

Cytotoxic synergy between the multikinase inhibitor sorafenib and the proteasome inhibitor bortezomib *in vitro*: induction of apoptosis through Akt and c-Jun NH₂-terminal kinase pathways

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Abstract

This study was undertaken to characterize preclinical cytotoxic interactions for human malignancies between the multikinase inhibitor sorafenib (BAY 43-9006) and proteasome inhibitors bortezomib or MG132. Multiple tumor cell lines of varying histiotypes, including A549 (lung adenocarcinoma), 786-O (renal cell carcinoma), HeLa (cervical carcinoma), MDA-MB-231 (breast), K562 (chronic myelogenous leukemia), Jurkat (acute T-cell leukemia), MEC-2 (B-chronic lymphocytic leukemia), and U251 and D37 (glioma), as well as cells derived from primary human glioma tumors that are likely a more clinically relevant model were treated with sorafenib or bortezomib alone or in combination. Sorafenib and bortezomib synergistically induced a marked increase in mitochondrial injury and apoptosis, reflected by cytochrome *c* release, caspase-3 cleavage, and poly(ADP-ribose) polymerase degradation in a broad range of solid tumor and leukemia cell lines. These findings were accompanied by several biochemical changes, including decreased phosphorylation of vascular endothelial growth factor receptor-2, platelet-derived growth factor receptor- β , and Akt and increased phosphorylation of stress-related c-Jun NH₂-terminal kinase (JNK). Inhibition of Akt was required for synergism, as a constitutively active Akt protected cells against apoptosis induced by the combination. Alternatively, the JNK inhibitor SP600125 could also protect cells from apoptosis induced by the combination,

indicating that both inhibition of Akt and activation of JNK were required for the synergism. These findings show that sorafenib interacts synergistically with bortezomib to induce apoptosis in a broad spectrum of neoplastic cell lines and show an important role for the Akt and JNK pathways in mediating synergism. Further clinical development of this combination seems warranted. [Mol Cancer Ther 2006;5(9):2378–87]

Introduction

Sorafenib (BAY 43-9006) is an oral bisarylurea derivative that was initially developed as a potent inhibitor of c-Raf kinase (IC₅₀ = 6 nmol/L) but was later found to inhibit wild-type and mutant B-Raf (IC₅₀ = 22 and 38 nmol/L, respectively) *in vitro* as well (1). The Raf proteins are an integral component of the Ras/Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK mitogen-activated protein kinase signaling cascade with a well-established role in cell transformation (2–4). Oncogenic mutations in this pathway are common, including B-Raf mutations in ~60% of malignant melanomas (5). Therapeutic targeting of the Ras/Raf/mitogen-activated protein/ERK kinase/ERK pathway has been an area of intense investigation. Sorafenib shows broad activity against various tumor cell lines *in vitro* and in xenograft models (1). In addition to its effect on Raf, sorafenib also potently inhibits receptor tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR)-2 (IC₅₀ = 90 nmol/L) and murine platelet-derived growth factor receptor (PDGFR)- β (IC₅₀ = 57 nmol/L; ref. 1). Thus, the anticancer activity of sorafenib is likely due to its effects on multiple targets. Sorafenib recently received Food and Drug Administration approval for the treatment of renal cell carcinoma based on results from a phase III study showing an improvement in progression-free survival relative to placebo (6). Preliminary evidence of single-agent activity has also been observed in malignant melanoma and hematologic malignancies (7, 8). As the clinical application of sorafenib evolves, there is increasing interest in defining the mechanisms underlying its antiproliferative activity as well as examining the effects of this agent in combination with other anticancer drugs.

Proteins destined for degradation are ubiquitinated by a series of ubiquitin ligases and shuttled to the proteasome where they undergo proteolytic degradation (9). The proteasome degrades a wide variety of proteins important for cell cycle progression and apoptosis induction, including p21^{Cip1}, p27^{Kip1}, p53, Bax, and I κ B (9). Inhibition of the proteasome leads to accumulation of these proteins,

Received 5/1/06; revised 7/12/06; accepted 7/26/06.

