

## Sorafenib, a Multikinase Inhibitor, Enhances the Response of Melanoma to Regional Chemotherapy

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### Abstract

Melanoma responds poorly to standard chemotherapy due to its intrinsic chemoresistance. Multiple genetic and molecular defects, including an activating mutation in the BRAf kinase gene, are associated with melanoma, and the resulting alterations in signal transduction pathways regulating proliferation and apoptosis are thought to contribute to its chemoresistance. Sorafenib, a multikinase inhibitor that targets BRAf kinase, is Food and Drug Administration approved for use in advanced renal cell and hepatocellular carcinomas. Although sorafenib has shown little promise as a single agent in melanoma patients, recent clinical trials suggest that, when combined with chemotherapy, it may have more benefit. We evaluated the ability of sorafenib to augment the cytotoxic effects of melphalan, a regional chemotherapeutic agent, and temozolomide, used in systemic and regional treatment of melanoma, on a panel of 24 human melanoma-derived cell lines and in an animal model of melanoma. Marked differences in response to 10  $\mu\text{mol/L}$  sorafenib alone were observed *in vitro* across cell lines. Response to sorafenib significantly correlated with extracellular signal-regulated kinase (ERK) downregulation and loss of Mcl-1 expression ( $P < 0.05$ ). Experiments with the mitogen-activated protein kinase/ERK kinase inhibitor U0126 suggest a unique role for ERK downregulation in the observed effects. Sorafenib in combination with melphalan or temozolomide led to significantly improved responses *in vitro* ( $P < 0.05$ ). In the animal model of melanoma, sorafenib in combination with regional melphalan or regional temozolomide was more effective than either treatment alone in slowing tumor growth. These results show that sorafenib in combination with chemotherapy provides a novel approach to enhance chemotherapeutic efficacy in the regional treatment of in-transit melanoma. *Mol Cancer Ther*; 9(7); 2090–101. ©2010 AACR.

### Introduction

Melanoma is a potentially life-threatening skin cancer with nearly 160,000 new cases worldwide each year (1). In addition to the dramatic increase in incidence, melanoma accounts for one of the highest rates of productive years of life lost from malignancy due to its propensity to occur in younger individuals (2). To date, the most effective single-agent chemotherapies for the management of metastatic melanoma are the alkylating

agents dacarbazine and temozolomide, for systemic disease, and melphalan, for regionally advanced disease of the extremity (3–5). Long-term response rates, especially to systemically administered chemotherapeutic agents, however, are generally poor. Recent evidence suggests that alterations in signal transduction pathways regulating proliferation and apoptosis may contribute to the intrinsically chemoresistant phenotype associated with melanoma (6).

One of the most common genetic alterations, observed in 50% to 70% of melanomas, is a mutation that results in substitution of glutamate for valine at codon 600 of the gene encoding the BRAf serine/threonine kinase (7). This mutation leads to constitutive activation of BRAf kinase with the resultant unchecked stimulation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase and the ERK pathways leading to increased activation of proliferative, survival/antiapoptotic, and angiogenic pathways and, ultimately, to enhanced growth and progression of melanomas (7). The high incidence of this activating BRAf mutation and the multitude of downstream effectors that enhance tumor growth and metastasis make BRAf an attractive therapeutic target.

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Sorafenib (Nexavar), an inhibitor of multiple kinases including the Raf kinases, is approved by the Food and Drug Administration for the treatment of renal cell and inoperable hepatocellular carcinomas (8, 9). Sorafenib blocks cell proliferation, cell survival, and angiogenesis, all of which can be driven by the Raf-MAPK/ERK kinase-ERK pathway (7). Sorafenib may also lead to apoptosis via downregulation of the antiapoptotic protein Mcl-1 and inhibition of the nuclear translocation of apoptosis-inducing factor (10–12). In addition, sorafenib effectively inhibits the activity of vascular endothelial growth factor receptors, platelet-derived growth factor receptor  $\beta$ , FLT-3, and c-Kit (9). Several of these receptors have been implicated in melanoma biology (13, 14), making sorafenib an attractive agent for melanoma therapy. Whereas sorafenib treatment of melanoma cell lines and tumor xenografts results in cell death and tumor growth delay (15, 16), its use as a single agent in the treatment of patients with metastatic melanoma has yielded disappointing clinical results (17).

Despite its relative ineffectiveness as a single agent in melanoma, the ability of sorafenib to alter cell proliferation and/or survival suggests that it may be useful in combination with other cytotoxic therapies. Indeed, sorafenib can enhance the effects of radiation, rapamycin, and the small-molecule inhibitor ABT-737, which inhibits the Bcl-2 protein (18–20). In phase I and II studies, melanoma patients treated with sorafenib combined with carboplatin and paclitaxel, temozolomide, dacarbazine, or IFN $\alpha$ -2a showed a greater response compared with historical control and, in one study of dacarbazine alone, with little enhancement of toxicity (14, 21–23). To date, however, no studies have examined whether sorafenib can augment the response to the alkylating agents melphalan and temozolomide in the unique setting of regional therapy of advanced in-transit melanoma using either isolated limb infusion (ILI) or isolated limb perfusion.

This study was designed to address this important question. We characterized a panel of human melanoma-derived cell lines for the presence of the V600E BRAf mutation and examined their response to either sorafenib alone or sorafenib in combination with melphalan or temozolomide chemotherapy, both *in vitro* and *in vivo*, in a rat model of regional isolated-limb infusion chemotherapy. The results presented also provide insight into some of the mechanisms by which sorafenib enhances the cytotoxicity of chemotherapy and the molecular changes that occur in melanoma cells in response to treatment.

## Materials and Methods

### Cell culture

Melanoma cell lines were maintained at 37°C, 5% CO<sub>2</sub> in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin. Duke melanoma (DM) cell lines, a generous gift from Dr. Hilliard Seigler (Duke

University Medical Center, Durham, NC), were derived from human melanoma patient samples under an institutional review board–approved protocol. A2058 and SkMel28 were purchased from the American Type Culture Collection (2006). All cell lines were confirmed *Mycoplasma*-free.

### Reagents

Melphalan and U0126 were purchased from Sigma. Sorafenib was provided by Bayer Pharmaceuticals. Temozolomide was provided by Schering-Plough. Antibodies against  $\beta$ -actin, ERK, phosphorylated T202/Y204-ERK (E10), and Bim were purchased from Cell Signaling Technology. Antibodies against Mcl-1 and BRAf were obtained from Santa Cruz Biotechnology. For RNA interference studies, lipid transfection reagents were obtained from Dharmacon (Dharmafect 2) or Invitrogen (Lipofectamine 2000). Control (AllStars Negative Control siRNA) and Mcl-1–specific siRNA (Hs\_Mcl1\_6) were obtained from Qiagen.

