the increased phosphorylation of MAPK/Erk kinase (Mek) and Erk and increased expression of cyclin D1 in melanoma cells (8). Moreover, animal models support the notion that BRAFV600E is oncogenic when introduced into melanocytes, leading to constitutive activation of Erk and resulting in tumorigenicity in nude mice (9) and zebrafish (10). Expression of BRAFV600E triggers the constitutive activation of the transcription factor nuclear factor-κB (NF-κB; ref. 11), which regulates the expression of many...
The constitutive activation of NF-κB also contributes to the intrinsic resistance of melanoma cells to systemic biotherapy and/or chemotherapy (13). We have shown and documented that NF-κB is a key player in human melanoma tumorigenesis (14, 15). Consequently, the MAPK and NF-κB signal pathways may play crucial roles in the development of melanoma and are therefore the ideal therapeutic targets. However, insight into these two signal pathways that coordinately regulate the apoptosis machinery remains poorly defined. Toward this aim, we have studied drugs or inhibitors that target these pathways in melanoma. For example, BAY 54-9085/sorafenib tosylate is a multikinase inhibitor that selectively blocks the Raf kinase isoform–triggered MAPK cascade (IC₅₀ 25 nmol/L for wild-type B-Raf, 38 nmol/L for mutant B-Raf, and 6 nmol/L for C-Raf; ref. 16) and also indirectly inhibits NF-κB in vitro (11). The IkB kinase (IKK) inhibitor, BMS-345541, has a dramatic efficacy for melanoma in preclinical settings (17).

Figure 1. Drugs with antimelanoma activity. A, chemical structure. TMZ is metabolically activated to MTIC [3-methyl-(triazen-1-yl)imidazole-4-carboxamide] by chemical decarboxylation and hydrolysis; BAY 54-9085 is the tosylate salt of BAY 43-9006; BMS-345541, 4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline)-4,5-dihydro-1,8-dimethylimidazo(1,2-a)quinoxalin-4-one-2-carboxylic acid. B, induction of melanoma cell death through targeting of B-Raf and IKK. Melanoma cells (2 × 10⁵) of either Hs294T, Sk-Mel-28, or A375 melanoma cell line were cultured in serum-free medium in six-well plates in triplicate and treated with 10 μmol/L of TMZ, BMS, or BAY for 48 h. Subsequently, adherent cells were trypsinized, collected, and counted. The percentage of surviving cells in the drug treatment group was normalized to the mock treated controls. Columns, mean percent change in cell growth; bars, SD. *, P < 0.05; **, P < 0.01, compared with corresponding vehicle control (Student’s t test). BMS, BMS-345541; BAY, BAY 54-9085. C, Erk lacks the synergic role on BMS-induced cell death. Hs294T melanoma cells (2 × 10⁵) were exposed to 10 μmol/L BMS and/or 20 μmol/L PD 98-059 for 24 h. The viable cells were counted and normalized to the DMSO vehicle-treated control. There was no statistical difference between the survival of cells treated with BMS versus BMS plus PD. D, analysis of promoter transcription activity. Melanoma cells (1 × 10⁵) were cultured in serum-free medium in 12-well plates in triplicate, and each well was transfected with 0.5 μg DNA vector encoding NF-κB-Gluc, AP-1-Gluc, or p53-Gluc reporter using FuGENE 6 Transfection Reagent as described in Materials and Methods. Twenty-four hours after transfection, the cells in serum-free medium were treated with the indicated concentrations of TMZ, BMS, or BAY. Twenty-four hours after treatment, the activity of Gaussia luciferase in 10 μL of the culture medium was measured. The promoter activity was calculated as fold of the reporter activity in the vehicle control group. *, P < 0.05; **, P < 0.01, versus corresponding vehicle control (Student’s t test).
elements of melanoma resistance to current chemotherapy. Therefore, the efficacy of chemotherapy against melanoma depends on the regulation of expression of NF-κB–mediated antiapoptotic genes. In contrast, our preclinical study in mice reported here using Hs294T human melanoma xenografts in nude mice shows that drug targeting of the Erk/MAPK pathway is ineffective for melanoma therapy when NF-κB is not also targeted.