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doi:10.1158/1535-7163.MCT-06-0235

resulting in dysregulation of cell cycle control and activation of apoptotic pathways. Because neoplastic cells seem to be more susceptible to proteasome inhibition than nontransformed cells (10, 11), there has been extensive investigation of possible proteasome inhibitors. Bortezomib is a small, cell-permeable dipeptidyl boronic acid derivative that reversibly inhibits the chymotrypsin-like activity of the proteasome by binding tightly to the active sites of the enzyme ($K_i = 0.6$ nmol/L; ref. 12). Notably, inhibition of 20S proteasome activity can be reliably and directly detected after treatment with bortezomib *in vitro*, *in vivo*, and *ex vivo* in cells and tissues (13). In the NCI-60 cell line screen, bortezomib showed growth inhibitory and cytotoxic activity for many human tumor cell types, with an average GI_{50} of 3.8 nmol/L across the 60 cell lines (13). The pattern of growth inhibition and cytotoxicity was unique, suggesting that bortezomib represented a novel class of cytotoxic compound. Preclinical activity of bortezomib has been shown in multiple myeloma (14, 15), myeloid leukemia (16), lymphoma (17), and solid malignancies (18–20). Although clinical development of bortezomib is ongoing in multiple areas, Food and Drug Administration approval has been granted for treatment of multiple myeloma based primarily on striking phase II data (21, 22). In addition, activity has been shown in mantle cell lymphoma and non-small cell lung cancer (23, 24).

Both sorafenib and proteasome inhibitors are broadly targeted, well-tolerated, clinically active anticancer agents. With the goal of inhibiting multiple signaling pathways involved in tumor growth, we tested the effects of the sorafenib/bortezomib combination *in vitro*. The present results show cytotoxic synergy of this combination in a broad spectrum of cultured human malignant cell lines derived from solid tumors and hematologic malignancies as well as in primary glioma cell lines developed from patient samples. In further studies, we show that the cytotoxic synergy involves modulation of Akt and c-Jun NH_2 -terminal kinase (JNK) signaling cascades, leading to enhanced apoptosis.

Materials and Methods

Materials

Sorafenib and bortezomib were kindly provided by Bayer Pharmaceutical Corp. (West Haven, CT) and Millennium Pharmaceuticals (Cambridge MA), respectively. Boc-D-fmk was purchased from Enzyme System Products (Livermore, CA). Glycogen synthase kinase inhibitors VIII and IX, SB216763, and SP600125 were purchased from Calbiochem (San Diego, CA). Wortmannin and LY294002 were supplied by Sigma Chemical Co. (St. Louis, MO).

Cells

A549, 786-O, MDA-MB-231, U251, Jurkat, and K562 cell lines were purchased from the American Type Culture Collection (Manassas, VA). MEC-2 cells, a cell line originally derived from a B-cell chronic lymphocytic leukemia patient, were obtained from Dr. Neil Kay. D37

cells were obtained from Dr. Jann Sarkaria. Short-term cultures of patient-derived glioblastoma multiforme cells have been described previously (25). All cells were grown following instructions provided by suppliers.

Cell Culture

Subconfluent, logarithmically growing cells were placed in sterile plastic T-flasks, allowed to adhere, supplemented with the designated drugs, and incubated in a humidified incubator with 5% CO_2 at 37°C for various time intervals as indicated.

Assessment of Apoptosis

Following drug treatment, apoptotic cells were detected by 4',6-diamidino-2-phenylindole (DAPI) staining, which allowed identification of apoptotic nuclear changes as described previously (26). Briefly, cells were washed with PBS and fixed with 1% glutaraldehyde at room temperature for 30 minutes. After washing with PBS, cells were resuspended in 20 μ L PBS and mixed with 5 μ L of 10 μ g/mL DAPI. Cell suspensions were mounted on slides and subjected to fluorescence microscopic examination. To further confirm the morphologic results, Wright-Giemsa stains were done as described previously (27). In brief, after drug treatment, cells were transferred to slides by cytocentrifugation, fixed, stained, and evaluated under light microscopy for apoptotic cells. Apoptotic cells were identified by classic morphologic features (i.e., nuclear condensation, cell shrinkage, and formation of apoptotic bodies). Five or more randomly selected fields, encompassing a total of ≥ 500 cells per slide, were quantified for the percentage of apoptotic cells. To confirm the results of morphologic analysis, Annexin/propidium iodide (BD PharMingen, San Diego, CA) analysis was carried out as per the manufacturer's instruction.