### BRAf and NRas mutation assay

To analyze the BRAf V600E and the NRas (codon 61) mutations, DNA was isolated from melanoma cells lines using the DNeasy kit (Qiagen). PCR amplification was done using HotStart Taq DNA polymerase (Qiagen) in a 50- $\mu$ L reaction volume. See Supplementary Table S1 for primer sequences and Supplementary Methods for thermocycle settings. The PCR products were purified (Qiaquick PCR Purification Kit, Qiagen) and sequenced by the Duke University DNA Analysis Facility using the Applied Biosystems Dye Terminator Cycle Sequencing system with AmpliTaq DNA Polymerase and ABI 377 PRISM DNA sequencing instruments and analysis software.

### Cell survival assay

The sensitivity of each cell line to drug treatment was measured with a colorimetric assay using WST-1 cell proliferation reagent (Roche). Temozolomide cytotoxicity was assessed using a 12-day assay, which, as previously described (24), yields *in vitro* responses to temozolomide that more closely mimic *in vivo* response. Briefly,  $1 \times 10^5$  cells/mL treated with temozolomide for 2.5 hours were plated in 96-well plates at 50 to 500 cells/100  $\mu$ L culture medium per well. The culture medium was changed after 6 days, and cell survival measured after 12 days with the WST-1 assay as described above. For testing sorafenib in combination with temozolomide, cells were treated with 10  $\mu$ mol/L sorafenib (or 0.1% DMSO control) for 2 hours, followed by temozolomide (d0) for 2.5 hours, and plated as described above. After 6 days (d6), the culture medium was replaced with fresh medium containing 0.1% DMSO or 10  $\mu$ mol/L sorafenib. For melphalan sensitivity, cells were plated at 3,000 to 5,000 cells/100  $\mu$ L per well of a 96-well plate and incubated overnight; DMSO (vehicle control) or drugs (melphalan

and/or sorafenib and/or U0126 in DMSO) were added at the required concentrations. In the combination treatment, sorafenib was added 2 hours before melphalan. The final DMSO concentration in each well was  $\leq 1\%$ . Cell line sensitivity to melphalan treatment was measured after 48 hours of incubation, as described above. Cell survival, defined as the absorbance of the treatment group divided by the absorbance of the control group, was plotted as a function of temozolomide or melphalan concentration (see Supplementary Fig. S1), and the area under the dose-response curve ( $AUC_{drug}$ ) was computed using GraphPad Prism v4.0 software over a concentration range of 0 to 0.5 mmol/L for temozolomide and 0 to 125  $\mu\text{mol/L}$  for melphalan. The level of resistance to chemotherapy was defined as  $R_{drug}$  (fraction resistant to drug), where

$$R_{drug} = AUC_{drug}/AUC_{max}$$

$AUC_{max}$  represents no loss of cell survival at the drug doses tested ( $AUC_{max} = 0.5$  for temozolomide assay and 125 for melphalan assay).

### Western blot analysis

Subconfluent cultures of melanoma cell lines were treated with DMSO or sorafenib (10  $\mu\text{mol/L}$  in DMSO) in growth medium for 5 or 24 hours. Cells were washed with ice-cold PBS and lysed in a modified radioimmunoprecipitation assay buffer (see Supplementary Methods). For tumor lysis, pieces of excised tumor were homogenized on ice with a hand-held homogenizer in radioimmunoprecipitation assay buffer. Clarified cell and tumor lysates (10–25  $\mu\text{g}$  protein) were analyzed by SDS-PAGE, followed by Western blotting and detection using the Visualizer Western Blot Detection Kit (Upstate Cell Signaling Solutions). Blots were visualized and quantified using a Bio-Rad Versa Doc 4000 and Quantity One Image software.

### siRNA transfection

A suspension of cells ( $41 \times 10^4/\text{mL}$ ) prepared in Opti-MEM (Invitrogen) was incubated with 100 nmol/L siRNA and 4  $\mu\text{g/mL}$  lipid transfection reagent. An equal volume of full medium was added after 5 hours and cells were cultured overnight. Cells were plated 24 hours after transfection into 96-well plates at a density of 2,000 cells/well and incubated overnight. Cells were treated with drugs 48 hours after transfection and cell survival was measured after 48 hours (4 days after transfection) as described above.

### Measurement of apoptosis

Subconfluent cultures of cells were treated with DMSO or sorafenib (10  $\mu\text{mol/L}$ ) for 2 hours before the addition of melphalan or DMSO (vehicle control). After 24 to 48 hours, cells were harvested by trypsinization and then washed, and the level of apoptosis was measured using the APO-BrdU TUNEL Assay Kit (Invitrogen), as directed

by the manufacturer with some modifications (see Supplementary Methods). Samples were analyzed by fluorescence-activated cell sorting using a FACScan flow cytometer (BD Biosciences) and results were analyzed using FloJo software (Tree Star, Inc.).

### Isolated limb infusion

Tumor initiation was done essentially as we described previously (25) by a s.c. injection of DM443 cells ( $5 \times 10^6$  in 2:1 PBS/Matrigel; BD Biosciences) into the right hind limb (see Supplementary Methods). Xenografts were measured with Vernier calipers and tumor volume was calculated as  $(\text{length} \times \text{width}^2)/2$ . Once the tumor volume had reached approximately 1  $\text{cm}^3$ , daily treatment with sorafenib or vehicle (60 mg/kg by oral gavage) for 10 days was initiated. See Supplementary Methods for details of drug preparations. On day 8, ILI with saline, melphalan, or temozolomide was done as previously described (see also Supplementary Methods; ref. 25). Stock solutions of melphalan and temozolomide were prepared fresh each day. Tumor volume was followed every other day, as described above, for 30 additional days. All animal protocols were approved by the Duke University Medical Center and the Durham Veterans Affairs Medical Center Institutional Animal Care and Use Committees. For each treatment arm, group size was five to six animals. Tumor growth data are calculated as fold change in tumor volume from that measured at the time of ILI (d0) and plotted as a function of time following infusion. Quintupling time is calculated as the number of days for tumor volume to increase 5-fold over untreated volume (d0).

### Immunohistochemical analysis of tumors

Immunohistochemistry was done as we previously described (26, 27). The MP5/73 antibody to detect melphalan adducts was kindly provided by Dr. M.J. Tilby (Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; ref. 28). Tumor specimens were also stained for microvascular density using the anti-CD31 mouse monoclonal antibody (Dako) as well as for apoptosis with Klenow Frag-EL (EMD) according to the manufacturer's specifications.

### Statistics

Statistical analysis of data from cell lines was done using GraphPad Prism 4 software. Differences in tumor growth rate were compared by taking the log transformation of the fold change in tumor volume and calculating the slopes for each treatment group. The log transformation was chosen to improve the linearity of the time trajectory in the fold change in tumor volume. To account for possible dependency among repeated tumor volume measurements, two-sided  $P$  values were calculated using the generalized estimating equation method (29, 30) implemented by Proc Genmod of SAS with the working independent correlation structure.

*P* values  $\leq 0.05$  were considered significant. Because of the sample size, no multiple testing adjustments were done when comparing the slopes between different treatment groups.

## Results

### ***In vitro* response to sorafenib, melphalan, and combination therapy**

Of the 24 human melanoma-derived cell lines evaluated, 15 were shown to harbor the V600E BRAf mutation. Of the 9 BRAf wild-type (WT) cell lines, 5 had an activating mutation at codon 61 of the NRas gene (see Table 1). The frequency of mutations in this panel of human melanoma-derived cell lines (62.5% BRAf mutation and 20.8% NRas mutation) was consistent with previously reported rates of mutation for both genes in melanoma (7).