**Materials and Methods**

**Reagents and Generation of Constructs**

TMZ (Temodal/Temodar), purchased from Schering Corp., was dissolved in distilled water. BAY 54-9085, provided by Bayer Corp., was dissolved in Cremophor EL/ethanol/water (12.5:12.5:75) for both in vitro and in vivo experiments. BMS-345541 was provided by the Bristol-Myers Squibb Pharmaceutical Research Institute. BMS-345541 was dissolved in DMSO to make up a 50 mmol/L stock solution for in vitro experiments or was dissolved in distilled water for in vivo experiments. Antibodies to phospho-Erk (sc-7383), Erk (sc-94), phospho-c-jun NH2-terminal kinase (JNK; sc-6254), JNK2 (sc-572), phospho-Mek1/2 (S218/222), cyclin D1 (sc-718), p53 (sc-6243), and actin (sc-1616) were purchased from Santa Cruz Biotechnology, Inc. Antibodies to phospho-Akt (Ser473, 587F11), Akt, phospho-p65 (Ser536), Bcl-2, and Bax were purchased from Cell Signaling Technology, Inc. The melanoma cell lines, Hs294T, SK-MEL-28, and A375, originally established from human metastatic melanoma, were purchased from American Type Culture Collection. These cell lines were selected and cultured in serum-free DMEM/Ham’s F-12 medium containing 2 mmol/L L-glutamine, 100 mmol/L MEM nonessential amino acids (Invitrogen Corp.), and 1 mmol/L sodium pyruvate (Sigma-Aldrich). Construction of NF-κB and activator protein (AP-1) Gaussia luciferase reporter gene vectors was done by inserting the Gaussia luciferase gene from the pCMV-Gluc-1 vector (Targeting System) at Hin6III and XbaI restriction sites to replace the luciferase gene of pNF-κB-Luc and the pAP1-Luc vectors (BD Biosciences Clontech). The two vectors were named as pNF-κB-Gluc and pAP1-Gluc, respectively. For generating

![Figure 2](image_url)

Figure 2. Molecular determinants of drug antitumor efficiency. **A**, analysis of phospho-Erk. Hs294T melanoma cells (1 x 10⁶) were cultured in serum-free medium in 10-cm culture dishes and treated with TMZ, BMS, BAY, or PD at 10 or 20 μmol/L concentrations. Twenty-four hours after treatment, cell lysates were prepared and subjected to immunoblotting for phospho-Erk. **B**, molecular responses to therapeutic agents. Hs294T melanoma cells (1 x 10⁶) were cultured in serum-free medium and treated with 10 μmol/L BMS and/or 10 μmol/L BAY. After 24-h treatment, cells were lysed and immunoblotted to detect the phosphorylation of Erk1/2, Akt, NF-κB/p65, and JNK as well as the expressed levels of Bax, p53, and cyclin D1. The densitometry values below the bands are fold change as compared with control. Data are representative of at least three independent experiments. **C**, cytochemistry of phospho-Erk. Hs294T cells were exposed to 10 μmol/L BMS or 10 μmol/L BAY for 24 h. The overlap of phospho-Erk (green) and nuclear propidium iodide staining (red) is noted by the yellow color.
the p53-Gluc vector, two copies of the p53-consensus binding site concatemer (80 bp; ref. 20) in the pGL3-promoter vector (Promega Biosciences, Inc.) were used to replace the NF-κB sequence at NheI/HindIII sites within pNF-κB-Gluc vector.

Western Blot Analysis
Proteins from cell extracts or tumor tissues were subjected to Western blotting following the protocol previously described (17).

Immunocytochemistry
Hs294T cells were cultured on coverslips in six-well plates, treated with individual drugs, fixed in 4% paraformaldehyde (Electron Microscopy Sciences), and blocked with 2% bovine serum albumin in 1× PBS containing 0.5% Triton X-100. Cells were incubated with the anti–phospho-Erk monoclonal antibody at a dilution of 1:800. After 2 h of incubation, cells were washed twice with 1× PBS and incubated with the secondary antibody conjugated with FITC for 1 h. Nuclei were stained with propidium iodide (0.5 μg/mL) for 10 min. After washing thrice, coverslips were mounted onto microscope slides. The slides were analyzed using a confocal laser-scanning microscope.

Transfection and Reporter Gene Assay
Melanoma cells (2 × 10^5) were cultured in 10% fetal bovine serum medium in six-well tissue culture plates and transfected with 0.5 μg vector DNA of the promoter-specific Gaussia luciferase reporter gene using FuGENE 6 Transfection Reagent (Roche Diagnostics Corp.) following the manufacturer’s protocol. Twenty-four hours after transfection, the cells were placed in serum-free medium and cultured for an additional 24 h; conditioned medium was collected and stored at −20°C. Media were replaced and cells were treated with each individual drug at varying concentrations for 24 h. Gaussia luciferase activity in 10 μL of medium from either before or after treatment was measured using Gaussia Luciferase Assay kit (Targeting System) according to the manufacturer’s instruction. The activity of Gaussia luciferase was detected from a 10-s constant light emission by the Monolight TM 3010 luminometer (BD Biosciences Pharmingen). The promoter reporter activity in the drug-treated cells was normalized to Gaussia luciferase activity in the cells 24 h post-transfection before treatment.