Immunoblot Analysis

Following drug treatment, cell lysates were prepared and subjected to immunoblot analysis using 30 μ g of cellular protein using standard techniques. The following primary antibodies were used at 1:1,000 dilution unless otherwise indicated: rabbit polyclonal antibodies to caspase-9, cleaved caspase-3, X-linked inhibitor of apoptosis, phosphorylated ERK1/2 (Thr²⁰²/Tyr²⁰⁴), phosphorylated p38 (Thr¹⁸⁰/Tyr¹⁸²), phosphorylated p70S6K (Thr³⁸⁹), and phosphorylated c-Jun (Ser⁷³) from Cell Signaling Technology (Beverly, MA); murine anti-phosphorylated JNK (Thr¹⁸³/Tyr¹⁸⁵) and murine monoclonal anti-Mcl-1 from PharMingen; rabbit anti-Bcl-x_L, phosphorylated Akt (Ser⁴⁷³), Akt, VEGFR, phosphorylated PDGFR- β (Tyr⁸⁵⁷), and PDGFR from Santa Cruz Biotechnology (Santa Cruz, CA); and rabbit polyclonal phosphorylated VEGFR-2 (Tyr¹⁰⁵⁴/Tyr¹⁰⁵⁹) from Biosource (Camarillo, CA). To ensure equivalent loading and transfer, blots were stripped and reprobbed with anti-actin antiserum. Western blots are representative of three independent experiments.

Assessment of Cytochrome c Release from Mitochondria

Following drug treatment, the release of cytochrome c from mitochondria was analyzed by a selective digitonin permeabilization method. For these assays, 4×10^6 cells

per condition were resuspended in 50 μ L of permeabilization buffer containing 75 mmol/L NaCl, 8 mmol/L Na_2PO_4 , 1 mmol/L NaH_2PO_4 (pH 7.4), 250 mmol/L sucrose (added fresh before use), 1 mmol/L EDTA, and 700 $\mu\text{g}/\text{mL}$ digitonin (final concentration of digitonin, 35 $\mu\text{g}/4 \times 10^6$ cells). After incubation at room temperature for 1 minute, cells were pelleted by centrifugation for 3 minutes at $13,000 \times g$ and the supernatants containing cytochrome *c* protein were obtained.

Analysis of Combined Drug Effects

The effect of combining sorafenib and bortezomib was analyzed using the median effect method of Chou and Talalay (28) using Calcsyn software (Biosoft, Oxford, United Kingdom) as reported previously (29).

Generation of a Stably Transfected Cell Line

K562 cells were transfected with the pUSEamp vector containing myr-Akt1-myc (constitutively activated, CA-Akt1-myc; Upstate, Lake Placid, NY) by a method described previously (30). Stably transfected Akt1 clones were selected with 800 mg/L G418, cloned by limiting dilution, screened for Akt by immunoblotting, and propagated in medium supplemented with 500 mg/L G418.

Statistical Analysis

All of the data were expressed as mean \pm SD from three individual experiments. Differences between groups were determined by using the Student's *t* test for unpaired observations. $P < 0.05$ was considered significant.