The 24 cell lines were evaluated for baseline sensitivity to sorafenib. The results showed modest sensitivity to 5  $\mu\text{mol/L}$  sorafenib across all cell lines (Supplementary Fig. S1A) at 48 hours; however, at 10  $\mu\text{mol/L}$  sorafenib (Fig. 1A), there was a broad spectrum of response ranging from very sensitive (DM833; cell survival, 27% of control) to resistant (DM751; cell survival, 98% of

control). No correlation was observed between sensitivity to sorafenib alone measured at 48 hours and BRAf mutation status ( $P > 0.50$ ), consistent with previous reports (19, 31, 32).

The results of the analysis of responsiveness to melphalan chemotherapy in melanoma examined at 48 hours following treatment with melphalan alone and in combination with 5 or 10  $\mu\text{mol/L}$  sorafenib are summarized in Fig. 1B and C. Response to melphalan alone ranged from moderately sensitive (DM422;  $R_{\text{melph}} = 0.41$ ) to resistant (DM598;  $R_{\text{melph}} = 0.93$ ) and showed no relationship to either BRAf mutation status ( $P > 0.40$ ) or sensitivity to sorafenib ( $P > 0.10$ ; Fig. 1B; Supplementary Fig. S1B). Following a 2-hour exposure to 10  $\mu\text{mol/L}$  sorafenib, sensitivity to melphalan was significantly increased over control (two-tailed unpaired *t* test,  $P < 0.05$ ) in all cell lines with the exception of DM751, which was the most resistant to sorafenib (Fig. 1C; Supplementary Fig. S1C). The increase in sensitivity to melphalan with 10  $\mu\text{mol/L}$  sorafenib correlated significantly ( $P < 0.0001$ ) with response to sorafenib alone (Fig. 2A, left) but not with BRAf mutation status ( $P > 0.90$ ) or baseline melphalan sensitivity ( $P > 0.50$ ; Fig. 2A, right).

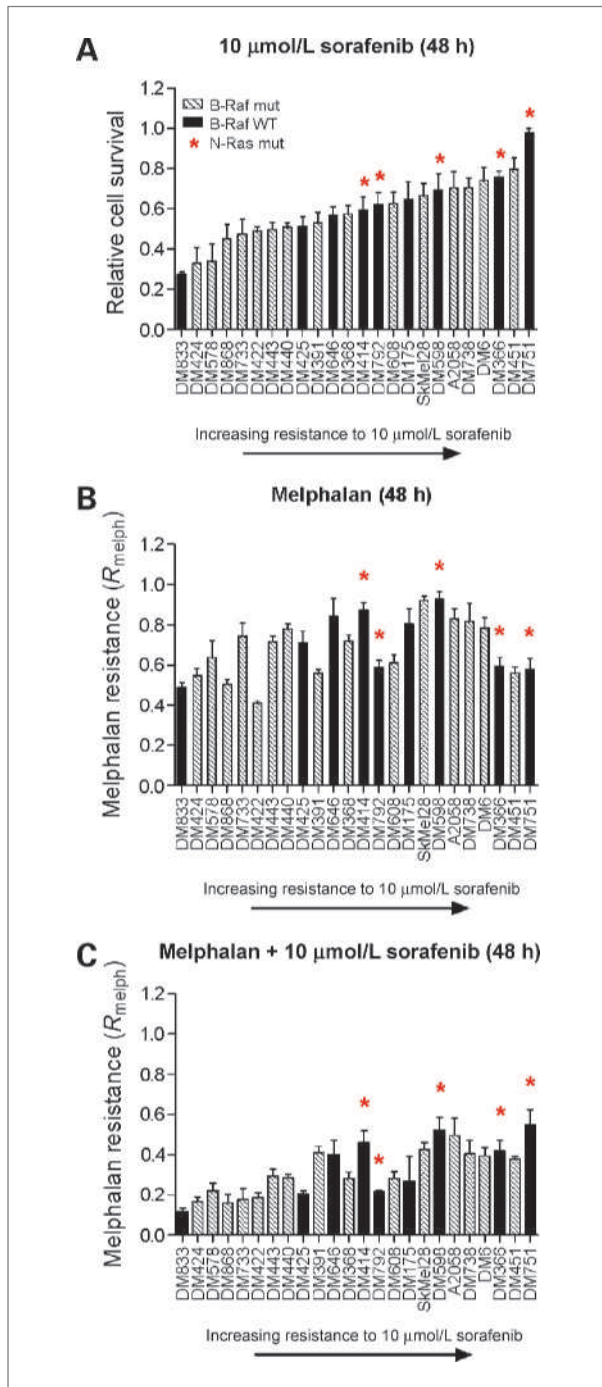
### **Effects of sorafenib on *in vitro* response to temozolomide**

Response to temozolomide alone and in combination with 10  $\mu\text{mol/L}$  sorafenib was evaluated using the 12-day assay. As shown in Fig. 2B, sensitivity to sorafenib measured with the 12-day assay was significantly ( $P < 0.04$ ) more pronounced than that observed at 48 hours across 10 of the 13 cell lines tested (see also Supplementary Fig. S2). Response to sorafenib treatment measured at day 12 showed a slight but significant difference between cell lines with the BRAf mutation and those with the NRas mutation ( $P < 0.02$ ; see Supplementary Fig. S2B).

In all cell lines, 10  $\mu\text{mol/L}$  sorafenib enhanced the response to temozolomide chemotherapy at day 12 (Fig. 2B). DM751, the most sorafenib-resistant cell line, when measured at 48 hours (see Fig. 1A), showed a 1.7-fold increase in sensitivity (among the lowest) to temozolomide with 10  $\mu\text{mol/L}$  sorafenib ( $P = 0.038$ ). DM440, the most temozolomide-sensitive cell line, showed little increase in temozolomide sensitivity with sorafenib (fold increase, 1.68). Across all cell lines, there was a significant correlation ( $P < 0.04$ ) between baseline sensitivity to sorafenib, measured at day 12, and the fold increase in temozolomide sensitivity with 10  $\mu\text{mol/L}$  sorafenib (Fig. 2C, left). Among the seven cell lines with the BRAf mutation, there was a significant correlation ( $P < 0.02$ ) between the fold increase in temozolomide sensitivity with 10  $\mu\text{mol/L}$  sorafenib and baseline sensitivity to temozolomide such that temozolomide-sensitive cells showed less enhancement with sorafenib compared with temozolomide-resistant cells (Fig. 2C, right). This pattern was not observed in the six BRAf WT cell lines.

