The Raf Inhibitor BAY 43-9006 (Sorafenib) Induces Caspase-Independent Apoptosis in Melanoma Cells

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Abstract

Mitogen-activated protein kinase (MAPK) is activated in the majority of melanomas, and its activity is essential for cell survival. In this report, we examined the effects of a novel raf inhibitor BAY 43-9006 on melanoma cell viability and intracellular signaling and found that it induces apoptosis through a caspase-independent mechanism. At concentrations that suppress extracellular signal-regulated kinase (ERK) phosphorylation, BAY 43-9006 dephosphorylates Bad on Ser75 and Ser99, activates Bak and Bax, and reduces the mitochondrial transmembrane potential. BAY 43-9006 (sorafenib) down-modulates the levels of Bcl-2 and Bcl-X₁ in a MAPK-independent manner in A2058 and SKMEL5 melanoma cells but not in the more resistant A375 cells. Of the three lines tested, only A375 cells were rescued from BAY 43-9006–induced apoptosis by knocking down Bad. BAY 43-9006 induced poly(ADP-ribose) polymerase cleavage and the mitochondrial release of cytochrome c and SMAC. However, the pan-caspase inhibitor Z-VAE-fmk had only a modest protective effect against the drug, suggesting that BAY 43-9006–induced apoptosis is largely caspase independent. BAY 43-9006 but not the MAP/ERK kinase inhibitors PD98059 or U0126 induced the nuclear translocation of apoptosis-inducing factor (AIF) in A2058 and SKMEL5 cells, and the introduction of a small interfering RNA (siRNA) for AIF partially protected these cells from BAY 43-9006–induced apoptosis. The AIF siRNA had little effect in A375 cells, in which drug-induced AIF release was negligible. These data indicate that in sensitive cell lines, BAY 43-9006–induced apoptosis is independent of Bad dephosphorylation and caspase activation and largely mediated through the nuclear translocation of AIF. (Cancer Res 2006; 66(3): 1611-9)

Introduction

Over 60% of cutaneous melanomas and a smaller fraction of papillary thyroid and other carcinomas have an activating mutation in the gene encoding the serine/threonine kinase B-raf (1–5). Approximately 80% to 90% of these mutations involve a T1796A transversion in exon 15 that results in the substitution of a glutamate for a valine at position 599 (V599E) in the B-raf kinase domain. Prior knock-down studies have shown that suppressing extracellular signal-regulated kinase (ERK) phosphorylation, BAY 43-9006 dephosphorylates Bad on Ser75 and Ser99, activates Bak and Bax, and reduces the mitochondrial transmembrane potential. BAY 43-9006 (sorafenib) down-modulates the levels of Bcl-2 and Bcl-X1 in a MAPK-independent manner in A2058 and SKMEL5 melanoma cells but not in the more resistant A375 cells. Of the three lines tested, only A375 cells were rescued from BAY 43-9006–induced apoptosis by knocking down Bad. BAY 43-9006 induced poly(ADP-ribose) polymerase cleavage and the mitochondrial release of cytochrome c and SMAC. However, the pan-caspase inhibitor Z-VAE-fmk had only a modest protective effect against the drug, suggesting that BAY 43-9006–induced apoptosis is largely caspase independent. BAY 43-9006 but not the MAP/ERK kinase inhibitors PD98059 or U0126 induced the nuclear translocation of apoptosis-inducing factor (AIF) in A2058 and SKMEL5 cells, and the introduction of a small interfering RNA (siRNA) for AIF partially protected these cells from BAY 43-9006–induced apoptosis. The AIF siRNA had little effect in A375 cells, in which drug-induced AIF release was negligible. These data indicate that in sensitive cell lines, BAY 43-9006–induced apoptosis is independent of Bad dephosphorylation and caspase activation and largely mediated through the nuclear translocation of AIF. (Cancer Res 2006; 66(3): 1611-9)

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These findings suggest that melanoma cell death resulting from raf inhibition (with a specific siRNA or with the pharmacologic inhibitor BAY 43-9006) might similarly depend on the inactivation of p90<sub>rbk</sub> and the dephosphorylation of Bad.