Results

Synergistic Induction of Apoptosis in K562 Cells After Exposure to Proteasome Inhibitors and Sorafenib

Both sorafenib and the proteasome inhibitor bortezomib have pleiotropic mechanisms of action. To assess the effect of combining these two agents, K562 cells were treated with the drugs individually or in combination and examined by Annexin V/propidium iodide staining to assess toxicity (Fig. 1A and B). At the concentrations tested, neither sorafenib nor bortezomib elicited significant cytotoxicity as a single agent, but the combination induced apoptosis in $\sim 75\%$ of the cells. To confirm the induction of apoptosis by this combination, we analyzed cell extracts for expression of biological markers of apoptosis. The combination resulted in marked cleavage of procaspase-9, procaspase-3, and poly(ADP-ribose) polymerase (PARP), whereas the individual agents did not (Fig. 1C, *left*). The combination also induced release of cytochrome *c* from mitochondria (Fig. 1C, *right*). Further experiments indicated that the broad-spectrum caspase inhibitor Boc-D-fmk did not block cytochrome *c* release but blocked caspase-3 cleavage and apoptosis, suggesting that cytochrome *c* release is upstream of caspase activation. Boc-D-fmk also blocked apoptosis induced by sorafenib and bortezomib (Fig. 1D). These results show that the combination of sorafenib and bortezomib synergistically activates apoptosis in K562 cells by release of cytochrome *c* and subsequent activation of caspases.

Sorafenib and Proteasome Inhibitors Are Synergistic in A549 Cells

To ensure that the effects seen in K562 were not cell line specific, we examined the combination in A549 cells. Exposure of A549 cells to sorafenib or bortezomib alone induced apoptosis in only 10% to 15% of the cells (Fig. 2A). However, the combination induced apoptosis in $\sim 90\%$ of the cells. To gain further insight into this interaction, dose-response studies were done in Fig. 2B. There was a dose-dependent increase in the degree of apoptosis caused by the combination of sorafenib and bortezomib. To do mathematical analysis by the median effect method, A549 cells were treated with different concentrations of sorafenib and bortezomib at a fixed ratio, and the combination index values on apoptosis induction were determined using the method of Chou and Talalay (28). As shown in Fig. 2C, the combination index values were < 1 , indicating a synergistic interaction. To test whether the synergistic interaction between sorafenib and bortezomib extended to combinations of sorafenib and other proteasome inhibitors, A549 cells were treated with sorafenib and/or the proteasome inhibitor MG132 for 48 hours. As seen in Fig. 2D, a significant augmentation in apoptosis occurred with sorafenib and MG132, suggesting that the synergism is a class effect and not specific for bortezomib.

Synergistic Cytotoxicity After Exposure to Bortezomib and Sorafenib in Multiple Solid Tumor and Leukemia Cell Lines

To determine the breadth of synergistic cytotoxicity observed with sorafenib and bortezomib, we tested a variety of different cell lines, including cell lines derived from solid tumors and leukemia cells. Solid tumor cell lines tested included 786-O (renal cell carcinoma), HeLa (cervical carcinoma), and MDA-MB-231 (breast; Fig. 3A). In all cases, enhanced apoptosis for these cell lines was detected after exposure to both agents. Importantly, the synergism seemed to be independent of p53 status, as it was observed in A549 and 786-O cells (wild-type p53) as well as in HeLa (inactivated p53) and MDA-MB-231 (mutant p53). In addition, synergy seemed independent of RAS mutational status because A549 and MDA-MB-231 harbor mutant K-Ras, whereas the other cell lines harbor wild-type Ras.

To examine whether this synergy also occurs in other leukemia cells, Jurkat (T-cell acute lymphoblastic leukemia) and MEC-2 (B-cell chronic lymphocytic leukemia) cell lines were exposed to the drug combination. Similar enhancement of apoptosis was observed in both cases (Fig. 3B). We have also tested the combination of sorafenib and bortezomib in several established glioma cell lines (Fig. 3C) and in cell lines derived from the Mayo glioblastoma xenograft panel (Fig. 3D) and found synergistic cytotoxicity in all cases. The cell lines derived from glioblastoma xenografts are short-term cultures developed from excised flank xenografts that were initially created from patient samples (25). These cells maintain the invasive characteristics of glioblastomas when injected