**Table 1.** Mutation status across 24 human melanoma-derived cell lines

Cell line	BRAF (bp 1799)	NRAS (bp 1336)
DM175	WT	WT
DM366	WT	CAA>CGA (Q>R)
DM368	GTG>GAG (V>E)	WT
DM391	GTG>GAG (V>E)	WT
DM414	WT	CAA>CGA (Q>R)
DM422	GTG>GAG (V>E)	not determined
DM424	GTG>GAG (V>E)	WT
DM425	WT	WT
DM440	GTG>GAG (V>E)	WT
DM443	GTG>GAG (V>E)	WT
DM451	GTG>GAG (V>E)	WT
DM578	GTG>GAG (V>E)	not determined
DM598	WT	CAA>CGA (Q>R)
DM6	GTG>GAG (V>E)	WT
DM608	GTG>GAG (V>E)	WT
DM646	WT	WT
DM733	GTG>GAG (V>E)	WT
DM738	GTG>GAG (V>E)	WT
DM751	WT	CAA>CTA (Q>L)
DM792	WT	CAA>CTA (Q>L)
DM833	WT	WT
DM868	GTG>GAG (V>E)	WT
A2058	GTG>GAG (V>E)	WT
SkMel28	GTG>GAG (V>E)	WT



**Figure 1.** Sorafenib enhances *in vitro* response to melphalan chemotherapy. Response of 24 human melanoma-derived cell lines to treatment with sorafenib (A; 10 µmol/L for 48 h), increasing doses of melphalan (B; measured after 48 h), or increasing doses of melphalan in combination with sorafenib (C; 10 µmol/L sorafenib for 2 h followed by melphalan measured after 48 h). The genotype of each cell line is indicated: B-Raf WT (closed columns), B-Raf mutant (hatched columns), and N-Ras mutant (red asterisks). Columns, average of at least three experiments; bars, SEM. Response to melphalan (B and C;  $R_{\text{melph}}$ ) is plotted for each cell line and is calculated as described in Materials and Methods. For reference,  $R_{\text{melph}} = 1$  represents maximal resistance to melphalan.

### ***In vivo* response to sorafenib in combination with melphalan or temozolomide isolated limb infusion**

The ability of sorafenib to enhance the effects of high-dose regionally infused chemotherapy was examined in our rat xenograft model of extremity melanoma (33, 34) using the DM443 cell line, which was moderately sensitive to sorafenib *in vitro* (Fig. 1A), moderately sensitive to melphalan both *in vitro* (Fig. 1B) and *in vivo* (25), and resistant to temozolomide both *in vitro* (Fig. 2B) and *in vivo* (25). We also examined the *in vivo* ERK kinase activity, which is regulated downstream of the Raf kinase, as well as the levels of Mcl-1, an antiapoptotic protein which has been shown to be downregulated by sorafenib via a Raf-independent mechanism (11, 12). Systemic treatment with 20 mg/kg sorafenib alone reduced both ERK activation and Mcl-1 protein levels (Fig. 3A). In an effort to enhance response without additional toxicity (19), a dose of 60 mg/kg sorafenib was administered over 10 days. Treatment with vehicle alone resulted in a substantial increase in tumor volume of nearly 7-fold over the course of 30 days (Fig. 3B and C); relative to vehicle, tumor growth was slowed by treatment with sorafenib ( $P = 0.0034$ ), temozolomide ( $P < 0.0001$ ), or melphalan ( $P = 0.0024$ ). Combination of sorafenib with melphalan in the ILI setting resulted in stabilization of tumor growth for up to 16 days, whereas sorafenib with temozolomide ILI resulted in marked slowing of tumor growth. At day 30, the growth of tumors treated with melphalan plus sorafenib was reduced significantly relative to saline alone ( $P < 0.0001$ ) or sorafenib alone ( $P < 0.0001$ ); relative to melphalan alone, growth was slowed but this did not reach statistical significance ( $P = 0.1135$ ). Temozolomide plus sorafenib significantly slowed tumor growth relative to saline alone ( $P < 0.0001$ ), sorafenib alone ( $P = 0.0026$ ), and temozolomide alone ( $P = 0.0025$ ). Additional measures of tumor growth such as quintupling time showed a trend toward improved tumor response with combination therapy over chemotherapy alone (see Supplementary Table S2).

### **Sorafenib enhances melphalan-induced apoptosis both *in vivo* and *in vitro***

Tumor specimens from rats treated with either melphalan ILI alone or melphalan ILI plus systemic sorafenib were examined by immunohistochemistry for sorafenib-related changes in apoptosis, melphalan-DNA adduct formation, and microvascular density. Tumor specimens harvested after 9 days of sorafenib treatment and 24 hours after ILI with melphalan showed marked increases in apoptosis (Fig. 4A, image 4) compared with tumors treated with melphalan alone (Fig. 4A, image 3). The formation of melphalan adducts, as determined by immunohistochemistry using the melphalan-DNA adduct-specific monoclonal antibody MP5/73, was relatively similar between the tumors treated with melphalan alone and those treated with melphalan plus sorafenib (Fig. 4A, images 5 and 6, respectively), as was the

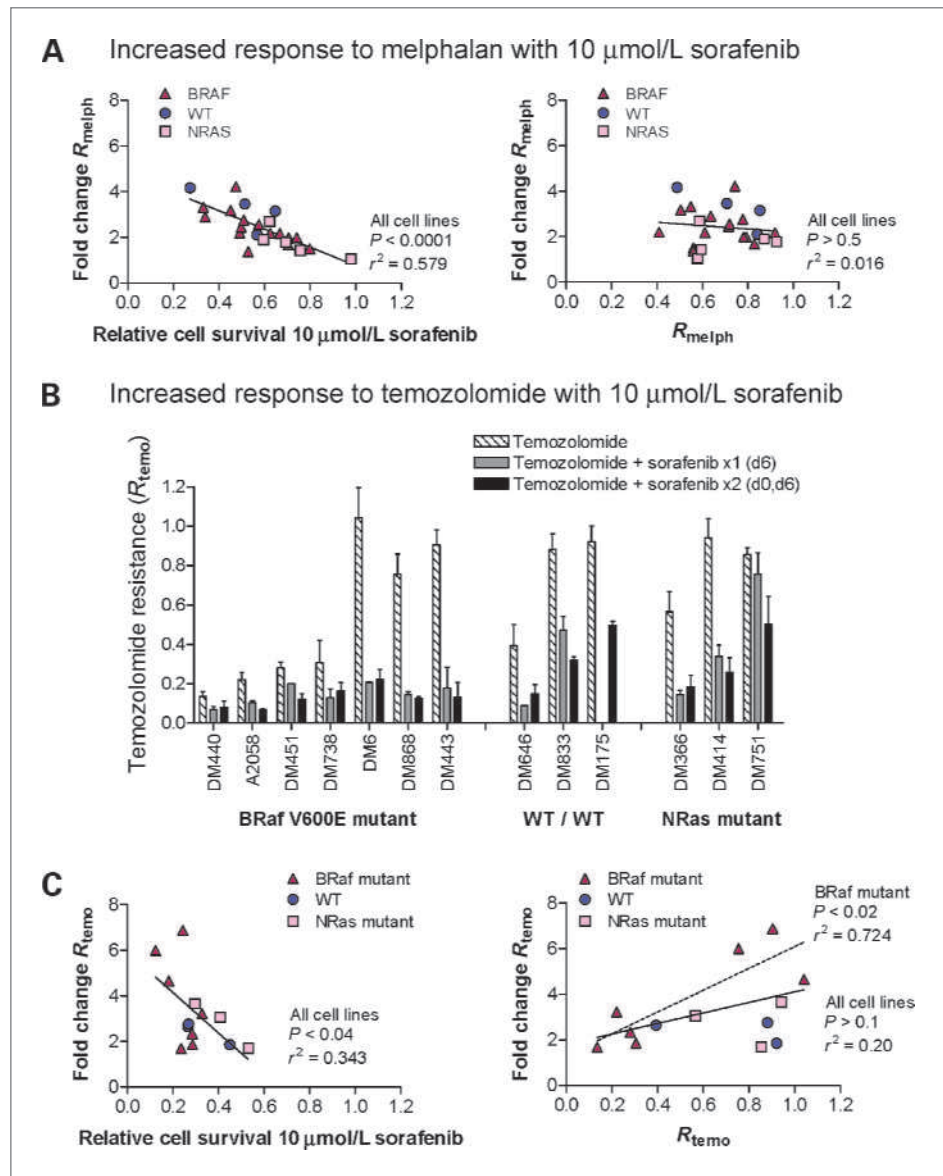
microvascular density (Fig. 4A, images 7 and 8) measured using an anti-CD31 monoclonal antibody.

