

GSK-3 β Inhibition Enhances Sorafenib-induced Apoptosis in Melanoma Cell Lines*

Received for publication, June 29, 2007, and in revised form, November 7, 2007 Published, JBC Papers in Press, November 8, 2007, DOI 10.1074/jbc.M705343200

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Glycogen synthase kinase-3 β (GSK-3 β) can participate in the induction of apoptosis or, alternatively, provide a survival signal that minimizes cellular injury. We previously demonstrated that the multikinase inhibitor sorafenib induces apoptosis in melanoma cell lines. In this report, we show that sorafenib activates GSK-3 β in multiple subcellular compartments and that this activation undermines the lethality of the drug. Pharmacologic inhibition and/or down-modulation of the kinase enhances sorafenib-induced apoptosis as determined by propidium iodide staining and by assessing the mitochondrial release of apoptosis-inducing factor and Smac/DIABLO. Conversely, the forced expression of a constitutively active form of the enzyme (GSK-3 β^{S9A}) protects the cells from the apoptotic effects of the drug. This protective effect is associated with a marked increase in basal levels of Bcl-2, Bcl-x_L, and survivin and a diminution in the degree to which these anti-apoptotic proteins are down-modulated by sorafenib exposure. Sorafenib down-modulates the pro-apoptotic Bcl-2 family member Noxa in cells with high constitutive GSK-3 β activity. Pharmacologic inhibition of GSK-3 β prevents the disappearance of Noxa induced by sorafenib and enhances the down-modulation of Mcl-1. Down-modulation of Noxa largely eliminates the enhancing effect of GSK-3 inhibition on sorafenib-induced apoptosis. These data provide a strong rationale for the use of GSK-3 β inhibitors as adjuncts to sorafenib treatment and suggest that preservation of Noxa may contribute to their efficacy.

The multikinase inhibitor sorafenib blocks B- and c-Raf signaling and induces apoptosis in melanoma cell lines through a caspase-independent mechanism (1). This drug-induced programmed cell death is mediated at least in part by the down-modulation of Bcl-2, Bcl-x_L, and Mcl-1 and the nuclear translocation of apoptosis-inducing factor (AIF)² (1, 2). The precise mechanism by which these anti-apoptotic Bcl-2 family members are down-modulated by sorafenib and the extent to which

their disappearance contributes to the lethality of the drug are unknown.

The down-modulation of Mcl-1 in response to apoptotic stimuli is due to proteasomal degradation triggered by either a destabilizing interaction with the BH3-only Bcl-2 family member Noxa (3–5) or by phosphorylation (6). In some situations (e.g. apoptosis induced by growth factor withdrawal), the kinase responsible for this phosphorylation is glycogen synthase kinase-3 β (GSK-3 β) (6). The catalytic activity of this constitutively active kinase is held in check primarily through an inhibitory phosphorylation on Ser⁹ (7), and several kinases involved in growth or cell cycle progression, including the ERK substrate p90^{RSK} (ribosomal S6 kinase), are known to phosphorylate this site (7–10). Because sorafenib blocks ERK phosphorylation, we hypothesized that it might activate GSK-3 β and that the activation of this kinase might play a role in the down-modulation of Mcl-1 and in the programmed cell death induced by the drug.

GSK-3 β resides in several distinct subcellular fractions (e.g. nuclear, mitochondrial, and cytosolic), each with access to a unique array of substrates (7, 11–13). Apoptotic stimuli such as exposure to DNA-damaging chemotherapy, for example, often activate only the nuclear or a combination of the nuclear and mitochondrial fractions (12, 13). For these reasons, in our analyses of the effects of sorafenib on GSK-3 β activity, mitochondrial, nuclear, and cytosolic fractions were evaluated separately.

The catalytic activity of GSK-3 β for a particular substrate is influenced not only by the extent to which the kinase is phosphorylated (on Ser⁹) but also by its association with other proteins (e.g. axin) and by the degree to which the substrate is already phosphorylated (7, 14). The ideal phospho-acceptor site for GSK-3 β is, in fact, a serine or threonine 4 residues upstream of an already phosphorylated hydroxyamino acid (14–16). GSK-3 β -mediated phosphorylation is therefore frequently preceded by an obligatory preparatory phosphorylation by one of the casein kinases. This tandem action of casein kinase and GSK-3 β permits substantial variation in substrate phosphorylation by GSK-3 β that may not be reflected by the extent to which GSK-3 β is phosphorylated on Ser⁹. For these reasons, our assessments of the effects of sorafenib on GSK-3 β activity were based on analyses of the effects of the drug on the phosphorylation of known GSK-3 substrates as well as that of GSK-3 β itself.

Although GSK-3 β activation is intimately involved in the apoptotic response to growth factor withdrawal (6), mTOR (mammalian target of rapamycin) pathway inhibition (17), and other noxious stimuli, recent studies have demonstrated that in some circumstances, the kinase exerts a predominantly anti-apoptotic effect. For example, in a recent synthetic lethal siRNA

* This work was supported by the NCI Specialized Programs of Research Excellence in Skin Cancer Grant P50CA93683 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: AIF, apoptosis-inducing factor; GSK-3 β , glycogen synthase kinase-3 β ; PI, propidium iodide; GFP, green fluorescent protein; Smac, second mitochondrion-derived activator of caspase; HA, hemagglutinin; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase.

library screen, the siRNA for GSK-3 β was specifically identified as the one that most enhanced the toxicity of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in tumor cell lines (18). This finding suggests that constitutive GSK-3 β activity can limit the apoptotic response to death receptor signaling. The recent observation that certain GSK-3 β inhibitors are by themselves able to induce apoptosis in tumor cells through a p53-dependent mechanism (19, 20) further corroborates the view that GSK-3 β activity can have a pro-survival effect in some circumstances.