Other studies, however, suggest that the proapoptotic effects of raf inhibition may be more complex. For example, Erhardt et al. have shown that cytchrome c does not activate caspases when added to lysates of cells overexpressing B-raf (23). This finding suggests that B-raf may function downstream of the mitochondria to prevent apoptosis. Although raf-1 knockout mice die during early embryogenesis, mice homozygous for a dual raf-1 mutation (Y340F/Y341F) that has no in vitro kinase activity toward MEK are phenotypically normal (24). These and other studies lend support to the notion that the predominate antiapoptotic effects of the various raf isoforms may be independent of MEK/ERK signaling (24–27), and the apoptosis induced by raf inhibition may not be due to the loss of MEK/ERK activity.

Regardless of the mechanism by which raf inhibition induces apoptosis, the data cited above underline the potential value of MAPK inhibitors in the treatment of melanoma. Indeed, several such agents have demonstrable activity in animal models and have recently entered clinical trials (28, 29). Perhaps the best characterized of these new agents is BAY 43-9006, a potent inhibitor of c-raf and B-raf as well as other potentially important kinase targets, such as the platelet-derived growth factor (PDGF) receptor and c-kit (29). This agent has substantial activity in human tumor xenograft models and in phase II clinical trials, especially when administered in conjunction with carboplatin and paclitaxel (30). These data lend credence to the notion that the MAPK pathway may be a clinically exploitable Achilles’ heel for an otherwise untreatable malignancy.

The studies reported in this article were undertaken to determine the mechanism by which BAY 43-9006 induces apoptosis in melanoma cells and in particular, the extent to which the suppression of ERK and Bad phosphorylation contributes to the process. Our results suggest that biochemical events independent of MAPK inhibition may be the more important determinants of the lethality of this novel agent for melanoma cells.

Materials and Methods

Cell lines and reagents. The human melanoma cell lines A375, A2058, and SKMEL5 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 10% fetal bovine serum (Sigma, St. Louis, MO), 2 mmol/L glutamine, and 50 μg/mL gentamicin at 37°C in 5% CO<sub>2</sub>. The A2058 and SKMEL5 cells are heterozygous for the constitutively active B-raf<sup>y99fs</sup> mutation (1), whereas the A375 line is homozygous as determined by sequence analysis. BAY 43-9006 was provided by Bayer Pharmaceuticals (New York, NY). The MEK inhibitors PD 98059 and U0126 were obtained from Cell Signaling. The antibody used to detect p-Ser75 in Bad was generated against a murine Bad phosphopeptide spanning Ser112, the equivalent site in the murine molecule.

Preparation and transfection of siRNAs for Bad and AIF. siRNAs for Bad and AIF were obtained from Santa Cruz Biotechnology. Cells were transfected with the Ds-Red Express construct (BD Clontech) alone or jointly with one of the siRNAs with the siRNA present in a 1:10 molar excess. The transfections were carried out using the TransIT-TKO Transfection reagent (Mirus Corp., Madison, WI) following the manufacturer’s protocol. After 48 hours, a portion of the cells were sorted for the Ds-Red–positive fraction using a MoFLO cell sorter (DakoCytomation, Ft. Collins, CO). The sorted red-fluorescent cells were then lysed and analyzed by Western blot to confirm that the siRNA had the desired effect on the level of the protein of interest. The remaining cells were treated with BAY 43-9006 (20 μmol/L) for 6 hours, stained with FITC–Annexin V, and then analyzed for red (FL2) and green (FL1) fluorescence using a Coulter FC 500 Flow cytometer as described above. The data were reported as the percentage of red-fluorescent cells staining with FITC–Annexin V.

Detection of cytochrome c, SMAC, and AIF release from the mitochondria. Mitochondria-enriched and cytosolic fractions were isolated from Dounce-homogenized cells using the ApoAlert Cell Fractionation Kit (BD Clontech). The quality of the mitochondria-enriched fractions was validated by Western blots using an antibody for the mitochondrial protein cytochrome c oxidase subunit IV (Cox4, BD Clontech; ref. 32). Cytosolic fractions were obtained during the isolation of the mitochondrial lysates from the mitochondrial and cytosolic fractions were analyzed for cytochrome c (BD Clontech) and SMAC/Diablo by Western blot as described above. Nuclei were isolated according to a standard protocol.
lysed, and analyzed along with the corresponding cytosolic fractions for AIF by Western blot as described above.