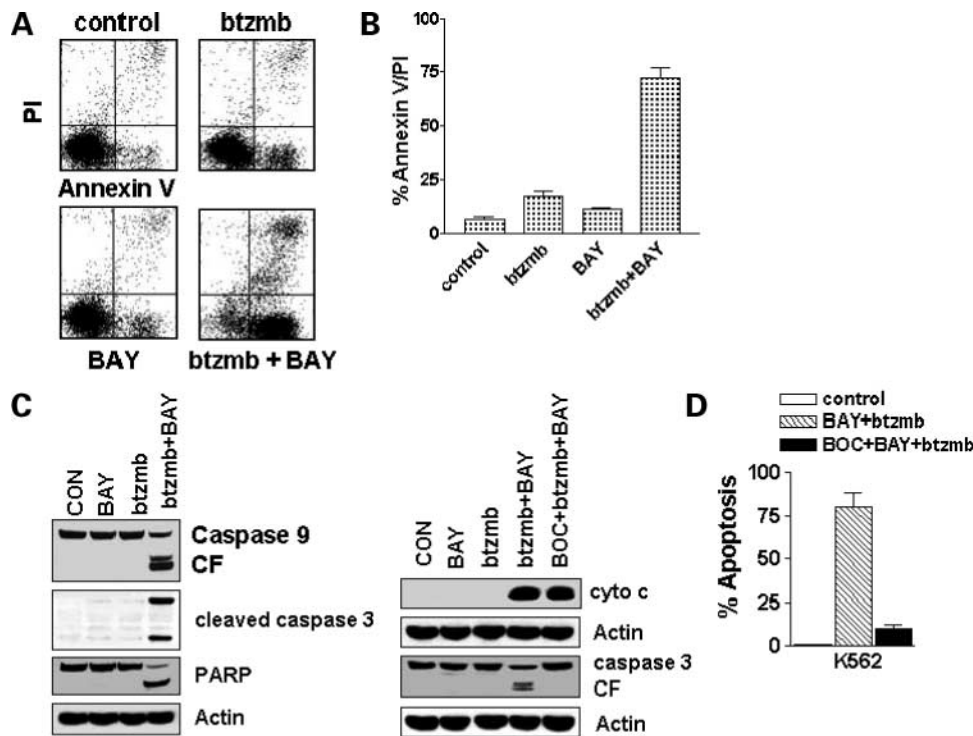


Figure 1. Apoptotic changes induced by exposure of K562 cells to a combination of sorafenib and bortezomib. **A**, K562 cells were treated with control medium, 2.5 $\mu\text{mol/L}$ sorafenib (BAY) alone, 6.0 nmol/L bortezomib (btzmb) alone, or the combination for 48 h. The extent of apoptosis was determined by flow cytometric analysis of Annexin V/propidium iodide staining. *Bottom right quadrant*, early apoptotic cells (Annexin V positive); *top right quadrant*, later apoptotic cells (Annexin V/propidium iodide positive). **B**, after K562 cells were treated as above for 48 h, the percentage of total apoptotic cells was determined as in (A). *Columns*, mean of three replicate determinations; *bars*, SD. **C**, protein extracts from K562 cells treated as indicated for 48 h were analyzed by Western blotting for caspase-9, cleaved caspase-3, PARP in whole-cell lysates, and cytochrome *c* (*cyto c*) from the cytosolic fraction (CF). The drug concentrations were as follows: 2.5 $\mu\text{mol/L}$ sorafenib, 6.0 nmol/L bortezomib, and 50 $\mu\text{mol/L}$ Boc-D-fmk (BOC). **D**, K562 cells were treated with 2.5 $\mu\text{mol/L}$ sorafenib and 6.0 nmol/L bortezomib with or without 50 $\mu\text{mol/L}$ Boc-D-fmk for 48 h. The percentage of apoptotic cells was determined by Wright-Giemsa staining.

intracranially and are likely a more clinically relevant *in vitro* model than tumor cell lines maintained in culture for long periods. Overall, sorafenib and bortezomib exhibited synergy in a wide variety of solid tumor and leukemia cell lines.