The studies reported herein were undertaken to determine whether sorafenib activates GSK-3 β and whether the kinase either facilitates or hinders sorafenib-induced programmed cell death in melanoma cell lines. Our data indicate that the kinase is indeed activated by the drug and that this activation reduces sorafenib lethality.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The human melanoma cell lines A375, A2058, and SKMEL5 were obtained from American Type Culture Collection and maintained in RPMI 1640 medium containing 10% fetal bovine serum (Sigma), 2 mM glutamine, and 50 μ g/ml gentamycin at 37 °C in 5% CO₂. The A2058 and SKMEL5 cells are heterozygous for the constitutively active B-Raf^{V599E} mutation (21), whereas the A375 line is homozygous as determined by sequence analysis. Sorafenib was provided by Bayer Pharmaceuticals, New Haven, CT. The GSK-3 inhibitor GSK-3 IX ((2'Z,3'E)-6-bromoindirubin-3'-oxime) was obtained from Calbiochem.

Western Blots—The melanoma cells were treated as described under "Results" and then lysed in lysis solution (Cell Signaling Technology, Danvers, MA) supplemented with sodium fluoride (10 μ M; Fisher) and phenylmethylsulfonyl fluoride (100 μ g/ml; Sigma-Aldrich). Lysates were fractionated in either 8 or 12% SDS-polyacrylamide gels, and the separated proteins were transferred to nitrocellulose. The blots were probed for the proteins of interest with specific antibodies followed by a second antibody-horseradish peroxidase conjugate and then incubated with SuperSignal chemiluminescence substrate (Pierce). Because of low levels of expression, the maximum sensitivity substrate was required for the imaging of GSK-3 α , which was scarcely detectable with the conventional substrate. The blots were then exposed to Kodak X-Omat Blue XB-1 film. The anti-ERK, anti-c-Myc, anti-Mcl-1, and anti-AIF antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-phospho-ERK, anti-GSK-3 α , anti-total and anti-phospho-GSK-3 β (Ser⁹), anti-Bcl-2, anti-Bcl-x_L, anti-Noxa, anti-phospho-Myc, anti-phosphoglycogen synthase, anti-total and anti-phospho-p53 (Ser³³), and anti-hemagglutinin (HA) antibodies were purchased from Cell Signaling. The anti-vinculin antibody was obtained from Sigma; the anti-Cox4 (cytochrome c oxidase subunit IV) antibody was obtained from Clontech; the anti-Smac/DIABLO antibody was purchased from eBioscience (San Diego, CA); and the anti-survivin antibody was from R&D Systems (Minneapolis, MN).

Cell Death Assay—In each assay, the adherent cells were detached from the underlying plastic by treatment with 20 mM EDTA in phosphate-buffered saline for 5 min and then com-

bined with the floating, non-adherent cells. Propidium iodide (5 ng/ml; Sigma) was added to the cell pool, and after 20 min at room temperature the cells were analyzed by flow cytometry with a Coulter FC 500 cytometer. The percentage of cells staining with propidium iodide (PI) was recorded, and each experiment reported was carried out at least three times. Data are reported as the means \pm S.E. for each experimental condition. In the studies examining apoptosis induction in cells into which an siRNA had been previously introduced, the percent of PI-positive cells (orange fluorescence, FL3) was computed by gating on the GFP-positive population (green fluorescence, FL1).

Subcellular Fractionation—Mitochondrial and cytosolic fractions were isolated from Dounce-homogenized cells using the ApoAlert cell fractionation kit (Clontech). The quality of the mitochondrial fractions was validated by Western blotting using an antibody for the mitochondrial protein Cox4 (22). Cytosolic fractions were obtained during the isolation of the mitochondria. Nuclei were isolated according to a standard protocol (23).

Generation of Cell Lines Expressing a Constitutively Active GSK-3 β —The constitutively active GSK-3 β vector GSK-3 β ^{S9A} (24) was a gift from Morris Birnbaum (University of Pennsylvania). To generate stable transfectants, SKMEL5 human melanoma cells were transfected with the vector (tagged with HA) using TransIT-TKO transfection reagent (Mirus, Madison, WI). Stable transfectants were selected and maintained in G418. Individual G418-resistant clones were further screened for expression of the GSK-3 β construct by Western blotting using an anti-HA antibody.

GSK-3 and Noxa Knockdown—The siRNA for GSK-3 used in these studies (obtained from Cell Signaling Technology) targets both the GSK-3 α and - β isoforms. Cells were cotransfected with the siRNA and a GFP construct ("Monster GFP," Promega, Madison, WI) in a 10:1 molar ratio or with GFP alone using the TransIT-TKO transfection reagent following the manufacturer's protocol. 48 h were allowed to elapse before the cells were used in any study assessing the effect of GSK-3 down-modulation on the susceptibility to sorafenib-induced apoptosis. The down-modulation of GSK-3 β by the siRNA was confirmed by Western blotting as described above. The siRNA for Noxa was obtained from Santa Cruz Biotechnology. This siRNA was cotransfected with DsRed-Express (Clontech) in a 10:1 ratio, and 48 h later, the cells were sorted using a MoFlo high-performance cell sorter (Cytomation, Fort Collins, CO). The red fluorescent cells were then used to assess the effects of Noxa down-modulation on the susceptibility to apoptosis induced by sorafenib and GSK-3 IX. As with the GSK-3 siRNA, the Noxa knockdown was confirmed by Western blot analysis.