To determine if AIF translocation to the nucleus could be detected in intact cells by immunohistochemistry, A2058 cells were plated onto eight-well Tissue Culture Treated slides (BD Falcon, Bedford, MA). The cells were treated with BAY 43-9006 (20 μmol/L), PD98059 (50 μmol/L), or U0126 (20 μmol/L) for 5 hours and fixed with 0.5 % zinc chloride and 0.5 % zinc acetate for 15 minutes. The cells were then treated with rabbit anti-AIF (1:100, Santa Cruz Biotechnology) followed by goat-anti-rabbit coupled to Alexa Fluor 488 (1:100; Molecular Probes, Eugene, OR). Nuclei were detected with Bisbenzimide H33342 (Alexis Biochemicals, San Diego, CA) as previously described (34). The slides were mounted and analyzed using a Zeiss Axiosplan fluorescence microscope. The anti-AIF (green colored) and nuclear (blue colored) stains were photographed and analyzed using Adobe Photoshop.

Statistical analysis. As previously stated, the apoptotic effects of drug exposure and the modulatory effects of transfection with any of several siRNAs on drug-induced apoptosis were quantified by measuring the percentage of cells staining with Annexin V alone or with both Annexin V and propidium iodide. In each of these studies, >1.0 x 10^7 events were analyzed. Studies comparing the percentage of Annexin or Annexin/propidium iodide staining cells within two disparately treated populations were analyzed with the two-sample Z test. Studies comparing the mean fluorescence intensities of two populations were analyzed with the two-sample t test.

Results

BAY 43-9006 induces apoptosis in melanoma cell lines. The concentration of BAY 43-9006 required to inhibit the MAPK pathway in tumor cell lines is quite variable (29). To determine the sensitivity of melanoma cell lines to the drug, A375, A2058, and SKMEL5 human melanoma cells were exposed to BAY 43-9006 at concentrations ranging from 0 to 20 μmol/L for 4 hours. The cells were then lysed, and the lysates were analyzed for MEK and ERK phosphorylation by Western blot as described in Materials and Methods. MEK phosphorylation was inhibited with 3 μmol/L BAY 43-9006 in A2058 cells and completely suppressed in all three cell lines at a concentration of 10 μmol/L. ERK phosphorylation was completely suppressed with 10 μmol/L BAY 43-9006 in the A2058 cells, whereas SKMEL5 cells required 20 μmol/L. A375 cells proved to be the most resistant in that trace residual ERK2 (p42) phosphorylation was detectable at 4 hours even at 20 μmol/L (Fig. 1A).

Figure 1. A, inhibition of MEK and ERK phosphorylation by BAY 43-9006. Melanoma cells were exposed to the drug at the indicated concentrations for 4 hours. The cells were then lysed, and the lysates were analyzed by Western blot. B, induction of apoptosis in melanoma cell lines by BAY 43-9006. Melanoma cells were exposed to the drug at the indicated concentrations for 20 hours, stained with FITC-Annexin V and propidium iodide, and then analyzed by flow cytometry. Numbers, percentage of cells staining with both Annexin V and propidium iodide.
To assess the lethality of BAY 43-9006 for melanoma cell lines, A375, A2058, and SKMEL5 cells were exposed to the drug at concentrations ranging from 0 to 20 μmol/L for 20 hours and then analyzed by flow cytometry with FITC-conjugated Annexin V and propidium iodide. As shown in Fig. 1B, BAY 43-9006 increased the fraction of cells staining with Annexin V and propidium iodide in a concentration-dependent manner for each of the cell lines studied. In the case of the SKMEL5 cells, >80% of the cells stained with Annexin V/propidium iodide after exposure to 20 μmol/L BAY 43-9006.

To assess the effects of BAY 43-9006 on mitochondrial polarity, cells were incubated with the drug (20 μmol/L for 20 hours). The Mitosensor reagent was added, and the cells were then analyzed by flow cytometry for polymeric (intramitochondrial) and monomeric (cytosolic) forms of the Mitosensor. As shown in Fig. 2A, exposure to BAY 43-9006, markedly increased the amount of monomeric Mitosensor within the cells, indicating a diminution in mitochondrial uptake and transmembrane potential. The mean fluorescence intensity (MFI) for the monomer was increased 4-fold in A375 cells, 5-fold in A2058 cells, and ~2-fold in SKMEL5 cells as a result of exposure to BAY 43-9006. The two MEK inhibitors PD98059 and U0126 had no effect on the subcellular distribution of the Mitosensor in A375 and A2058 cells and only a modest effect in SKMEL5 cells at concentrations that inhibited ERK phosphorylation (50 and 20 μmol/L, respectively). The BAY 43-9006–induced accumulation of the Mitosensor in the cytosol was highly significant (P < 0.001 for each cell line).