Cell-Based IKK Activity Assay
Melanoma cells expressing an IκB-fused Gaussia luciferase (IκB-Gluc) protein were used to measure IKK activity. To assemble the IκB-Gluc reporter, the Kozak consensus sequence and human IκBα gene (accession no. BC002601) lacking a stop codon were ligated with Gaussia luciferase gene that had been lacking start codon amplified from the pCMV-Gluc-1 vector (Targeting System). The IκB-Gluc gene was cloned into the NheI and XhoI sites of the pTracer-CMV/Bsd vector (Invitrogen). IKK reporter cell lines were created from the human melanoma cell line Hs294T expressing the stable IκB-Gluc fusion protein by transfection with Scal linearized vector followed by selection with 2 μg/mL blasticidin. The fusion protein of IκBα-Gaussia luciferase has been characterized for secretion into the culture medium and catalytic reaction with its substrate, coelenterazine. IKK reporter values were assayed over time by addition of 100 μmol/L of coelenterazine substrate and normalized to Gaussia luciferase activity that was determined before treatment. The luciferase readings at each time point are the percentage of the luciferase reading obtained from the culture medium before treatment.

Approaches for Drug Delivery and Tumor Measurement
Animal experimentation was done according to protocols approved by the Institutional Animal Care and...
Use Committee at Vanderbilt University. BALB/c-nu/nu female mice (10 per group) of ages 8 to 10 wk were s.c. inoculated with $2 \times 10^6$ Hs294T melanoma cells suspended in 200 μL of PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na₂HPO₄, 1.47 mmol/L KH₂PO₄, and adjusted to a final pH of 7.4). The delivery of drugs and tumor measurement were carried out as we have previously described (17).
Pharmacokinetic Analysis of Drug-Drug Interactions

Plasma samples (25 μL) were deproteinized with 2 volumes of acetonitrile containing 1 μg/mL BMS-724656 as internal standard. After centrifugation to remove precipitated protein, a 5-μL portion of clear supernatant was analyzed by high-performance liquid chromatography (HPLC)-tandem mass spectrometry. The HPLC system consisted of an Agilent model 1100 HPLC/Autosampler combination. The column used was a Phenomenex Luna C18 (2) 50 × 2 mm × 2 μm particles, maintained at 40°C and a flow rate of 0.4 mL/min. The mobile phase consisted of 5 mmol/L ammonium formate in 90% water/10% acetonitrile, pH 3.75 (A) and acetonitrile (B). The initial mobile phase composition was 80%A/20%B. After sample injection, the mobile phase was changed to 25%A/75%B over 1 min and held at that composition for an additional 3 min. The mobile phase was then returned to initial conditions, and the column re-equilibrated. The HPLC was interfaced to a Finnigan LCQ ion-trap mass spectrometer operated in the positive APCI, full tandem mass spectrometry mode. For BMS-345541, fragmentation of m/z 256 [M+H]+ yielded daughter ion for quantitation at m/z 239. For the internal standard, m/z 406 [M+H]+ was fragmented to yield quantitation ion of m/z 364. Helium was the collision gas. The retention times for BMS-345541 and the internal standards were 3.4 and 4.0, respectively. The standard curve ranged from 8 nmol/L to 31 μmol/L and was fitted with a quadratic regression weighed by reciprocal concentration (1/x). The lower limit of quantification for the purposes of this assay was 21 nmol/L. Quality control samples at two levels in the range of the standard curve were used to accept individual analytic sets.

For the analysis of BAY 54-9085, the HPLC was interfaced to a Finnigan LCQ ion-trap mass spectrometer operated in the positive electrospray, full tandem mass spectrometry mode. Fragmentation of m/z 465 [M+H]+ yielded daughter ion for quantitation at m/z 270. For the internal standard, m/z 406 [M+H]+ was fragmented to yield quantitation ion of m/z 364. Helium was the collision gas. The retention times for BAY 54-9085 and the internal standards were 3.8 and 3.4 respectively. The standard curve ranged from 5 nmol/L to 17 μmol/L and was fitted with a quadratic regression weighed by reciprocal concentration (1/x). The lower limit of quantification for the purposes of this assay was 20 nmol/L. Quality control samples at two levels in the range of the standard curve were used to accept individual analytic sets.