Statistical Analysis—Data depicted as bar graphs represent means \pm S.E. from at least three separate experiments. For most of the studies shown, the significance of an apparent difference in mean values for any parameter (e.g. the percent of cells staining with propidium iodide) was validated by a Student's paired *t* test, and the difference was considered significant if *p* < 0.05. Studies involving comparisons between several clones were analyzed with analysis of variance and a post hoc Dunnett's *t* test.

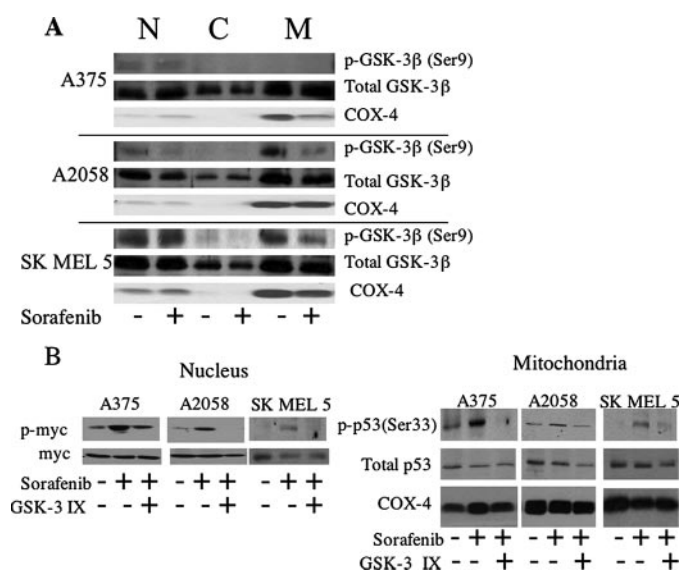


FIGURE 1. A, total and phospho (p)-GSK-3 β (Ser⁹) in the nuclei (N), cytosol (C), and mitochondria (M) of untreated and sorafenib-treated melanoma cell lines. Cox4 was used as a mitochondrial marker. B, effect of sorafenib on the phosphorylation of c-Myc (Thr⁵⁸) and p53 (Ser³³) in the nuclear and mitochondrial subcellular fractions, respectively. The enhanced phosphorylation of these substrates induced by sorafenib is blocked by the GSK-3 inhibitor GSK-3 IX.

RESULTS

Activation of GSK-3 β by Sorafenib—To assess the effects of sorafenib on GSK-3 β activity in melanoma cells, we first examined the effect of the drug on GSK-3 β Ser⁹ phosphorylation. A375, A2058, and SKMEL5 cells were exposed to the drug (20 μ M) for 5 h. Treated and untreated cells were then mechanically disrupted, and the lysates were centrifuged as described under “Experimental Procedures” to generate mitochondrial, nuclear, and cytosolic fractions from each cell preparation. The GSK-3 β activity of each fraction was then determined by Western blotting using antibodies specific for total and phospho-GSK-3 β (Ser⁹).

As shown in Fig. 1A, GSK-3 β was present in all three subcellular fractions in each cell line examined. The extent of Ser⁹ phosphorylation (*i.e.* GSK-3 β inactivation) differed, however, among the lines and individual subcellular fractions. For example, GSK-3 β Ser⁹ phosphorylation was absent in the cytosolic and mitochondrial fractions and scarcely detectable in the nuclear fraction of the A375 cells, suggesting that the enzyme is catalytically active in all subcellular fractions. In A2058 cells, Ser⁹ phosphorylation was evident in the nuclear and mitochondrial (but not cytosolic) fractions, but markedly reduced in response to sorafenib exposure, suggesting drug-induced activation of the kinase at these sites. The kinase was most abundantly phosphorylated in the SKMEL5 cells, and in this cell line the Ser⁹ phosphopeptide was detectable even in the cytosolic fraction. As in the A2058 cells, exposure to sorafenib reduced the extent of Ser⁹ phosphorylation in the mitochondrial fraction.

In addition to inferring GSK-3 β activity from the extent of Ser⁹ phosphorylation, we assessed the phosphorylation of several known GSK-3 β substrates present in the various subcellular fractions. For example, GSK-3 β phosphorylates (and desta-

bilizes) the nuclear transcription factor c-Myc on Thr⁵⁸ (25). As shown in Fig. 1B, exposure to sorafenib enhanced c-Myc phosphorylation in all three cell lines. This effect was blocked by the GSK-3 inhibitor GSK-3 IX (3 μ M), suggesting that the enhanced phosphorylation was GSK-3-mediated.

In addition to its role as a nuclear transcription factor, p53 has been shown to associate with Bax in the mitochondria and to enhance its pro-apoptotic activity (26). GSK-3 β phosphorylates p53 on Ser³³ (27). As shown in Fig. 1B, sorafenib increased p53 Ser³³ phosphorylation, and this effect was blocked by the inhibitor GSK-3 IX. Thus, the activities of both nuclear and mitochondrial fractions of GSK-3 β are enhanced by sorafenib.