Effects of the Sorafenib/Bortezomib Combination on Signaling Pathways

To evaluate the mechanistic basis for synergistic cytotoxicity between sorafenib and bortezomib, we determined the effects of this combination on various signaling and apoptosis regulatory proteins in K562 cells after treatment for 16 hours (Fig. 4), a time point when the combination induces apoptosis in $\sim 30\%$ of cells (data not shown). Interestingly, sorafenib or bortezomib alone had little effect on levels of phosphorylated PDGFR- β , phosphorylated VEGFR-2, and phosphorylated Akt. However, exposure of cells to the combination of sorafenib and bortezomib resulted in a marked attenuation of phosphorylated PDGFR- β , phosphorylated VEGFR-2, and phosphorylated Akt. In addition, there was a marked increase in levels of phosphorylated JNK and phosphorylated c-Jun, whereas either drug alone only had minimal effects on these signaling molecules.

Sorafenib induced down-regulation of Mcl-1 and phosphorylated p38. These effects were reversed by coexposure to bortezomib. We have previously reported that the cytotoxicity of sorafenib is mediated in part by enhanced proteasome-directed Mcl-1 degradation (26). Our results here confirm those findings and also suggest that, despite restoration of Mcl-1 protein levels, alternative pathways are able to overcome this antiapoptotic pathway to induce cell death. The effect on Mcl-1 by bortezomib also shows effective proteasome inhibition at doses where synergism is observed. There were no significant changes in other proteins, such as phosphorylated p70S6K, phosphorylated ERK1/2, Bcl-x_L, and X-linked inhibitor of apoptosis, after treatment with either drug alone or the combination.

To rule out the possibility that these signaling changes were indirectly related to caspase activation, we exposed cells to the drug combination in the presence of the caspase inhibitor Boc-D-fmk. Results shown in Fig. 4C show that both down-regulation of phosphorylated Akt and activation of JNK still occurred when K562 cells were treated with sorafenib and bortezomib in the presence of Boc-D-fmk. Focusing on Akt and JNK, modulation of these