The degree to which sorafenib enhanced apoptosis *in vitro* was measured by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay for the moderately sorafenib-sensitive cell lines DM868 (BRAF mutant), DM175 (BRAF WT), and DM443 (BRAF mutant) (Fig. 4B). Treatment of cells with sorafenib enhanced the ability of melphalan to induce apoptosis, relative to sorafenib alone, consistent with *in vivo* observations. Treatment of DM443 cells with 10  $\mu\text{mol/L}$  sorafenib and 125  $\mu\text{mol/L}$  melphalan for 24 hours increased the percentage of TUNEL-positive cells (39.0%) compared with 125  $\mu\text{mol/L}$  melphalan alone (3.0%) and 10  $\mu\text{mol/L}$  sorafenib alone (3.0%; Fig. 4B, top). Sorafenib alone increased the level of apoptosis slightly after

48 hours of treatment; however, the percentage of TUNEL-positive cells 48 hours after treatment with sorafenib in combination with 125  $\mu\text{mol/L}$  melphalan was still markedly higher compared with melphalan or sorafenib alone (32.0% melphalan plus sorafenib compared with 6.0% melphalan and 15.0% sorafenib). Similar results were seen with the DM175 cell line (Fig. 4B, middle). Results with the DM868 cell line were less dramatic, with only small increases in the percentage of TUNEL-positive cells observed after 24 hours of treatment with 125  $\mu\text{mol/L}$  melphalan (Fig. 4B, bottom).

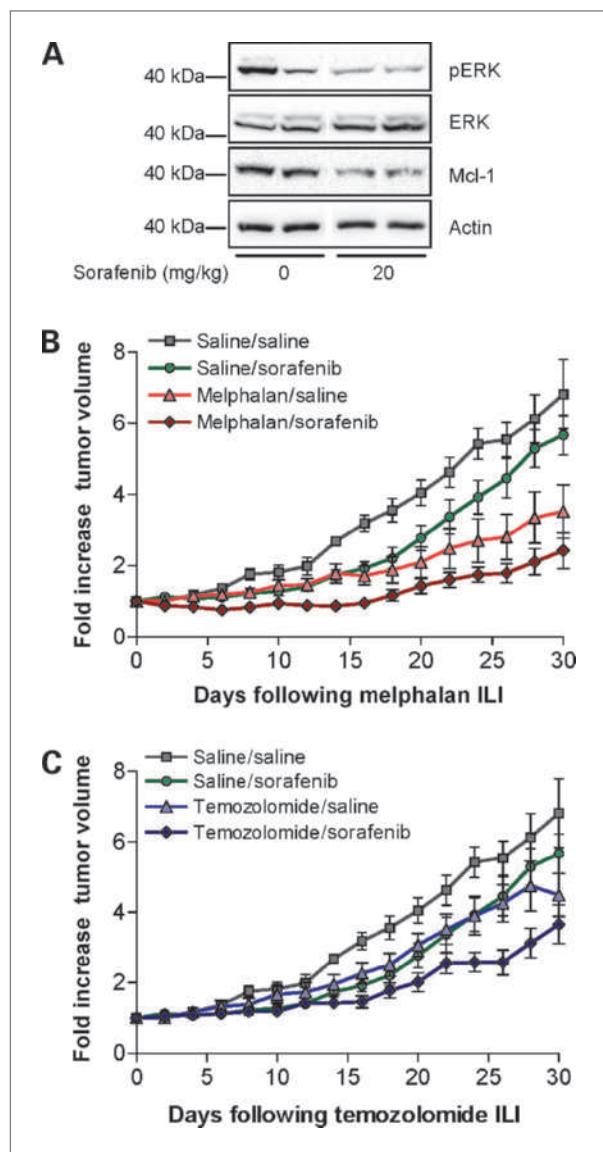
These results show that sorafenib can enhance response to melphalan and lead to increased apoptosis both *in vitro* and *in vivo*, consistent with previous reports where sorafenib has been shown to enhance apoptosis induced by other agents (32).

**Figure 2.** A, left, fold increase in melphalan response ( $R_{\text{melph}}/R_{\text{melph}} + \text{Soraf}$ ) is plotted as a function of cell survival in 10  $\mu\text{mol/L}$  sorafenib (shown as fraction of control). The line represents a linear regression analysis. Right, fold increase in melphalan response compared with  $R_{\text{melph}}$ . The line represents a linear regression analysis. B, response of 13 human melanoma-derived cell lines to treatment with increasing doses of temozolomide alone (hatched columns) or in combination with one dose of 10  $\mu\text{mol/L}$  sorafenib given at day 6 (solid gray column) or two doses of 10  $\mu\text{mol/L}$  sorafenib given at day 0 (2 h before temozolomide) and day 6 (solid black column). See Materials and Methods for details. The cells are shown grouped by genotype BRAf WT, BRAf mutant, and NRas mutant. Columns, average of at least three experiments; bars, SEM. Response to temozolomide ( $R_{\text{temo}}$ ) is plotted for each cell line and is calculated as described in Materials and Methods. For reference,  $R_{\text{temo}} = 1$  represents maximal resistance to temozolomide. C, left, fold increase in temozolomide response ( $R_{\text{temo}}/R_{\text{temo}} + \text{Soraf}$ ) is plotted as a function of cell survival in 10  $\mu\text{mol/L}$  sorafenib (shown as fraction of control) measured at day 12. The line represents a linear regression analysis. Right, fold increase in temozolomide response compared with  $R_{\text{temo}}$ . The line represents a linear regression analysis.