To determine if BAY 43-9006–induced mitochondrial depolarization might be associated with the activation of the proapoptotic Bcl-2 family members Bak or Bax, melanoma cells were treated with the drug (20 μmol/L for 4 hours), permeabilized with digitonin, and then analyzed by flow cytometry using antibodies specific for the NH2 termini of Bak and Bax. In viable cells, the NH2 termini of both Bak and Bax are concealed within the hydrophobic core of the respective proteins. In response to an apoptotic stimulus, however, the proteins refold, exposing NH2-terminal epitopes that can be detected by flow cytometry (20, 35). As shown in Figure 2B, BAY 43-9006 increased the amount of monomeric Mitosensor within the cells, indicating a diminution in mitochondrial uptake and transmembrane potential.

Figure 2. A, drug-induced mitochondrial depolarization in melanoma cell lines as determined with the Mitosensor. MFI and SE for the monomer (bottom right quadrant). B, activation of Bak and Bax by BAY 43-9006 as determined by flow cytometry. The open and shaded curves were generated from BAY 43-9006–treated and untreated cells, respectively. MFI and SE of the untreated (U) and treated (T) cells (top right quadrant). C, Western blot analysis of cell lysates from untreated and BAY 43-9006–treated melanoma cells.
Figure 3. *A*, effects of BAY 43-9006, PD98059, and U0126 on Bad Ser\(^{75}\) and Ser\(^{99}\) phosphorylation. Indicated time points refer to the changing of the culture medium. *B*, effect of Bad siRNA transfection on the lethality of BAY 43-9006 in melanoma cells. Apoptosis was assessed in untreated (open columns) and BAY 43-9006–treated (shaded columns) cells by Annexin V staining. The Bad knockdown was confirmed by Western blot.

in Fig. 2B, exposure to BAY 43-9006 resulted in the activation (i.e., refolding) of both Bak and Bax in all three lines. The MFIs for Bak increased ~2-fold in all cell lines as a result of drug exposure and that of Bax increased from 1.4-fold (in A2058 cells) to 2.9-fold (in A375 cells). This increase in Bak and Bax staining was not due to an increase in protein levels as no increase in either protein could be detected by Western blot (Fig. 2C). These increases in MFI were highly significant by \(t\) test (\(P < 0.0001\) for both Bak and Bax in each cell line).

**Effects of BAY 43-9006 on proapoptotic and antiapoptotic members of the Bcl-2 family.** The accumulation of dephosphorylated Bad in the mitochondria has been proposed as a mechanism by which MEK inhibitors induce programmed cell death in melanoma cells (16). To determine if theraf inhibitor BAY 43-9006 similarly inhibits Bad phosphorylation, melanoma cells were exposed to the drug (20 \(\mu\)mol/L for 20 hours) and lysed, and the lysates were then analyzed by Western blot using 12% SDS-PAGE. Cells treated with the MEK inhibitors PD98059 (50 \(\mu\)mol/L) or U0126 (20 \(\mu\)mol/L) were similarly analyzed. For these studies, we used a phospho-specific antibody that recognizes p-Ser\(^{75}\), the site phosphorylated by the ERK substrate p90rsk, and an antibody specific for p-Ser\(^{99}\), the site phosphorylated by Akt and the mammalian target of rapamycin target p70rsk (16, 36). As shown in Fig. 3A, BAY 43-9006 as well as the two MEK inhibitors each suppressed Bad phosphorylation on Ser\(^{75}\) in all three cell lines. Ser\(^{99}\) was also dephosphorylated by BAY 43-9006, although this effect was delayed relative to the dephosphorylation of Ser\(^{75}\) and not observed in cells treated with either of the MEK inhibitors, suggesting that BAY 43-9006–induced Ser\(^{99}\) dephosphorylation cannot be attributed to MAPK inhibition.