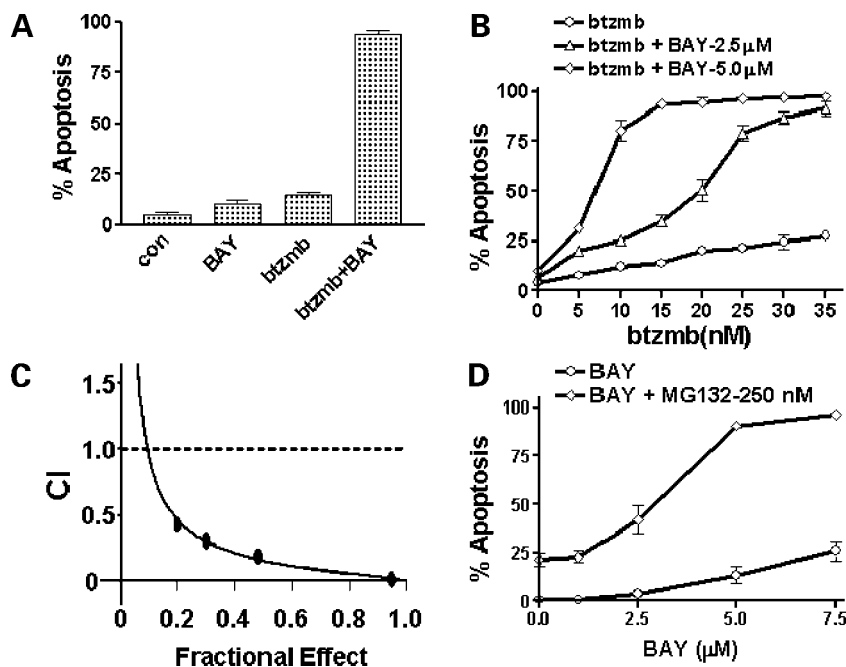


Figure 2. Synergistic cytotoxicity after exposure of A549 cells to sorafenib and bortezomib. **A**, A549 cells were treated with 5.0 μmol/L sorafenib with or without 20 nmol/L bortezomib for 48 h. The percentage of apoptotic cells was determined by DAPI staining. **B**, after exposure to the indicated concentrations of bortezomib with or without 2.5 or 5.0 μmol/L sorafenib in A549 cells for 48 h, the percentage of apoptotic cells was determined by DAPI staining. **C**, A549 cells were treated with various concentrations of sorafenib (2.5–5.0 μmol/L) and/or bortezomib (10–20 nmol/L) at a fixed ratio of 250:1. After the percentage of apoptotic cells was determined in each condition, the combination index was calculated as described in Materials and Methods. A combination index <1 represents synergism. **D**, after exposure to 250 nmol/L MG132 with or without indicated concentrations of sorafenib in A549 cells for 48 h, the percentage of apoptotic cells was determined by DAPI staining.

pathways is not cell line specific, as similar results were observed in Jurkat cells (Fig. 4D). Collectively, these results indicate that synergism between sorafenib and bortezomib reflects inhibition of Akt and activation of JNK that occurs upstream of caspase activation.

A Constitutively Active Akt Protects Against Apoptosis Induced by the Combination of Sorafenib and Bortezomib

To determine whether the observed changes in signaling were causative, K562 cells were stably transfected with myc epitope-tagged constitutively active Akt1 (CA-Akt1-myc) and cloned by limiting dilution. Clones 6 and 10 expressed almost undetectable CA-Akt1-myc levels, whereas clones 11 and 22 expressed roughly physiologic

levels of CA-Akt1-myc (Fig. 5A). We next examined the sensitivities of these clones to the combination of sorafenib and bortezomib. As seen in Fig. 5B, ~15% of the K562 cells expressing high levels of CA-Akt1 (clones 11 and 22) became apoptotic after exposure to sorafenib and bortezomib compared with ~90% of the cells expressing low CA-Akt1 levels (clones 6 and 10; $P < 0.001$). These data show that CA-Akt1 protects cells from sorafenib/bortezomib-induced apoptosis and support the idea that the attenuation in levels of phosphorylated Akt by the combination of sorafenib and bortezomib contributes to the enhanced induction of apoptosis.

Further analysis of clones 10 and 11 (Fig. 5C) revealed that overexpression of CA-Akt1 blocked cytochrome *c*