Role of GSK-3 β Activation in Sorafenib-induced Apoptosis—To assess the contribution of GSK-3 β activation to sorafenib-induced apoptosis, A375 melanoma cells were exposed to increasing concentrations of the GSK-3 inhibitor GSK-3 IX for 20 h in the presence or absence of sorafenib (20 μ M). The cells were then stained with propidium iodide and analyzed for drug-induced cell death by flow cytometry as described under “Experimental Procedures.” As shown in Fig. 2A, GSK-3 IX by itself was nontoxic at concentrations of \leq 3 μ M. This concentration was, however, sufficient to enhance the lethality of sorafenib ($p < 0.0191$) and was therefore employed in our subsequent studies with other melanoma cell lines. As shown in Fig. 2B, GSK-3 IX (3 μ M) increased the lethality of sorafenib in all three cell lines tested. The PI staining induced by the sorafenib/GSK-3 IX combination was significantly greater than that induced by sorafenib alone for each cell line ($p < 0.018$, < 0.0098 , and < 0.029 for A375, A2058, and SKMEL5 cells, respectively). These data suggest that the GSK-3 β activation induced by sorafenib not only did not contribute to the apoptotic effect of the drug, but exerted a protective effect. The inhibition of ERK and glycogen synthase phosphorylation by sorafenib and GSK-3 IX, respectively, is shown in Fig. 2C. The anti-phosphoglycogen synthase antibody used in these Western blots is specific for Ser⁶⁴¹, one of three known phosphorylation sites of GSK-3 β (28). Glycogen synthase is constitutively phosphorylated in intact cells by several kinases (14, 15). The higher molecular weight band seen in the sorafenib-treated cells is thought to indicate the phosphorylation of additional glycogen synthase sites by GSK-3 β . This view is supported by the disappearance of this upper band in the lanes corresponding to the GSK-3 IX-treated cells.

The effect of GSK-3 β inhibition on sorafenib-induced cell death was also assessed using cells in which GSK-3 β was down-modulated with an siRNA. These siRNA studies were carried out with an siRNA preparation that targets both the GSK-3 α and β isoforms. The decision to use this siRNA was based on our observation that it down-modulates GSK-3 β far more effectively than a β -isoform-specific siRNA (data not shown). Furthermore, GSK-3 α was scarcely detectable in the melanoma cell line used in these studies, and its expression was unaffected by the GSK-3 α/β siRNA (Fig. 2D). As described under “Experimental Procedures,” cells expressing the GSK-3 siRNA were cotransfected with a GFP construct, and all determinations of PI staining for these cells were based on analysis, gating on the GFP-positive population.