Results

In vitro Antitumor Activity Associated with NF-κB but not Erk/AP-1

Hs294T cells derived from metastatic melanoma possess all malignant properties including abnormal nuclear morphology, lack of anchorage dependence for growth, aggressive growth on a contact-inhibited monolayer, and tumorigenicity in athymic mice (21). Hs294T cells were chosen for the experimental model due to highly constitutive Erk/AP-1 and IKK/NF-κB (22, 23). When cultured melanoma cells were treated with TMZ alone at a concentration of 10 μmol/L, TMZ was not effective in inducing cell death. This failure to observe biological activity for TMZ in vitro was surprising because, normally, TMZ spontaneously undergoes conversion to its active MTIC [3-methyl-(triazlen-1-yl)]imidazole-4-carboxamide] form in physiologic conditions. However, a pH greater than 7.0 facilitates the conversion to its active form, and this occurs more readily in tissue (especially tumor tissue) than in plasma, offering a possible synergistic effect. For BAY 54-9085, the HPLC was interfaced to a Finnigan LCQ ion-trap mass spectrometer operated in the positive APCI, full tandem mass spectrometry mode. Fragmentation of m/z 465 [M+H]+ yielded daughter ion for quantitation at m/z 270. For the internal standard, m/z 406 [M+H]+ was fragmented to yield quantitation ion of m/z 364. Helium was the collision gas. The retention times for BAY 54-9085 and the internal standards were 3.8 and 3.4 respectively. The standard curve ranged from 5 nmol/L to 17 μmol/L and was fitted with a quadratic regression weighed by reciprocal concentration (1/x). The lower limit of quantification for the purposes of this assay was 20 nmol/L. Quality control samples at two levels in the range of the standard curve were used to accept individual analytic sets.

Real-time Intra-melanoma NF-κB Activity Assay

To examine the real-time intratumoral NF-κB activity via the luminescent imaging approach, coelenterazine (10 μg/g body weight) was i.v. injected to each mouse immediately before luminescent imaging using the IVIS 200 Imaging System (Xenogen Corp.). The luminescent intensity of each tumor was quantified to reflect the relative NF-κB activity in vivo. To determine intratumor NF-κB activity via tissue lysate assay, tumor tissue lysate was prepared by sonicating tumor tissue in PBS, and the bioluminescence was immediately measured after addition of 100 μmol/L of coelenterazine substrate.

Statistical Analysis

Results are expressed as mean ± SD from three independent experiments. Statistical analysis was done using the unpaired Student t test. P < 0.05 was considered statistically significant.

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To test the potential role of inhibition of MAPK signaling on the induction of cell death by the IKK inhibitor, cells were exposed to BMS-345541 (10 μmol/L) plus 20 μmol/L of the Erk inhibitor, PD 98-059. Although 20 μmol/L of PD 98-059 alone resulted in a 13% reduction in the cell survival rate, it showed no synergic effect in combination with BMS-345541 (10 μmol/L; Fig. 1C).

Because abnormal cross talk between NF-κB, AP-1, and p53 is associated with tumorigenesis (25, 26), we postulated that inhibitor targeting of one of these pathways might produce a transcriptional response from the other pathways. To this end, promoter reporter DNA vectors for NF-κB, AP-1, or p53 were transiently transfected into Hs294T cells. Cells were treated with TMZ, BAY 54-9085, or BMS-345541 and the reporter Gaussia luciferase activity was determined. In contrast to TMZ, NF-κB transcriptional activity was significantly inhibited by 10 μmol/L of either BMS-345541 (77 ± 8% inhibition, P < 0.01) or BAY 54-9085 (65 ± 7% inhibition, P < 0.01); AP-1 activity was markedly elevated by 2.4 ± 0.41-fold with 10 μmol/L of BMS-345541 and declined by 84 ± 0.12% with 10 μmol/L of BAY 54-9085 (P < 0.01); p53 activity was induced by 28 ± 8% with 10 μmol/L of BMS-345541 (P < 0.05) and inhibited 33 ± 7% with 10 μmol/L of BAY 54-9085 (P < 0.05; Fig. 1D). These data indicate the diversity of drug effects on signal networks. However, the alteration of NF-κB transcriptional activity is more directly associated with drug antitumor efficacy (Fig. 1B).