To determine if the dephosphorylation of Bad might be sufficient to account for the lethal effects of BAY 43-9006 on melanoma cells, A375, A2058, and SKMEL5 cells were transfected with an siRNA for Bad and the Ds-Red Express construct (10:1 ratio) or with the Ds-Red Express construct alone. Cells were then exposed to BAY 43-9006 (20 \(\mu\)mol/L for 20 hours) and analyzed for Annexin V staining by flow cytometry, gating on the red-fluorescent (Ds-Red\(^{+}\)) cells. To confirm the Bad knockdown, the singly and dually transfected cells were sorted for Ds-Red expression. The Ds-Red\(^{+}\) cells were then lysed and analyzed for Bad expression by Western blot. As shown in Fig. 3B, BAY 43-9006 induced a 3.9-fold increase in the percentage of Annexin (+) Bad-replete A2058 cells and a 2.6-fold increase in the percentage of Annexin (+) Bad-deficient A2058 cells (\(P < 0.0001\) for both).

The difference between the Annexin staining of the drug-treated Bad-replete and Bad-deficient cells was not statistically significant (\(P = 0.33\)). BAY 43-9006 increased the percentage of Annexin (+) SKMEL5 cells by 6.2- and 2.5-fold in the Bad-replete and Bad-deficient preparations, respectively (\(P < 0.0001\)). The difference between the Annexin staining of the drug-treated Bad-replete and Bad-deficient SKMEL5 cells was significant (\(P < 0.0001\)), indicating some degree of protection, albeit modest, by the Bad...
siRNA. BAY 43-9006 increased the percentage of Annexin (+) Bad-replete A375 cells 1.7-fold ($P < 0.0001$) but that of the Bad-deficient cells only 1.1-fold, the latter of which did not reach statistical significance ($P = 0.52$), indicating that the Bad knockdown protected the A375 cells from BAY 43-9006–induced apoptosis. These data indicate that the contribution of Bad dephosphorylation to BAY 43-9006–induced apoptosis varies among melanoma cell lines and in only one line examined did the knockdown of Bad completely block the proapoptotic effects of the drug.

We also examined the effects of BAY 43-9006 on the expression of the antiapoptotic proteins Bcl-2 and Bcl-XL. As shown in Fig. 4, the drug had little effect on Bcl-2 and Bcl-XL levels in A375 cells. However, it nearly eliminated both proteins from the more sensitive A2058 and SKMEL5 cells. The down-modulation of Bcl-2 was evident within an hour of adding the drug, whereas that of Bcl-XL was more gradual. ERK phosphorylation was equally inhibited in all three cell lines in this study. Of note is the observation that neither of the two MEK inhibitors tested (PD98059 and U0126) induced the down-modulation of Bcl-2 or Bcl-XL, suggesting that this effect of BAY 43-9006 may not be attributable to the inhibition of the MAPK pathway. The data shown in Figs. 3 and 4 suggest that the down-modulation of Bcl-2 and Bcl-XL may obviate the need for Bad dephosphorylation in the induction of apoptosis and that the dephosphorylation of Bad may play an essential role in BAY 43-9006–induced cell death only in those cell lines that maintain high Bcl-2 and Bcl-XL levels in the presence of the drug.

**BAY 43-9006 induces mitochondrial cytochrome c and SMAC release and PARP cleavage.** To determine if the mitochondrial depolarization and Bak/Bax activation induced by BAY 43-9006 are associated with the release of proapoptotic proteins from the mitochondria, A375, A 2058, and SKMEL5 melanoma cells were exposed to the drug (20 μmol/L for 20 hours), disrupted by Dounce homogenization, and then separated into cytosolic and mitochondrial fractions. The individual fractions were detergent solubilized and analyzed by Western blot for cytochrome c and SMAC. As shown in Fig. 5A, BAY 43-9006 induced the leakage of cytochrome c from the mitochondria into the cytosol in all three cell lines. The release of the IAP-binding protein SMAC was less consistent and correlated with the relative sensitivities of the cell lines to BAY 43-9006 in that the release was scarcely detectable in A375 cells but nearly quantitative in the more sensitive A2058 cells (Fig. 5B).