### Intracellular signaling mechanisms

To evaluate the molecular mechanisms underlying sorafenib enhancement of temozolomide and melphalan sensitivity, we selected 12 melanoma cell lines to further



**Figure 3.** Sorafenib enhances *in vivo* response to regional ILI chemotherapy. **A**, irradiated nude rats harboring DM443 xenografts were treated with sorafenib at 20 mg/kg daily for 7 d. Two hours after the last dose of sorafenib, tumors were excised. Tumor lysates were analyzed by Western blot analysis using the indicated antibodies. Results represent protein levels in two independent tumors. **B** and **C**, irradiated nude rats harboring DM443 xenografts were treated with vehicle (control) or sorafenib (60 mg/kg) daily for 10 d. ILI with saline, melphalan (**B**), or temozolomide (**C**) was done on day 8 and tumors were measured every other day for 30 d. Tumor volume, measured every other day, was normalized to the starting volume measured on the day of ILI and plotted as a function of time (days) following ILI. **B** and **C**, saline/saline (black;  $n = 6$ ) and sorafenib/saline (green;  $n = 5$ ). **B**, saline/melphalan (light red;  $n = 5$ ) and sorafenib/melphalan (dark red;  $n = 6$ ). **C**, saline/temozolomide (light blue;  $n = 6$ ) and sorafenib/temozolomide (dark blue;  $n = 6$ ). Points, average; bars, SEM.

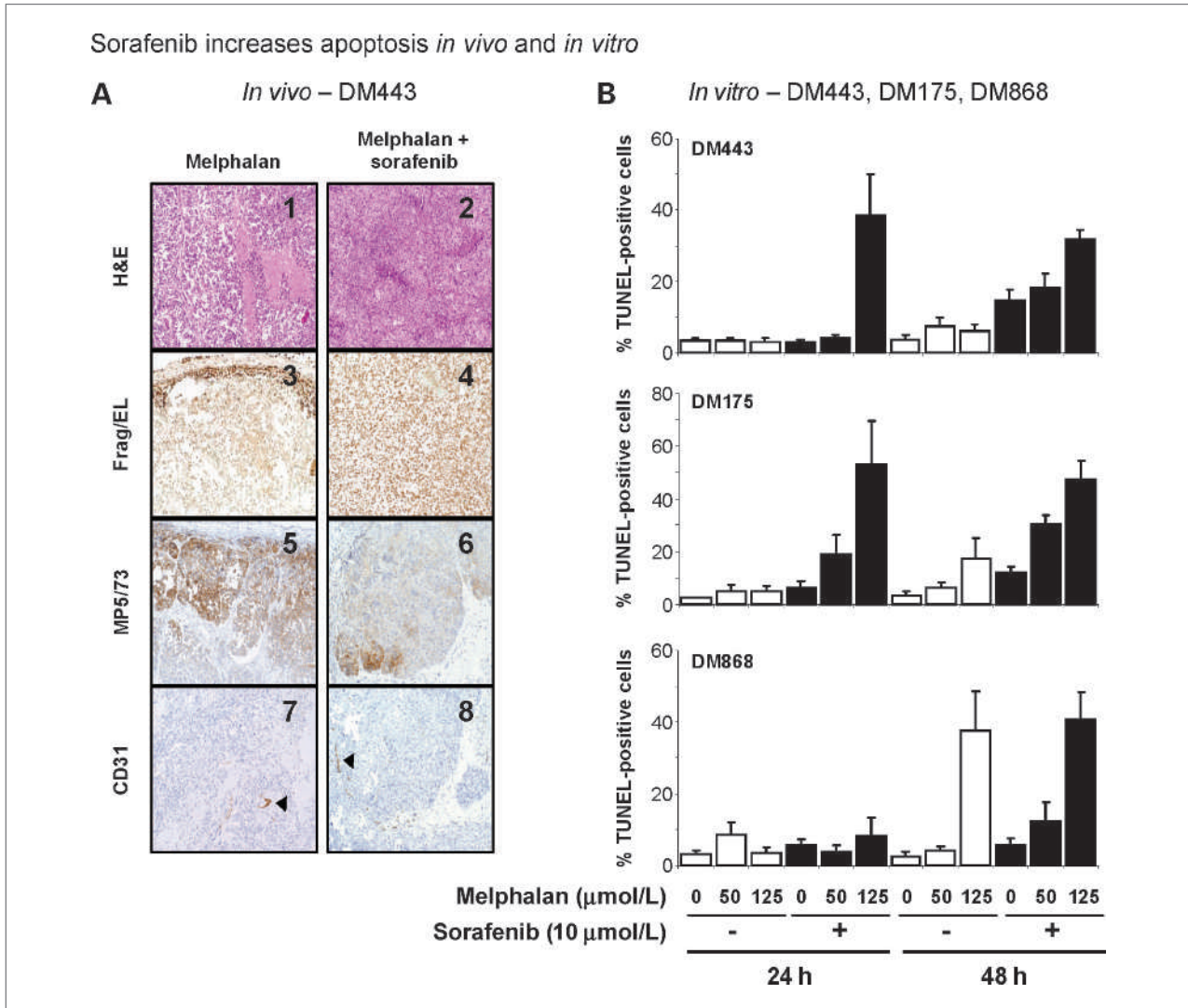
analyze for changes in signaling events in response to sorafenib treatment alone. These included seven V600E Braf mutant cell lines (A2058, DM6, DM440, DM443, DM451, DM738, and DM868), three NRas Q61 mutant cell lines (DM366, DM414, and DM751), and two cell lines that were WT for both NRas and Braf (DM175 and DM646).

There was a marked reduction in Mcl-1 levels after 5 hours of exposure to 10  $\mu\text{mol/L}$  sorafenib (49–86% reduction from control; Fig. 5A), which was sustained or further reduced after 24 hours in all cell lines tested (48–99%; see also Supplementary Fig. S3). Likewise, ERK activity, as measured by the phosphorylation of T202/Y204 of ERK, decreased after 5 hours of exposure to 10  $\mu\text{mol/L}$  sorafenib across all cell lines tested (52–88% reduction from control; Fig. 5A). Sustained inhibition of ERK activity (for up to 24 hours) was observed in only seven of the cell lines tested, with three (DM868, A2058, and DM451) showing further reduction at 24 hours (Fig. 5A; Supplementary Fig. S3). In five of the cell lines (DM366, DM751, DM738, DM646, and DM175), inhibition of ERK activity was not sustained over 24 hours.

Changes in ERK activation and Mcl-1 protein levels at 24 hours were quantified (see methods) and plotted as a function of cell survival at 48 hours and 12 days after initial drug treatment. Although a significant correlation was not seen at 48 hours (Fig. 5B, bottom), there was a trend toward decreased cell survival with decreasing ERK activation. A similar trend was seen with Mcl-1 expression. When cell survival was measured at day 12, a significant correlation ( $P < 0.05$ ) was observed in both the level of decrease in ERK activation at 24 hours and the decrease in Mcl-1 protein levels at 24 hours (Fig. 5B, top).