**Inhibition of the NF-κB, but not MAPK, Pathway Is Responsible for Cell Death**

To examine drugs that regulate the NF-κB, AP-1, and p53 pathways and their target protein levels of genes that are involved in the initiation of apoptosis, melanoma Hs294T cells were exposed to BAY 54-9085 and/or BMS-345541. Cytosolic proteins were analyzed by Western blot with the indicated antibodies. It was obvious that Erk phosphorylation of p65 and Erk in melanoma. Hs294T melanoma cells were exposed to BAY 54-9085 and/or BMS-345541 for 48 hours underwent massive cell death when the inhibitor concentrations were >10 μmol/L. (Fig. 1B).
lation was induced by treatment with BMS-345541 and inhibited by BAY 54-9085 (Fig. 2A). Total Erk protein served as a loading control. Hyperphosphorylation of Erk induced by BMS-345541 may account for the enhancement of AP-1 activity (Fig. 1D). However, this increase in AP-1 activity did not rescue cells from BMS-345541–induced death. Interestingly, BMS-345541, but not BAY 54-9085, reactivated the death pathways by increased expression of p53 (10 ± 3.9-fold), JNK (3 ± 1.8-fold), and Bax (1.8 ± 1.8) and down-regulated the phosphorylation of Akt and the p65 subunit of NF-κB (Fig. 2B). The expression of Bcl-2 was not significantly changed after the above treatments (data not shown). The response of these proteins in Hs294T cells on drug treatments is similar to that in Sk-Mel-28 cells (data not shown). We postulate that BAY 54-9085 works through an alternative pathway to indirectly inhibit NF-κB and deplete cyclin D1 expression, thus affecting the cell survival pathway in cell culture (11).

Reentry into the cell cycle from quiescence (G0) requires the activation of Erk. For instance, phosphorylated Erk is active and concentrated in the nucleus in cycling cells but is largely confined to the cytoplasm during G0 (27). We examined the physical location of the phosphorylated Erk when Hs294T cells were treated with 10 μmol/L BMS-345541 for 24 hours. As shown in Fig. 2C, BMS-345541–induced cytoplasmic hyperphosphorylation of Erk (pErk) failed to translocate to the nucleus. In contrast, BAY 54-9085 did not reduce nuclear pErk. These findings indicate that the nuclear compartment of pErk may play major role in cell survival. Alternatively, the cytosolic versus nuclear pErk may be more a result of BMS-345541 and BAY 54-9085 effects on cell cycle.

**BMS-345541, but not BAY 54-9085, Inhibition of NF-κB Is Erk-B Dependent**

Recent published work suggests that mutational activation of B-Raf triggers IKK activation and IκB degradation, Contributing to the constitutive NF-κB activation in human melanomas (11). Based on this result, we speculated that the B-Raf inhibitor BAY 54-9085 would inhibit NF-κB via the canonical pathway (11). To examine this concept, we used a cell-based IKK activity assay over the time course of inhibition treatment, allowing dynamic quantitation of inhibition of IKK activity, where experiments were done using Hs294T melanoma cells that stably express IκB-α-Gluc fusion protein. In this reporter system, the 16-amino-acid NH2-terminal signal sequence of Gluc remains in the fused IκB-α-Gluc protein, allowing Gluc to be secreted into the culture medium to serve as a reporter reflecting the cellular IκB-α level. This system offers a very sensitive approach for quantitation of the level of IκB-α by detecting the Gaussia luciferase activity in the culture medium. If IKK is inhibited and/or IκB-α is not degraded, then IκB-α-Gluc will remain constant or increase. We observed that the highly selective IKK inhibitor BMS-345541 steadily protected IκB-α from degradation in a time- and dose-dependent fashion (Fig. 3A and B). Cells exposed to 10 μmol/L of BMS-345541 for 24 hours exhibited a 6.54-fold increase in IκB-α reporter (Gluc secretion) in the culture medium. The intracellular IκB-α reporter increased by 12.3-fold in response to 10 μmol/L BMS-345541 (Fig. 3C). In contrast, cells treated with BAY 54-9085 for 24 hours failed to show increased IκB-α reporter secretion into the culture medium or retention of IκB-α-Gluc in cell lysates (P > 0.05; Fig. 3). Tumor necrosis factor α as an IKK stimulus produced significant degradation of IκB-α-Gluc at 2 hours treatment (P < 0.05; Fig. 3A). Taken together, these data suggest that BAY 54-9085 inhibition of B-Raf to reduce NF-κB activity (Fig. 1D) was potentially possible in part through effects on Akt activity (Fig. 2B, lane 3) rather than through effects on the IKK/IκB pathway (Fig. 3A and B). Our data indicate that activating B-Raf mutation induces NF-κB activation through an alternative pathway.