To determine if the cytochrome c and SMAC release resulted in caspase activation, cells were treated with BAY 43-9006 as above for various intervals and lysed. The lysates were then fractionated by 8% SDS-PAGE and analyzed by Western blot for PARP cleavage. As shown in Fig. 5C, an 89-kDa PARP proteolytic fragment could be detected in the lysates of each cell line at 24 hours and in A2058 and SKMEL5 at 4 hours. The extent to which PARP was cleaved correlated with the relative sensitivities of the cell lines to BAY 43-9006–induced apoptosis as determined by Annexin V/propidium iodide staining in that the cleavage was more extensive in A2058 than A375 cells and nearly quantitative in the most sensitive cell line SKMEL5.

To assess the contribution of caspase activation to the lethality of BAY 43-9006, the pan-caspase inhibitor Z-VAD-fmk (20 μmol/L) was added to the melanoma cells. Thirty minutes later, BAY 43-9006 (20 μmol/L) was added, and the cells were subsequently analyzed by flow cytometry for staining with Annexin V/propidium iodide. As shown in Fig. 5D, the caspase inhibitor had only a modest protective effect on the cells. In the case of the SKMEL5 cells, for example, Annexin V/propidium iodide staining after 24 hours of exposure to BAY 43-9006 was only slightly reduced (95-84%) by the inclusion of Z-VAD at a concentration that reduced the extent of PARP cleavage from nearly complete to scarcely detectable. For the purpose of statistical analysis, we took the percentage of Annexin/propidium iodide (+) Z-VAD alone–treated cells to represent the Annexin/propidium iodide staining that would be observed in cells treated with both BAY 43-9006 and Z-VAD in the event that Z-VAD completely inhibited BAY 43-9006–induced apoptosis (i.e., 100% inhibition) and that of BAY 43-9006 alone–treated cells, respectively, to represent that of cells treated with both drugs in the event Z-VAD had no inhibitory effect (0% inhibition). Using this criterion to quantify drug-induced apoptosis, the probability that Z-VAD inhibited BAY 43-9006–induced apoptosis (i.e., Annexin/propidium iodide staining) by even 50% was <0.001 for all three cell lines at both the 6- and 24-hour time points. These data indicate that Z-VAD at concentrations that block PARP cleavage failed to prevent BAY 43-9006–induced apoptosis in melanoma cells.

**BAY 43-9006 induces mitochondrial release and nuclear translocation of AIF.** To determine if AIF is translocated from the mitochondria to the nucleus in response to BAY 43-9006, melanoma cells were treated with the drug (20 μmol/L for

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**Figure 4.** Effect of BAY 43-9006, PD98059, and U0126 on Bcl-2 and Bcl-XL levels in melanoma cells. The ERK and pERK analyses were included to confirm that all three drugs were used at concentrations that totally suppress ERK phosphorylation.
20 hours), Dounce homogenized, and fractionated into subcellular components. The mitochondrial and nuclear fractions were then detergent solubilized, fractionated by 12% SDS-PAGE, and analyzed for AIF by Western blot. As shown in Fig. 6A, BAY 43-9006–induced nuclear translocation of AIF followed a pattern similar to that of the SMAC release depicted in Fig. 5B in that it was undetectable in the A375 cells but nearly quantitative in the A2058 and SKMEL5 cells. The accumulation of AIF in the nucleus was also readily demonstrable by immunohistochemistry, as shown in Fig. 6B. In this study, the cells were examined after only 5 hours of drug exposure. In the untreated cells, AIF seems to be scattered throughout the cytosol, but within 5 hours, nuclear staining is clearly evident, indicating that the nuclear translocation of AIF is an early event in the apoptosis induced by BAY 43-9006. Of note

Figure 6. A, BAY 43-9006–induced translocation of AIF from the mitochondria to the nucleus. Untreated melanoma cells (0) and cells treated with BAY 43-9006 (B), PD98059 (P), or U0126 (U) for 20 hours were Dounce homogenized, and the various subcellular fractions were isolated, detergent solubilized, and analyzed by Western blot for AIF. B, subcellular distribution of AIF as determined by immunohistochemistry. Nuclei were localized by staining with Bisbenzimide. Arrows, nuclei staining for AIF. In this study, A2058 cells were exposed to the drugs indicated for only 5 hours. C, effect of AIF depletion on BAY 43-9006–induced Annexin V staining. The down-modulation of AIF by siRNA was confirmed by Western blot.
is the observation that neither of the two MEK inhibitors PD98059 or U0126 at concentrations sufficient to suppress ERK phosphorylation (i.e., 50 and 20 μmol/L, respectively) induced appreciable AIF translocation, suggesting that this particular effect of BAY 43-9006 is not the result of MAPK inhibition.