These results suggest that sorafenib enhances sensitivity to chemotherapy by altering signaling in both the MAPK and the mitochondrial apoptotic pathways. To clarify the importance of the MAPK signaling pathway in sorafenib-induced melanoma cell line apoptosis, the specific MAPK/ERK kinase inhibitor U0126 was used. Marked inhibition of ERK activation was observed after only 4 hours of exposure with 10  $\mu\text{mol/L}$  U0126 (see Supplementary Fig. S4B) and was sustained for up to 24 hours. Although Mcl-1 protein expression was not altered by U0126 in the three melanoma cell lines tested (Braf mutant DM443, NRas mutant DM366, and WT DM646), an increase in Bim expression was observed after 24 hours of treatment with U0126 but not sorafenib. When cells were treated with melphalan in combination with U0126, there was a marked decrease in resistance ( $R_{\text{melph}}$ ) comparable to that observed with either sorafenib or U0126 plus sorafenib (see Supplementary Fig. S4A). These results suggest that sorafenib-induced enhancement of melanoma response to chemotherapy is at least in part dependent on inhibition of signaling in the MAPK pathway.

To evaluate the role of Mcl-1 downregulation in the sorafenib-enhanced response to chemotherapy, Mcl-1



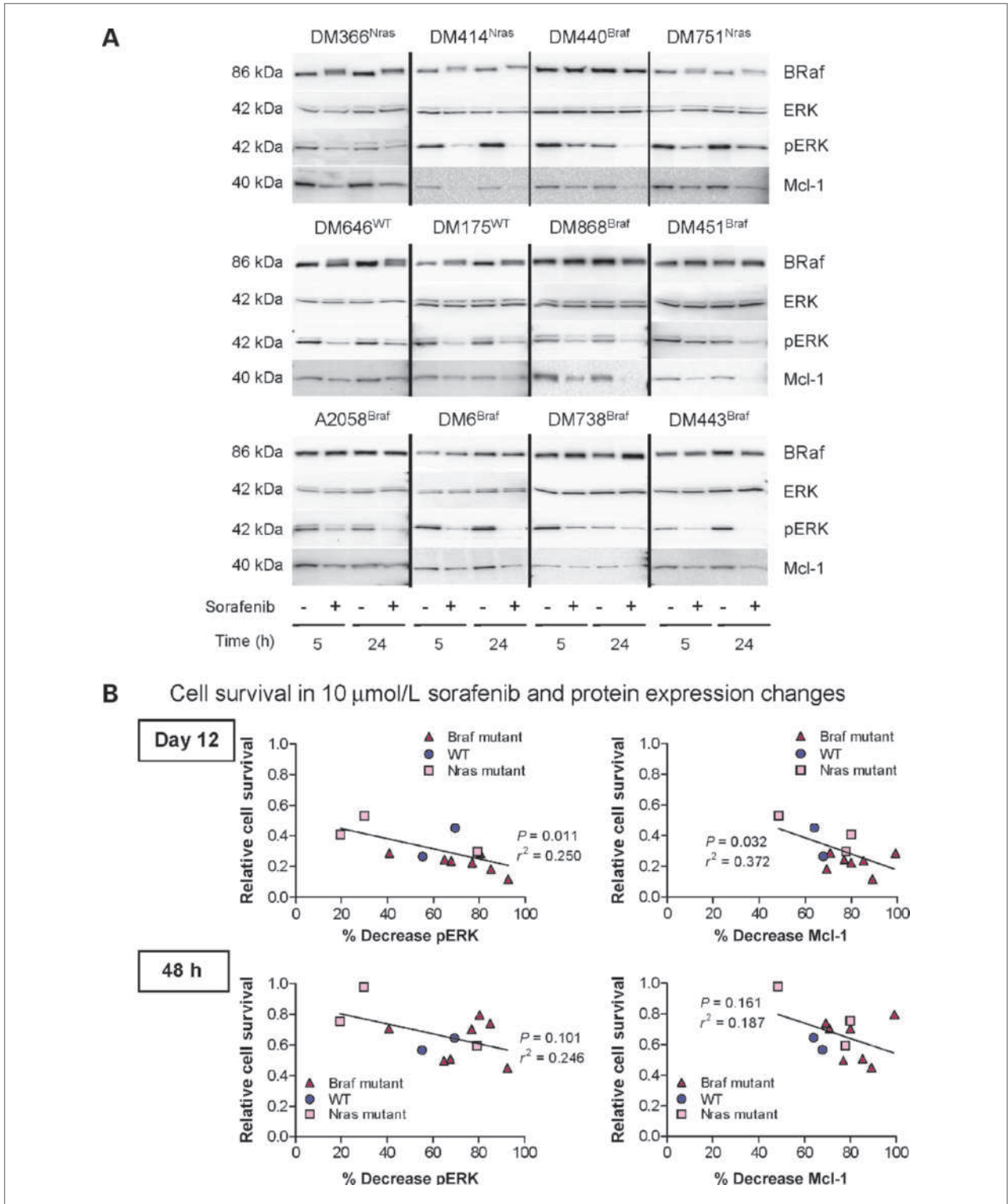
**Figure 4.** Treatment with sorafenib leads to increased apoptosis. **A**, immunohistochemical staining of representative DM443 tumor specimens treated with melphalan alone (left column) or melphalan plus sorafenib (right column). Images 1 and 2, H&E staining of the tumors. Images 3 and 4, Frag/EL staining for apoptosis. Images 5 and 6, staining for melphalan-DNA adducts using the specific monoclonal antibody MP5/73. Images 7 and 8, staining for CD31 (arrowheads) to measure microvascular density. **B**, melanoma cell lines DM443 (top), DM175 (middle), and DM868 (bottom) were treated with sorafenib (10 μmol/L) for 2 h before the addition of the indicated concentrations of melphalan. Apoptosis was measured 24 and 48 h after melphalan addition by TUNEL assay. Columns, average percent TUNEL-positive cells of at least three independent measurements; bars, SEM.

protein was selectively knocked down using siRNA (see Supplementary Fig. S5A) and response to melphalan alone and in combination with sorafenib was measured. Response to melphalan alone ( $R_{\text{melph}}$ ) was not altered across the five melanoma cell lines tested when Mcl-1 protein expression was reduced. Furthermore, sorafenib enhancement of the response to melphalan was not diminished when Mcl-1 protein expression was reduced and, in the case of the BRAf mutant cell lines DM440 and DM443, was slightly enhanced. These results suggest that the chemosensitizing actions of sorafenib are not dependent on Mcl-1 downregulation.