**In vivo Antitumor Activity of TMZ, BAY 54-9085, and/or BMS-345541**

To examine whether TMZ, BAY 54-9085, or BMS-345541 antitumor activity in vivo also occurs in vivo, Hs294T melanoma cells were s.c. inoculated into BALB/c nu/nu nude mice. When tumor size reached ~50 mm3, tumor-bearing mice received oral doses of TMZ (30 mg/kg b.i.d.), BMS-345541 (30 mg/kg b.i.d.), or BAY 54-9085 (40 mg/kg daily) alone or in a variety of combinations as indicated. The control group was treated with vehicle only. After 14 days of treatment, tumor size was determined. In contrast to the tumor size increase of 625% in the control group, mice-bearing tumors treated with TMZ or BMS-345541 showed only 20% or 68% increase in tumor size, respectively. Tumor-bearing mice treated 14 days with the combination of TMZ and BMS-345541 exhibited a 4.6-fold regression in tumor volume. In contrast, BAY treatment alone did not yield antitumor activity. Thus, BAY induction of melanoma tumor cell death in vivo was not equally effective in vivo. However, the combination of TMZ with BAY resulted in an ~30% inhibition in tumor growth over sorafenib alone (which was equivalent to control). Surprisingly, the combination of BAY 54-9085 with either TMZ or BMS-345541, both of which were effective as single agent, resulted in drastic reduction in the antitumor activity of TMZ or BMS-345541 alone (Fig. 4A). To rule out potential pharmacokinetic interaction as a cause of the apparent antagonism between BAY 54-9085 and BMS-345541, the concentrations of BMS-345541 and BAY 54-9085 were determined in the plasma and tumors of mice treated with the individual agents or the combination. The concentrations of BMS-345541 in both the plasma and tumors were higher in the combination group, thus establishing that reduction of drug exposure was not a cause of the observed antagonism (Fig. 4B). To further test the efficacy of the combination therapy on late-stage tumors, mice bearing larger tumors (570 ± 97 mm3) were p.o. given 30 mg/kg BMS-345541 and 30 mg/kg TMZ twice a day for 12 days. The tumor shrinkage was 27 ± 15% within the first 8 days of treatment and the average tumor size was reduced to 168 ± 46 mm3 with the 8-day treatment regimen. However, tumor volume did not decline further when treatments were continued for 4 additional days (Fig. 4C).
To characterize the histologic features of the treated or untreated tumors, melanoma tumor sections from mice treated with monotherapy or multiple therapy were stained with H&E. In contrast to tumors from control mice, tumors from BMS-345541–treated mice exhibited pyknotic nuclei that are recognized as a signature of apoptotic cells. There were deformed melanoma nuclei that might represent senescent cells (28) and fibrocytes invading into the melanoma tissues from TMZ-group mice, resulting in coincident fibrosis. We failed to observe the same alteration in sections of tumors from BAY 54-9085–treated mice. Surprisingly, tumor sections from the mice treated with the combination of BMS-345541 and TMZ showed gain of pigmentation and were fibrotic (Fig. 4D). To clarify the phenotype of fibroblast-like cells, tumor sections were stained with antibodies to vimentin (fibroblast marker) and keratin 14 (keratinocyte marker). Figure 4E shows that these fibroblast-like cells with positive vimentin staining are of fibroblast origin and were most likely from mesenchymal fibroblasts that mainly reside in the dermis.

**Drug Antitumor Activity In vivo Is NF-κB Dependent**

Because the agents tested in this study exhibited dramatically different antitumor activities in vitro and in vivo, we speculated that drug metabolism in the liver may affect the antitumor activity of drugs, such as TMZ. To determine whether the drugs administrated (BMS-345541, TMZ, and BAY 54-9085) affected NF-κB activity in tumors growing in mice, the melanoma Hs294T cells that stably express the NF-κB promoter reporter (Gaussia luciferase) were s.c. inoculated into nude mice. After tumor size reached ~400 mm³, tumor-bearing mice were p.o. given TMZ (30 mg/kg bi-daily), BMS-345541 (30 mg/kg bi-daily), or BAY 54-9085 (40 mg/kg daily). Four days after treatment, the real-time luminescent luciferase image was taken (Fig. 5A) after tail vein injection with the Gaussia luciferase specific substrate, coelenterazine, and quantified (Fig. 5B). Data shown here indicate that the intratumoral NF-κB activity was inhibited by either TMZ (44.9% inhibition, P < 0.05) or BMS-345541 (60.6% inhibition, P < 0.01) but was elevated by BAY 54-9085 (56%, P < 0.05). To further quantitate the intratumoral NF-κB activity, tumor tissue lysate from tumor-bearing mice was prepared and subjected to the luminescence assay in vitro. The quantified NF-κB activity (data not shown) exhibited a pattern highly similar to that of Fig. 5B. In contrast, BMS-345541, TMZ, or BAY 54-9085 showed totally different antitumor activity in vitro and in vivo as a single agent. The potential efficacy of these drugs is in part dependent on their regulation of intratumoral NF-κB activity. To clarify whether drug-mediated effects on NF-κB activity are through regulation of the IKK complex, Hs294T(IκB<sub>Gluc</sub>)–reporter cells were s.c. inoculated into nude mice. After tumor size reached 400 mm³, tumor-bearing mice received the same treatments as described above. Tumor lysate was prepared and IκB<sub>Gluc</sub> reporter-Gaussia luciferase activity was quantitated to reflect the intratumoral IKK activity. Results in Fig. 5C indicate that BMS-345541 showed a 6.2-fold protection of IκB<sub>Gluc</sub> from degradation, suggesting that BMS-345541, but not BAY 54-9085, regulation of NF-κB is IKK dependent. The interaction between TMZ and the IKK/NF-κB pathway in vivo needs further investigation. To examine the phosphorylation status of p65 and Erk in melanoma, Hs294T melanoma–bearing mice were given the same treatments as described for Fig. 5A. After a 4-day treatment, tumor tissue was homogenized and Western blot analysis was performed. Data in Fig. 5D indicate that in contrast to the in vitro inhibitory propensity, BAY 54-9085 promoted both NF-κB and Erk activity, confirming and perhaps explaining the unfortunate inefficiency of this drug in vivo. Moreover, the BAY 54-9085 reversal of BMS-345541 antitumor activity may be due to its attenuation of the inhibition of NF-κB activity by BMS-345541 in melanoma xenografts (data not shown). Thus, regulation of the intratumoral NF-κB by these agents is largely associated with antitumor efficacy in vivo.