To assess the contribution of AIF nuclear translocation to BAY 43-9006–induced apoptosis, melanoma cells were transfected with an siRNA for AIF and a Ds-Red expression vector (10:1 ratio) or the Ds-Red construct alone. The transfected cells were exposed to BAY 43-9006 (20 μmol/L for 20 hours) and then analyzed for Annexin V staining by flow cytometry, gating on the Ds-Red+ population. The down-modulation of AIF by the siRNA was confirmed by Western blot analysis of lysates prepared from cells sorted for red fluorescence. As shown in Fig. 6C, the AIF siRNA reduced the percentage of cells that stained with Annexin after exposure to BAY 43-9006 in each cell line. However, only in the A2058 cells did the knockdown block the increase in Annexin staining induced by the drug. The drug-induced 3.9-fold increase in Annexin positivity in the AIF-replete A2058 cells was highly significant (P < 0.0001) but that induced in the AIF-deficient A2058 cells was not (P = 0.143). In the case of the A375 cells, the drug increased the percentage of Annexin (+) cells in both the AIF-replete and AIF-deficient cells (P < 0.0001 for both). Moreover, the fold increase in Annexin positivity induced by BAY 43-9006 was actually higher (3.9-fold) in the AIF+ knockdown cells than in the AIF-replete cells (1.7-fold), indicating no protection. This result is consistent with the failure of the drug to induce AIF translocation in these cells (Fig. 6A). The data with the SKMEL5 cells were intermediate in that BAY 43-9006 increased the Annexin positivity of both the AIF-replete and AIF-deficient cells (P < 0.0001 for both), although the increase was reduced from 6.2-fold to 2.2-fold by the AIF knockdown. These data indicate that in some sensitive cell lines, BAY 43-9006 induces the nuclear translocation of AIF, and this translocation plays an essential role in the apoptosis induced by the drug.

**Discussion**

MAPK is constitutively activated in virtually all melanomas (8), and the disruption of the raf/MEK/ERK signaling pathway with a B-raf siRNA or with a MEK inhibitor results in caspase activation and programmed cell death (6, 16, 28). The raf inhibitor BAY 43-9006 is also lethal to melanoma cell lines, although its mechanism of action may be considerably more complex than that proposed for MEK inhibitors.

Exposure to BAY 43-9006 activates Bak and Bax. In healthy cells, Bak and Bax are in an inactive conformation in which their NH2 and COOH termini are folded into a hydrophobic pocket (18–20, 35). In response to apoptotic stimuli, the proteins unfold and form multimers in the mitochondrial membrane. The lethality of this multimerization is blocked through the formation of heterodimers with Bcl-2 or Bcl-XL (22) and favored by signaling events that render Bcl-2 and Bcl-XL unavailable to partner with Bak and Bax.

In A2058 and SKMEL5 cells, BAY 43-9006 down-modulates Bcl-2 and Bcl-XL, an effect predicted to favor Bak and Bax homodimerization and apoptosis induction. This down-modulation of Bcl-2 and Bcl-XL could not be duplicated with either of the MEK inhibitors PD98059 or U0126 and is therefore presumably not mediated through the inhibition of MAPK. In cells in which Bcl-2 and Bcl-XL levels are maintained (e.g., A375), heterodimerization with Bak and Bax is presumably prevented by competition with Bad. When present in the mitochondria, Bad binds Bcl-2 and Bcl-XL, rendering them unavailable for heterodimerization with Bak and Bax (17). The dephosphorylation and mitochondrial translocation of Bad induced by MAPK inhibition would therefore be expected to have the same consequences for cell viability as the down-modulation of Bcl-2 and Bcl-XL. The results of our experiments with the Bad siRNA are entirely consistent with this notion as the effect of Bad depletion on the susceptibility to BAY 43-9006–induced apoptosis correlated with Bad levels and the effects of the drug on Bcl-2 and Bcl-XL. A2058 and SKMEL5 cells, both of which down-modulate Bcl-2 and Bcl-XL in response to BAY 43-9006 and have relatively low levels of Bad, were not protected by the Bad knockdown, whereas the A375 cells, which maintain high Bcl-2 and Bcl-XL levels in the presence of the drug and have high levels of Bad expression, were protected. These data suggest the existence of at least two distinctive patterns of response to BAY 43-9006. In some melanoma cell lines, the drug down-modulates Bcl-2 and Bcl-XL through a process independent of MAPK inhibition. Other cell lines respond to BAY 43-9006 as they might to a MEK inhibitor, in which case the dephosphorylation of Bad serves as the primary means of Bak and Bax activation and apoptosis induction.