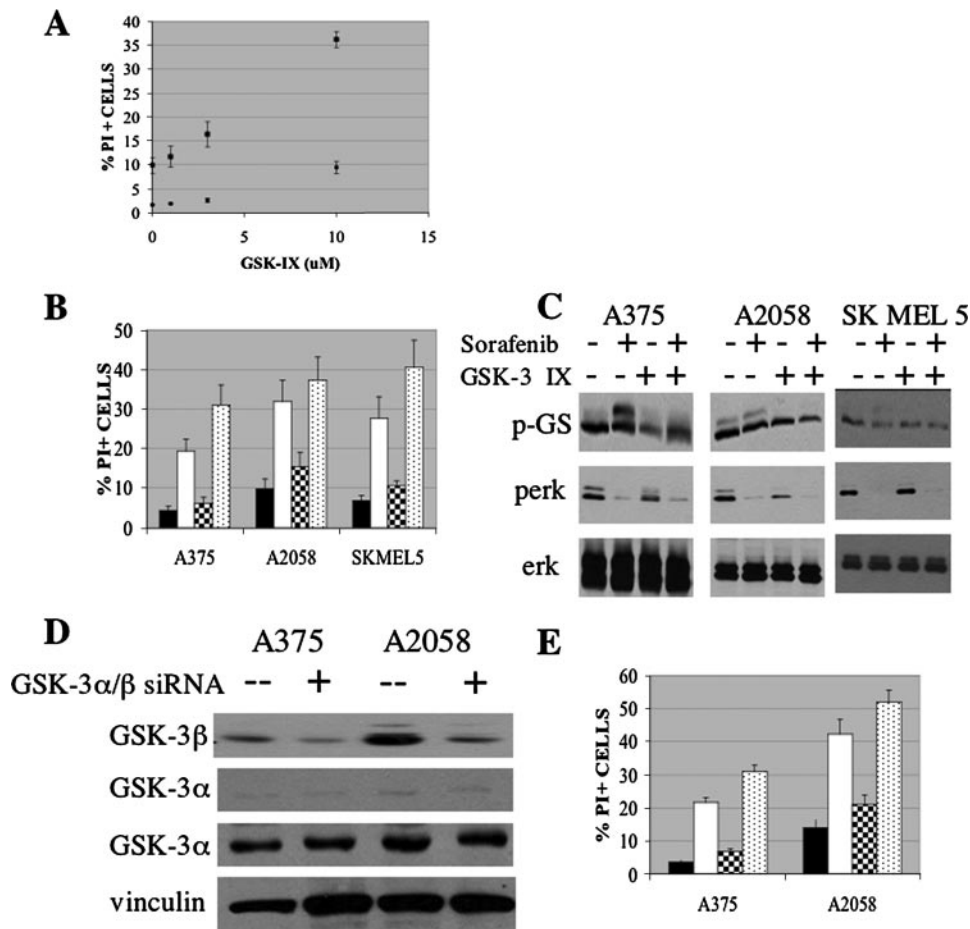


FIGURE 2. A, apoptotic effects of the GSK-3 inhibitor GSK-3 IX alone (●) or in combination with 20 μ M sorafenib (■) on A375 melanoma cells. Although low concentrations of GSK-3 IX (≤ 3 μ M) were nontoxic alone, they enhanced the lethality of sorafenib. Data were derived from seven separate experiments. B, effect of GSK-3 inhibition on sorafenib-induced cell death in melanoma cell lines. Cells were untreated (black bars), exposed to 20 μ M sorafenib alone (white bars), exposed to the GSK-3 inhibitor GSK-3 IX (3 μ M) alone (checked bars), or exposed to both agents (dotted bars) for 20 h; stained with PI; and then analyzed by flow cytometry. In the case of cells exposed to both drugs, the GSK-3 inhibitor was added 1 h prior to the addition of sorafenib. Data were derived from six separate experiments with A375 and A2058 cells and from three with SKMEL5 cells and are depicted as the mean \pm S.E. of each treatment group. C, Western blots verifying the inhibition of ERK and glycogen synthase (p-GS) phosphorylation by sorafenib and GSK-3 IX, respectively. D, validation of the down-modulation of GSK-3 β by the GSK-3 siRNA. The GSK-3 α/β siRNA down-modulated GSK-3 β but had no discernible effect on GSK-3 α expression as determined by Western blotting. GSK-3 α was scarcely detectable in A375 and A2058 cells even with prolonged exposure times using the conventional Western blot substrate and could be imaged as shown only with the maximum sensitivity substrate (see "Experimental Procedures"). E, effect of GSK-3 β down-modulation on sorafenib-induced apoptosis. Untreated and sorafenib-treated untransfected cells (black and white bars, respectively) and cells transfected with GSK-3 siRNA (checked and dotted bars, respectively) were stained with PI and analyzed by flow cytometry. Data were derived from three separate experiments.

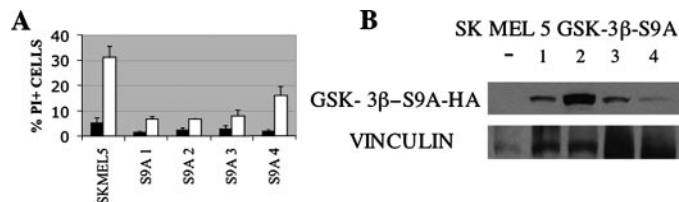


FIGURE 3. A, effect of GSK-3 activity on the susceptibility of SKMEL5 cells to sorafenib-induced cell death. Parent SKMEL5 cells as well as several GSK-3 β^{S9A} -transfected clones (designated S9A1–4) were exposed to sorafenib. The untreated (black bars) and treated (white bars) cells were then stained with PI and analyzed for cell death by flow cytometry as described. Data were derived from three separate experiments. B, Western blot showing the expression of the constitutively active GSK-3 β (S9A) tagged with HA in the SKMEL5 transfectants.

As shown in Fig. 2E, GSK-3 down-modulation was minimally toxic to the cells (*i.e.* had little effect on base-line PI staining). However, as was the case with GSK-3 IX treatment, expression of the siRNA increased the susceptibility of the cells to sorafenib-induced apoptosis. The PI staining induced by sorafenib in the untransfected cells was significantly less than in the transfected (*i.e.* GSK-3 β -depleted) cells for both cell lines studied ($p < 0.034$ for A375 and < 0.041 for A2058 cells).

The ability of GSK-3 β to suppress sorafenib-induced apoptosis was further corroborated by an analysis of SKMEL5 transfectants stably expressing a constitutively active (*i.e.* S9A) form of GSK-3 β (24). As shown in Fig. 3A, the susceptibility of each of several GSK-3 β^{S9A} transfectant clones to sorafenib-induced cell death was less than that of the parent SKMEL5 line. The PI staining of the sorafenib-treated parent SKMEL5 cells was significantly greater than that of any of the sorafenib-treated transfectants ($p < 0.01$ by analysis of variance; each < 0.05 for pairwise comparison *versus* the untransfected cells). In fact, the most sensitive of the clones (S9A4) hardly expressed the HA-tagged GSK-3 β^{S9A} construct as determined by Western blotting (Fig. 3B).

Effect of GSK-3 β Activity on Sorafenib-induced Smac and AIF Release from the Mitochondria—We previously demonstrated that even a brief (6-h) exposure to sorafenib is sufficient to induce the

release of Smac and AIF from the mitochondria of A2058 and SKMEL5 cells, but not from the more resistant A375 cells (1). To determine whether this divergent response to the drug might be attributable to differing basal levels of GSK-3 β activity, we exposed melanoma cells to sorafenib in the presence or absence of the GSK-3 inhibitor GSK-3 IX. After 5 h, the cells were disrupted and fractionated, and the various subcellular fractions were analyzed by Western blotting for Smac and AIF. As shown in Fig. 4, A375 cells released Smac into the cytosol in response to sorafenib only when GSK-3 was inhibited. AIF was likewise released and could be detected in the nuclei of cells exposed to both sorafenib and GSK-3 IX, but not in cells exposed to sorafenib alone. In contrast to A375 cells, SKMEL5 cells released Smac and AIF from the mitochondria in response

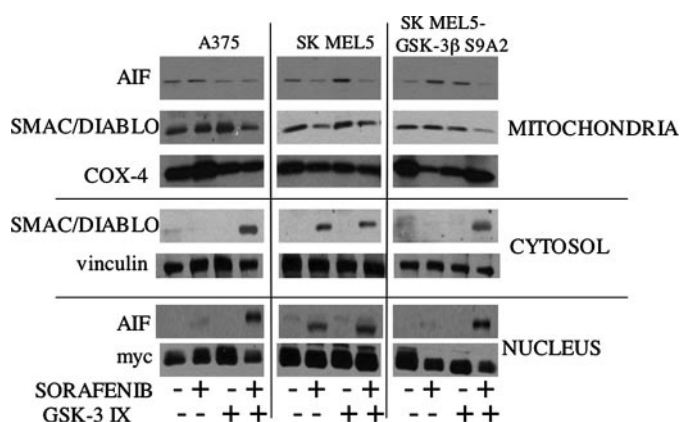


FIGURE 4. Western blot analyses of mitochondrial, cytosolic, and nuclear fractions of A375 and SKMEL5 (parent line) cells and an SKMEL5 GSK-3 β ^{S9A} transfectant (S9A2) before and after treatment with sorafenib \pm GSK-3 IX. Blots were probed for AIF and Smac/DIABLO. Cox4, vinculin, and c-Myc were used as markers for the mitochondrial, cytosolic, and nuclear subcellular fractions, respectively.

to sorafenib regardless of whether or not GSK-3 IX was present (1). This release was not observed in the SKMEL5 GSK-3 β ^{S9A} transfectant (S9A2), however, unless GSK-3 was inhibited pharmacologically. These data indicate that GSK-3 β inhibits the release of AIF and Smac from the mitochondria otherwise induced by exposure to sorafenib and suggest that the divergent apoptotic responses of various melanoma cell lines to this agent might be determined by constitutive GSK-3 β activity.