## Discussion

In-transit metastases occur in 5% to 10% of cases of melanoma limited to the extremity (35). Whereas treatment of small-volume localized disease is re-excision, most patients present with diffuse disease with multiple skin and subcutaneous nodules requiring a regional or systemic form of treatment. In the absence of effective systemic therapies, regional chemotherapy using the alkylating agent melphalan in hyperthermic isolated limb perfusion or ILI has been the primary treatment of choice for patients with this pattern of disease (3, 5). Regional therapy,





**Figure 5.** Intracellular signaling changes in response to treatment with sorafenib. **A**, melanoma cell lines were treated with sorafenib (10  $\mu\text{mol/L}$ ) for 5 and 24 h and the levels of Mcl-1 protein and ERK activation (phospho-ERK) were measured by Western blotting to assess response to sorafenib. Total ERK and BRaf are shown to indicate equal loading of protein. **B**, the level of activation of ERK (phospho-ERK relative to total ERK; left) and the levels of Mcl-1 protein (right) following treatment with 10  $\mu\text{mol/L}$  sorafenib for 24 h were quantified from Western blots as shown in **A** and the average across three separate experiments is plotted as a function of cell survival in 10  $\mu\text{mol/L}$  sorafenib (the average of at least three separate experiments) after 12 d (top) or 48 h (bottom). Lines represent a linear regression analysis.

which, because the drug is limited to the affected extremity, allows for the use of much higher levels of chemotherapy than achievable with standard systemic treatment, has led to response rates of 70% to 90% with about one half of these responses being complete (36, 37). Temozolomide is a second-generation alkylating agent with a mechanism of action similar to that of dacarbazine (DTIC), currently the drug of choice for the treatment of systemic melanoma and the only widely used drug that is Food and Drug Administration approved for melanoma. Recent clinical trials have shown that for systemic treatment of metastatic melanoma, temozolomide is as effective as DTIC (38). Furthermore, in an animal model of extremity melanoma, ILI with temozolomide was shown to be as effective or more effective than melphalan in four of five melanoma xenografts studied (25).

Earlier attempts to improve the response rate of regionally administered chemotherapy in melanoma focused on adding tumor necrosis factor (TNF) to the regional circuit. A completed randomized prospective phase III trial comparing melphalan alone to melphalan plus TNF was stopped at its interim analysis because no response advantage to TNF was identified and there were three times the number of grade 4 adverse events in the TNF arm (39). More recent approaches to combination therapy have been designed so that the targeted agent or modulator of drug resistance is administered systemically around a window of time during which high-dose regional chemotherapy is given. One such combination therapy approach has been to target cell signaling pathways involved in tumor growth and survival. In the present study, we show that the multikinase inhibitor sorafenib can increase the cytotoxic response of melanoma cells to the alkylating chemotherapeutics melphalan and temozolomide. *In vitro*, across a panel of 24 cell lines, the sorafenib-mediated augmentation of chemotherapy-induced cytotoxicity was independent of BRAf or NRas mutational status (see Supplementary Fig. S2C). *In vivo*, in the context of a high-dose regional chemotherapy rat model, sorafenib enhanced the cytotoxic response of a human melanoma xenograft to both melphalan and temozolomide. These results provide compelling evidence that combination therapy of systemic sorafenib with regional chemotherapy infusion could provide a novel regimen for augmenting the treatment of melanoma patients with localized in-transit extremity disease.

Sorafenib can induce cell cycle arrest and both caspase-dependent and caspase-independent apoptosis via a variety of mechanisms (10–12, 19, 20, 40). In our cohort of cell lines, treatment with sorafenib was associated with decreased levels of ERK activation and Mcl-1 protein levels. Furthermore, the level of response of cell lines to sorafenib measured at day 12 was significantly correlated with the magnitude to which both ERK activation and Mcl-1 protein levels were decreased. Although previously published reports using both tumor cell lines and xenografts in mice suggest a lack of correlation between response and alterations in the Raf/ERK signaling path-

way (10, 32, 40), our results are in agreement with those reported for liver cancer cells lines, in which the antitumor activity of sorafenib corresponded with downregulation of ERK activity (40), and suggest that in melanoma the ability of sorafenib to downregulate ERK activity is a critical factor in the chemosensitizing actions of the drug. With respect to Mcl-1, previous studies have implicated the negative regulation of Mcl-1 as one of the main mechanisms of action of sorafenib alone and in combination with other agents (11, 12, 41). However, more recent data suggest that downregulation of Mcl-1 alone is not sufficient to induce cell death or enhance the sensitivity of cells to chemotherapy or radiation (19, 42), consistent with our observations.

Systemic treatment of rats with a small peptide inhibitor of N-cadherin has recently been shown to increase the levels of melphalan adducts in tumor cells in our xenograft melphalan ILI animal model (43). Likewise, alterations in drug delivery have been reported for other vascular targeting agents, including Gleevec (imatinib) and Avastin (bevacizumab; refs. 44–47). These observations led us to hypothesize that sorafenib-mediated inhibition of the vascular endothelial growth factor receptor tyrosine kinase, which can lead to reduced microvessel density (16, 32), may similarly increase melphalan delivery to tumors. Although our results failed to similarly show alterations in microvascular density, it is possible that 9 days of treatment with sorafenib was not sufficient to elicit significant changes in microvascular density similar to that observed after 15 days of treatment in a mouse model (16). Our results suggest that, for at least some melanoma cells and tumor xenografts, sorafenib actions may be able to reduce the cellular threshold for induction of apoptosis, thereby making cells more susceptible to other apoptotic-inducing therapies such as melphalan (Fig. 4). Others have shown similar enhancements in cell death when sorafenib was used in combination with radiation, the platinum-based chemotherapy agents oxaliplatin and cisplatin, rapamycin, the proteasome inhibitor MG-132, the Bcl-2 family inhibitor ABT-737, and tumor necrosis factor-related apoptosis-inducing ligand (12, 18–20, 41, 42, 48).

It is noteworthy that responses to sorafenib are likely to be dependent on the context of the cells treated, as suggested by our observation that DM868 cells did not respond to the combination of sorafenib and melphalan compared with the level of enhancement of apoptosis that we observed with the DM175 and DM443 cell lines (Fig. 4B). Similarly, others have observed that liver cancer cell lines responded differentially to sorafenib in terms of caspase-independent versus caspase-dependent apoptosis (40) and that the effects of sorafenib on the cell cycle varied remarkably between cell lines (19). These observations suggest that although a broad range of cells and tumors may be responsive to sorafenib alone or in combination, the mechanism by which the response occurs may depend on the molecular background and biology of the individual cell line or tumor.

The promising results we describe here on the efficacy of combinations of sorafenib and chemotherapy provide a strong rationale for the development of other systemically administered targeted agents to be used in conjunction with regionally administered chemotherapeutics for treatment of regionally advanced in-transit melanoma. Our results show that this approach may be effective not only with regionally administered chemotherapeutic agents like temozolomide (currently being evaluated in a phase I dose escalation ILI trial) but also with other systemically administered targeted agents or modulators. In addition to sorafenib, as described here, preclinical studies using systemically administered agents that target the glutathione detoxification system, such as the reduced glutathione-depleting agent buthionine sulfoximine, and cell adhesion, such as the small peptide inhibitor of N-cadherin ADH-1 (43, 49), have proved very effective in improving tumor responses. Phase I/II clinical trials examining the efficacy of these agents, including sorafenib, to enhance the response of melanoma to ILI with melphalan are currently in progress or under development. In the case of ADH-1, marked improvements in complete response rates from 30% to 50% have already been observed in phase I clinical trials (50). Correlative science

components of these trials will aim to understand the mechanism by which these targeted therapeutics affect response to chemotherapy. Through a better understanding of the molecular mechanism underlying the ability of sorafenib to mediate chemosensitization in regional chemotherapy, we hope to provide the basis for developing strategies to use sorafenib more effectively in patients such that, ultimately, it can be optimized for use in systemic chemotherapy strategies for metastatic melanoma.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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