**Discussion**

Although numerous novel drugs have been developed and tested in melanoma, the systemic treatment of metastatic melanoma with these drugs is often largely ineffective, in part due to multiple mutations, genetic heterogeneity, and the deregulation of many signaling pathways in melanoma (29). For example, V600E mutation elevates B-Raf basal kinase activity, which up-regulates the MAPK pathway, evidenced by constitutive Mek/Erk (6, 30) and IKK/NF-κB activation (11), resulting in the expression of many antiapoptotic, pro-proliferative, and prometastatic genes (15, 31). Thus, the high frequency of BRAF mutation makes it an attractive therapeutic target for advanced-stage melanoma patients (8). In this model of Hs294T melanoma cells, we target the mutant B-Raf gene with BAY 54-9085 and target IKKβ with the IKKβ inhibitor BMS-345541. The reduced phosphorylation of Akt and p65 by both inhibitors contributes to the inhibition of NF-κB activation. However, BMS-345541 affects the canonical IKK/IκB pathway whereas BAY 54-9085 inhibits NF-κB through a pathway independent of IκB stabilization. The up-regulation of NF-κB target genes, such as antiapoptotic cyclin D1 in melanoma (Fig. 2B), seems to consistently contribute to antitumor activity. In contrast, Erk phosphorylation, which is up-regulated by BMS-345541 and down-regulated by BAY 54-9085, plays a minor role in melanoma cell survival in vivo. This point has been further illustrated by direct inhibition of Erk with PD 98-095 in our experiments as well as in other studies by Mhaidat et al. (28). Thus, inhibition of Erk activity in the present study does not potentiate killing of the melanoma cells. In contrast, tremendous Erk phosphorylation by BMS-345541 may contribute to the death of melanoma cells, which is in agreement with recent observations that excessive activation of the MAPK pathway drives cell cycle G2-M arrest (32) and results in retardation of melanoma tumor progression (33).
response to the BMS-345541—mediated Erk activation, AP-1 Gaus sia luciferase reporter activity is induced. It has been shown previously that NF-κB interacts with AP-1 by forming a ternary complex between the p65 subunit of NF-κB and the Fos-Jun heterodimers (25), resulting in p65 inhibition of AP-1 activity (34). When BMS-345541 inhibits nuclear localization of p65, this p65 inhibition of AP-1 is interrupted, further contributing to the activation of AP-1 promoter activity. Moreover, the interaction of NF-κB target genes with other signaling molecules in the JNK pathway may be a critical death determinant (35, 36). Our in vitro data show that inhibition of the NF-κB–mediated survival pathway by BMS-345541, but not by BAY 54-9085, is associated with increased expression of JNK, which is involved in the death pathway in melanoma cells (Fig. 2B). The biochemical mechanism for induction of cell death in vitro via targeting of B-Raf with BAY 54-9085 requires further investigation.

The importance of p53 function as a tumor suppressor is underscored by the fact that more than 60% human cancers have mutated or functionally inactive p53 (37). Cells lacking the response pathway of p53 are more susceptible to tumorigenesis and more resistant to chemotherapy. Pise-Masison et al. (26) recently reported that p53 inactivation induced by human T-cell lymphotropic virus type 1 Tax requires the NF-κB pathway. Jeong et al. (38) have shown that IKKβ plays an important role in Tax-induced p53 inhibition through phosphorylation of p65 at Ser536, suggesting that stable association of p65-p53 at the p53-responsive promoter is responsible for the inhibition of transcription in vivo (39). However, in the present study, we observed that in response to BMS-345541, phosphorylation of p65 at Ser536 is efficiently inhibited and the expression of p53 protein is elevated (Fig. 2B). In contrast to effects with overexpression of dominant negative IκBα mutant (26), BMS-345541–induced p53 expression is independent of p53 transcriptional activity (Fig. 1D). Thus, BMS-345541 regulation of p53 might involve inhibition of p53 degradation by a ubiquitin/26S proteasome pathway (40). Nevertheless, the targeting of B-Raf with BAY 54-9085 to induce the in vitro killing of melanoma cells seems to be p53 independent.