Effect of GSK-3 Activity on the Constitutive Expression and Sorafenib-induced Down-modulation of Anti-apoptotic Bcl-2 Family Members—In a previous report, we demonstrated that Bcl-2 and Bcl-x_L levels are low in melanoma cell lines that are highly susceptible to sorafenib-induced apoptosis (e.g. A2058 and SKMEL5) and that both of these proteins are rapidly down-modulated in response to the drug (1). Conversely, these proteins are both abundant in the more resistant melanoma cell line A375, and the levels are unaffected by sorafenib exposure. To determine whether these differences between melanoma cell lines might be attributed to variations in basal GSK-3 β activity, we compared the Bcl-2, Bcl-x_L, and Mcl-1 levels of SKMEL5 GSK-3 β ^{S9A} transfectants with those of the parent SKMEL5 cell line and with A375 cells. Of all of the Bcl-2 family members evaluated, Mcl-1 was the most abundantly expressed. As shown in Fig. 5A, the constitutive levels of Mcl-1 and Bcl-x_L were only slightly higher in the GSK-3 β ^{S9A} transfectants relative to the parent SKMEL5 line. On the other hand, Bcl-2 levels were markedly increased in the GSK-3 β ^{S9A} transfectants and approached levels seen in A375 cells (which have high constitutive GSK-3 β activity).

We also examined the effects of GSK-3 on sorafenib-induced down-modulation of these Bcl-2 family members. As shown in Fig. 5B, sorafenib had no effect on Bcl-2 or Bcl-x_L levels in A375 cells, even in the presence of the GSK-3 inhibitor GSK-3 IX. As previously reported (1), sorafenib down-modulated both Bcl-2 and Bcl-x_L in SKMEL5 cells. The SKMEL5 GSK-3 β ^{S9A} transfectant S9A2 maintained its Bcl-2 and Bcl-x_L levels in the presence of the drug, however, suggesting that chronic activation of GSK-3 β was able to prevent this particular sorafenib effect. Of note, the resistance to sorafenib-induced Bcl-2 and Bcl-x_L

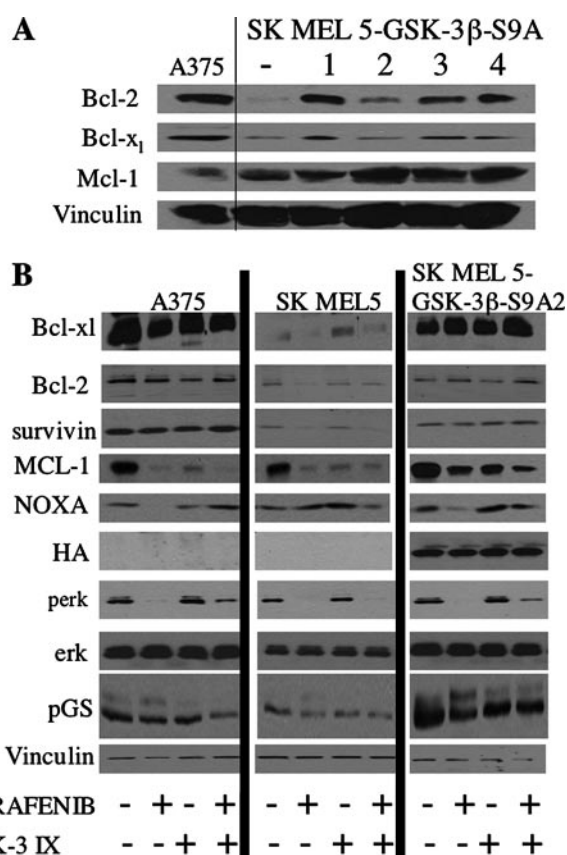


FIGURE 5. A, constitutive levels of Bcl-2, Bcl-x_L, and Mcl-1 in A375 and SKMEL5 cells and in SKMEL5 GSK-3 β ^{S9A} stable transfectants as determined by Western blotting. B, Western blot analyses of cell lysates from A375 and SKMEL5 (parent) cells and an SKMEL5 GSK-3 β ^{S9A} transfectant (clone S9A2) treated with sorafenib \pm GSK-3 IX. Blots were probed with antibodies for Bcl-2, Bcl-x_L, Mcl-1, Noxa, and survivin. An anti-HA antibody was used to validate the presence of the GSK-3 β ^{S9A} construct in the SKMEL5 transfectant. Antibodies against phospho-ERK (perk) and phosphoglycogen synthase (pGS) were used to confirm the biological potency of sorafenib and GSK-3 IX, respectively.

down-modulation observed in A375 cells and in the SKMEL5 GSK-3 β ^{S9A} transfectants was not reversed by GSK-3 IX.

Sorafenib was previously shown to down-modulate Mcl-1 in numerous tumor cell types (2). As shown in Fig. 5B, this effect was readily inducible in melanoma cell lines as well. Exposure to the GSK-3 inhibitor GSK-3 IX also down-modulated Mcl-1 levels in all lines examined, and this effect was additive to that of sorafenib.