Exposure to BAY 43-9006 resulted in cytochrome c release from the mitochondria and caspase activation (PARP cleavage) in all three cell lines examined. These events, however, played only a minor role in BAY 43-9006–induced apoptosis because the pan-caspase inhibitor Z-VAD-fmk had little effect on drug-induced Annexin V staining at concentrations sufficient to inhibit PARP cleavage. These data suggest that BAY 43-9006–induced apoptosis is largely caspase independent. BAY 43-9006 also induced the mitochondrial release of the IAP inactivator SMAC and AIF. The release of these proapoptotic proteins was highly variable from one cell line to another, scarcely detectable in the A375 cells but nearly complete in SKMEL5 and A2058 cells. As with the down-modulation of Bcl-2 and Bcl-XL, AIF release could not be induced with either of the MEK inhibitors, suggesting that this effect of BAY 43-9006 was not due to MAPK inhibition.

AIF is a flavoprotein with both oxidoreductase and DNA-binding domains but no intrinsic DNase activity (37–40). Depending on the circumstances, AIF can function to promote cell survival or induce apoptosis. In the mitochondria, AIF is involved in cellular respiration (37, 38) and is essential for cell survival, especially in retinal and cerebellar neurons (40). When translocated to the nucleus, AIF binds the DNase Endo G, another mitochondrial constituent released in response to apoptotic stimuli (39). This recruitment of EndoG results in DNA fragmentation and cell death (41, 42).

The release of AIF from the mitochondria and its subsequent translocation to the nucleus is one of the earliest events observed in melanoma cells after exposure to BAY 43-9006. These events were demonstrable by Western blot and immunohistochemistry within a few hours of drug exposure. To determine if AIF might be involved in the apoptosis induced by BAY 43-9006, we introduced an siRNA for AIF into melanoma cells and compared the responses of control and AIF-deficient cells to the drug. In each cell line, BAY 43-9006–induced Annexin V staining was reduced by the siRNA, suggesting that the AIF knockdown protected the cells from the drug. This protective effect was, however, highly variable depending on the cell line. The A2058 and SKMEL5 cells, which release AIF in response to the drug, were clearly protected by AIF depletion,
whereas the A375 cells (in which the knockdown was most successful) were the least protected by the AIF siRNA. This result is consistent with the fact that AIF release was nearly undetectable in the A375 cells.

The apoptosis induced in sensitive melanoma cell lines (e.g., A2058 and SKMEL5) by BAY 43-9006 has several features not shared with MEK inhibitors, including rapid onset, depolarization of the mitochondria, the down-modulation of Bcl-2 and Bcl-XL, the nuclear translocation of AIF, and the fact that the process is independent of Bad dephosphorylation. In the case of the more resistant A375 cells, BAY 43-9006-induced apoptosis is dependent on Bad dephosphorylation and is unaccompanied by either Bcl-2/ Bcl-XL down-modulation or AIF translocation and, in this respect, resembles that induced by MEK inhibitors. It is conceivable that the distinctions between the effects of raf and MEK inhibitors might be due to the ability of raf to promote cell survival through a mechanism independent of the MAPK pathway. Indeed, several non-MEK raf-binding proteins have been identified that clearly play a role in cell survival (20, 27). However, none of these alternative substrates are known to require phosphorylation by raf to exert their biological effects, and it is therefore unclear how their function might be affected by a raf kinase inhibitor, such as BAY 43-9006. It is of course possible that some of the apoptotic effects of BAY 43-9006 may be due to the inhibition of non-raf targets, such as c-kit or the PDGF receptor-β (29), and studies are currently under way to determine the extent to which the inhibition of these membrane tyrosine kinases contributes to BAY 43-9006-induced apoptosis and tumor regression.

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