Because hyperactivation of Erk downstream of BRAFV600E exists in up to 90% of human melanoma lesions (41), B-Raf is currently a focus of drug development for melanoma therapy (42). Targeting B-RafV600E by siRNA knockdown reduces metastasis (43). It is assumed that a B-Raf inhibitor should have the same effect. However, as monotherapy in phase II clinical trials, BAY 43-9006 (sorafenib) shows only modest efficacy for human melanoma (44). Fortunately, sorafenib in combination with dacarbazine (45) or TMZ (46) showed encouraging improvement in phase II study of patients with advanced melanoma. However, there was no benefit of sorafenib plus DTIC over DTIC alone when assessed by overall survival (46). Although we observed an ~30% inhibition of tumor growth with the combination of TMZ plus sorafenib, there was no significant inhibition of tumor growth with sorafenib alone. Apart from the B-Raf and NF-κB pathways, sorafenib also potentially targets p38 (IC50 38 nmol/L), C-kit (IC50 68 nmol/L), vascular endothelial growth factor receptor 1 (IC50 26 nmol/L), platelet-derived growth factor receptor (IC50 26 nmol/L), Mek (IC50 40 nmol/L), and other kinases (16). Thus, sorafenib serves as a multitargeted kinase inhibitor, and it is quite possible that responses derived from sorafenib in patients are associated in part with the inhibition of kinases other than B-Raf (47).

In our preclinical studies described here, BAY 54-9085 was not a successful treatment for melanoma xenografts in mice, and it even reversed the antitumor activities of other drugs such as BMS-345541 and TMZ. We observed that in contrast to the in vitro results, in vivo BAY 54-9085 induces intratumoral NF-κB activity and reverses BMS-345541 reduction of NF-κB activity. The B-Raf inhibitor may not directly act on NF-κB signaling. It activates glycogen synthase kinase-3β in melanoma cells (48), which may result in p65 phosphorylation and up-regulation of NF-κB–mediated transactivation (49). In contrast to the in vivo BAY 54-9085–reduced Erk phosphorylation, intratumoral Erk phosphorylation is elevated by treatment with BAY 54-9085. Interestingly, BMS-345541 retains both the antitumor activity and inhibition of NF-κB activity in vivo in spite of its induction of Erk activity. Our data suggest that failure of BAY 54-9085 as a single agent in clinical trials could be a result of induction of intratumoral NF-κB activity in response to BAY 54-9085, based on the real-time NF-κB activity assay used in our model.

TMZ, a DNA alkylating agent, is known to have clinical activity against melanoma mainly through a mechanism of adding a methyl group to the O6 position of guanine in genomic DNA (50). Our study indicates that TMZ-induced cell death in melanoma is not through induction of the apoptosis pathway but is possibly through induction of a senescent pathway (28). Here we show that the efficient antitumor activity of TMZ is strongly associated with inhibition of both NF-κB and Erk activities. Moreover, we also performed experiments in mice with a larger melanoma tumor burden at the time of treatment initiation, which more closely represent clinical patients with advanced melanoma. In these experiments, the synergic effect of BMS-345541 and TMZ produced rapid tumor regression.

We conclude that the response of melanoma tumor-bearing mice treated with BAY 54-9085, TMZ, or BMS-345541 depends on inhibition of NF-κB rather than inhibition of Erk activity. The synergic effect of combined BMS-345541 and TMZ therapy provides an important insight for development of clinical treatment of melanoma. The mechanism of either BAY 54-9085 or BMS-345541 action is linked to effects on p53, Bax, and cyclin D1, which are regulated by NF-κB to control cell cycle and the apoptosis machinery. Clearly, targeting NF-κB in melanoma offers promise for treatment of this dreaded tumor.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
5. Meyskens FL, Jr. American Society of Preventive Oncology Distin-
zines. 1. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl)imi-
22. Ikenoue T, Hikiba Y, Kanai F, et al. Different effects of point mutations within the B-Raf glycin-rich loop in colorectal tumors on mitogen-activated protein/extracellular signal-regulated kinase/mito-
38. Elmore E, Jain A, Siddiqui S, et al. Development and characteristics of the early phases of this project. We also thank Linda W. Horton and Sarah Palmer for excellent technical assistance and the Vanderbilt Editor Club for critical reading of the manuscript.
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