Mcl-1 is destabilized when associated with the BH3-only protein Noxa (3–5). GSK-3 β inhibition has been shown recently to induce the expression of Noxa through a p53-dependent mechanism (19, 20). To determine whether the combined suppressive effects of sorafenib and GSK-3 IX on Mcl-1 levels might be explained by drug-induced changes in Noxa levels, melanoma cells were exposed to sorafenib, GSK-3 IX, or both drugs, and the cells were analyzed by Western blotting. As shown in Fig. 5B, sorafenib profoundly down-modulated Noxa in both A375 cells and the SKMEL5 GSK-3 β ^{S9A} transfectant S9A2 (both of which have high basal GSK-3 β activity), but not in the parent SKMEL5 cell line (which has low activity). This down-modulation was blocked by the inhibitor GSK-3 IX. Contrary to what has been observed with other GSK-3 inhibitors (19, 20), GSK-3 IX alone had little effect on Noxa levels.

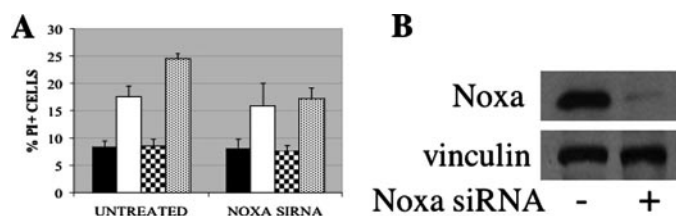


FIGURE 6. A, PI staining induced by sorafenib and GSK-3 IX in untransfected and Noxa-depleted A375 cells. The untransfected and Noxa-depleted cells were left untreated (black bars) or treated with sorafenib (white bars), GSK-3 IX (checked bars), or both drugs (dotted bars) for 20 h prior to PI staining. Data shown were derived from three separate transfection experiments. B, Western blot confirming the down-modulation of Noxa.

Effect of GSK-3 Activity on Sorafenib-induced Down-modulation of Survivin—In addition to its role as a caspase inhibitor and regulator of the G₂/M checkpoint (29), the IAP (inhibitor of apoptosis protein) survivin has been proposed to govern the release of AIF from the mitochondria (30). Because GSK-3 activity inhibits AIF release (Fig. 4), we considered the possibility that the kinase might achieve this protective effect indirectly through the up-regulation or stabilization of survivin. As shown in Fig. 5B, survivin is down-modulated by sorafenib in SKMEL5 but not A375 cells (which have higher basal GSK-3 β activity). The introduction of a constitutively active GSK-3 β (*i.e.* GSK-3 β^{S9A}) prevents the down-modulation of survivin that would otherwise occur in SKMEL5 cells in response to sorafenib. As was previously shown for Bcl-2 and Bcl-x_L, the stabilizing effect of chronic GSK-3 β activation on survivin was not reversed by exposure to the GSK-3 inhibitor GSK-3 IX.

Effect of Noxa Down-modulation on Sorafenib-induced Apoptosis—As shown in Fig. 5B, the down-modulation of Noxa by sorafenib was observed only in melanoma cell lines with high constitutive GSK-3 β activity (*e.g.* A375 and SKMEL5 GSK-3 β^{S9A} transfectants) and was blocked by GSK-3 IX, suggesting that the effect may be GSK-3 β -mediated. To assess the biological significance of the Noxa down-modulation induced by sorafenib, A375 cells were dually transfected with a Noxa siRNA and the fluorescent marker DsRed-Express as described under "Experimental Procedures," and the cells were then sorted. The red fluorescent (Noxa-depleted) cells were exposed for 20 h to various combinations of sorafenib (20 μ M) and GSK-3 IX (3 μ M) and then analyzed for PI staining by flow cytometry. As shown in Fig. 6A, the down-modulation of Noxa had no effect on baseline apoptosis or that induced by sorafenib but largely eliminated the enhancing effect of the GSK-3 inhibitor. The PI staining induced by sorafenib in the transfected (*i.e.* Noxa-depleted) cells was not significantly different from that induced in untransfected cells ($p < 0.78$). As previously shown, the PI staining induced by the sorafenib/GSK-3 IX combination was significantly greater than that induced by sorafenib alone in the untransfected A375 cells ($p < 0.032$), but this difference was lost in the Noxa-depleted cells ($p < 0.595$). The PI staining induced by the drug combination in the untransfected cells exceeded that induced in the Noxa-depleted cells ($p < 0.0367$). These data suggest that the enhancing effects of GSK-3 IX on sorafenib-induced apoptosis may be due substantially to its ability to prevent the down-modulation of Noxa that would otherwise be induced by sorafenib. Fig. 6B confirms the

down-modulation of Noxa in the transfectants by Western blotting.

DISCUSSION

GSK-3 β regulates numerous cellular processes, including programmed cell death (7). It phosphorylates a wide range of substrates, including transcription factors and Bcl-2 family members, many of which are destabilized and degraded in the proteasome as a result (7). The kinase can either play a critical role in apoptosis or promote cell survival, depending on the cell type and the nature of the cellular injury (6, 18). GSK-3 β activity is regulated by numerous upstream kinases, the inhibition of which leads to enzyme activation (7–10). On the basis of these considerations, we hypothesized that the Raf inhibitor sorafenib might activate GSK-3 β and that the constitutive as well as drug-enhanced activity of the kinase might affect the lethality of the drug in melanoma cells.

To assess the effects of GSK-3 β on the susceptibility of melanoma cells to sorafenib-induced apoptosis, we determined the degree to which drug exposure induced apoptosis (PI staining) in cells in which the activity of the kinase was suppressed by the pharmacologic inhibitor GSK-3 IX or genetically manipulated through the introduction of a GSK-3 siRNA or a constitutively active form of the kinase (GSK-3 β^{S9A}). Our data indicate that GSK-3 β activity protects against sorafenib-induced apoptosis. GSK-3 β inhibition or down-modulation increases sorafenib-induced apoptosis. Conversely, an increase in its activity, as seen in the SKMEL5 GSK-3 β^{S9A} transfectants, renders melanoma cells more resistant to the drug. This pro-survival effect of GSK-3 β is associated with the inhibition of sorafenib-induced AIF and Smac release from the mitochondria. A375 cells fail to release these pro-apoptotic proteins in response to sorafenib unless GSK-3 is inhibited. Conversely, SKMEL5 cells, which are otherwise prone to release AIF and Smac in response to sorafenib, fail to do so in the setting of chronic GSK-3 β activation (*i.e.* after transfection with the GSK-3 β^{S9A} construct).

The basis for the sorafenib resistance conferred upon melanoma cells by chronic GSK-3 β activation is unknown, but may involve increased expression of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x_L, or of the IAP survivin. Basal levels of these proteins are considerably lower in SKMEL5 cells (which have low basal GSK-3 β activity) than in A375 cells (Fig. 5B) and are down-modulated by sorafenib in SKMEL5 but not A375 cells. The introduction of an active form of GSK-3 β (*i.e.* GSK-3 β^{S9A}) into SKMEL5 cells not only increases the basal levels of Bcl-2, Bcl-x_L, and survivin, but also renders the cells resistant to sorafenib-induced down-modulation. These data suggest that high constitutive levels of Bcl-2, Bcl-x_L, and survivin, which are maintained in the presence of the drug, may account for the resistance to sorafenib seen in melanoma cell lines with high basal GSK-3 β activity.

Sorafenib was previously shown to down-modulate Mcl-1 in lung, colon, renal, and breast carcinoma cell lines (2). As shown in Fig. 5B, the drug has a similar effect in melanoma cell lines, including SKMEL5 GSK-3 β^{S9A} transfectants. The consistency with which Mcl-1 is down-modulated by sorafenib contrasts with the disappearance of Bcl-2 and Bcl-x_L, which is induced by the drug only in cells with low basal

GSK-3 β activity (e.g. SKMEL5 and A2058). The expression of an activated form of GSK-3 β in fact blocks the down-modulation of Bcl-2 and Bcl-x_L that would otherwise be induced by sorafenib in susceptible cells (e.g. SKMEL5). The basis for the differential effects of GSK-3 β on the susceptibility of these anti-apoptotic Bcl-2 family members to sorafenib-induced down-modulation is unknown.

In a previous study, the apoptosis induced in hematopoietic cell lines by interleukin-3 withdrawal was attributed to the activation of GSK-3 β and the resulting phosphorylation and degradation of the anti-apoptotic Bcl-2 family member Mcl-1 (6). The observation that Mcl-1 is a GSK-3 β substrate suggests that the inhibition of the kinase would likely increase Mcl-1 levels and reduce the susceptibility to apoptosis. However, as shown in Fig. 5B, exposure to the GSK-3 inhibitor GSK-3 β IX had the opposite effect and actually reduced Mcl-1 levels. Moreover, the down-modulation induced by the inhibitor was additive with that induced by sorafenib. The unexpected loss of Mcl-1 induced by the GSK-3 inhibitor suggests that the drug must have an effect on Mcl-1 that is unrelated to the predicted reduction in GSK-3-dependent Mcl-1 phosphorylation. Recent studies have shown that the acute inhibition of GSK-3 activates p53 and increases Noxa expression (19, 20), which would be expected to destabilize Mcl-1 (3–5). It is possible that in certain circumstances, this destabilizing effect of Noxa might override the diminution in Mcl-1 phosphorylation induced by GSK-3 inhibition, resulting in a decline in Mcl-1 levels and an increase in the susceptibility to apoptosis. Our data indicate that this is in fact the case in sorafenib-treated melanoma cells. We have noted that the down-modulation of Noxa with an siRNA largely eliminates the additive effect of GSK-3 inhibition on sorafenib-induced apoptosis (Fig. 6). These data suggest that the maintenance of Noxa levels in the presence of sorafenib (and the greater down-modulation of Mcl-1) might partly account for the enhancing effect of GSK-3 inhibition on sorafenib-induced apoptosis.

Our data indicate that sorafenib activates GSK-3 β in melanoma cell lines. Moreover, they suggest that the susceptibility of these cells to the lethal effects of sorafenib is regulated by GSK-3 β . Prolonged activation of the kinase is protective, possibly as a result of increased Bcl-2, Bcl-x_L, and survivin expression. For reasons yet to be determined, cells with high basal GSK-3 β activity also appear to be resistant to sorafenib-induced down-modulation of these anti-apoptotic proteins. Pharmacologic inhibition of GSK-3 β enhances sorafenib-induced apoptosis. The additive suppressive effects of sorafenib and GSK-3 β inhibition on Mcl-1 levels may partly account for this apparent synergy. Regardless of the biochemical mechanism by which GSK-3 β inhibition sensitizes melanoma cells to sorafenib, our data suggest that inhibitors of this kinase might

be useful adjuncts to Raf inhibitors in the treatment of metastatic melanoma.

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J. Biol. Chem. 2008, 283:726-732.

doi: 10.1074/jbc.M705343200 originally published online November 8, 2007

Access the most updated version of this article at doi: [10.1074/jbc.M705343200](https://doi.org/10.1074/jbc.M705343200)

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