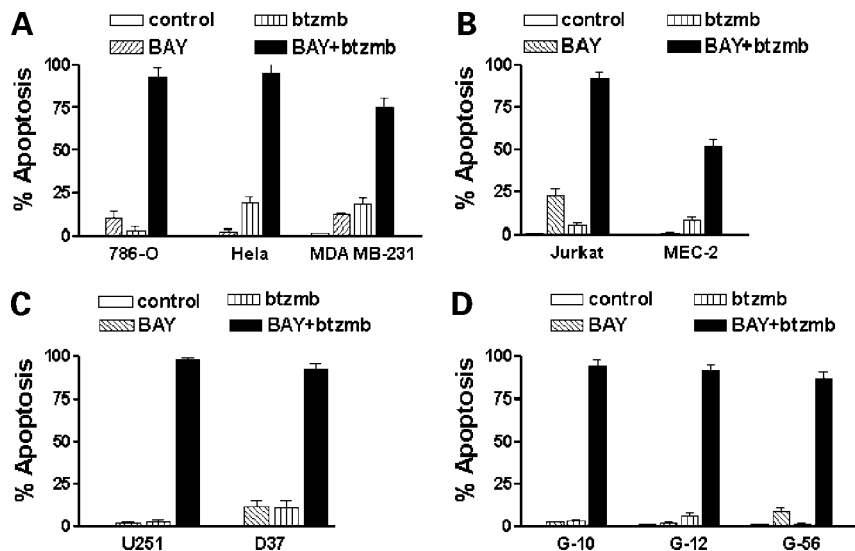
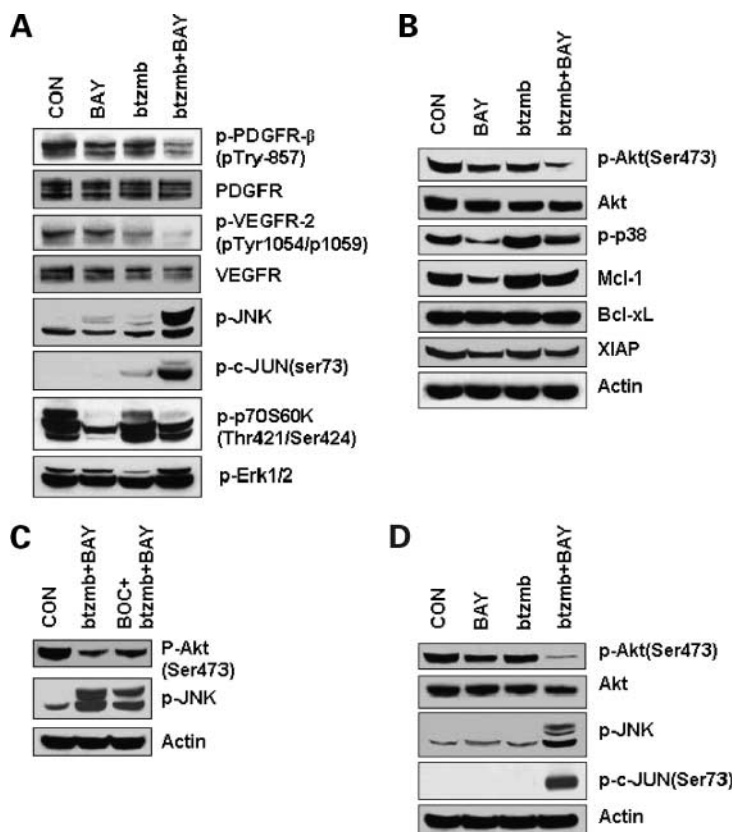


Figure 3. Sorafenib enhances proteasome inhibitor-induced apoptosis in a broad spectrum of cancer cell lines. Multiple tumor cell lines, including solid tumor (**A**), leukemia (**B**), glioma (**C**), and short-term patient glioblastoma multiforme cell lines (**D**), were treated with sorafenib and bortezomib, and the percentage of apoptotic cells was determined by DAPI staining. The conditions of cotreatment were as follows: 786-O, 2 d, 7.5 μmol/L sorafenib, 15 nmol/L bortezomib; HeLa, 2 d, 7.5 μmol/L sorafenib, 10 nmol/L bortezomib; MDA-MB-231, 3 d, 4 μmol/L sorafenib, 5 nmol/L bortezomib; Jurkat, 2 d, 5 μmol/L sorafenib, 5 nmol/L bortezomib; MEC-2, 2 d, 5 μmol/L sorafenib, 7.5 nmol/L bortezomib; U251, 2 d, 10 μmol/L sorafenib, 3 nmol/L bortezomib; D37, 3 d, 5 μmol/L sorafenib, 20 nmol/L bortezomib; G-10, 2 days, 10 μmol/L sorafenib, 10 nmol/L bortezomib; G-12, 4 d, 10 μmol/L sorafenib, 10 nmol/L bortezomib; and G-56, 4 d, 10 μmol/L sorafenib, 10 nmol/L bortezomib.

Figure 4. Effects on multiple signaling pathways in K562 and Jurkat cells after cotreatment with sorafenib and bortezomib. After exposure of K562 cells (**A** and **B**) to 2.5 $\mu\text{mol/L}$ sorafenib and 5 nmol/L bortezomib for 16 h, multiple signaling pathways as indicated were examined by Western blot analysis in whole-cell lysates. *XIAP*, X-linked inhibitor of apoptosis. **C**, K562 cells were treated with 2.5 $\mu\text{mol/L}$ sorafenib and 5 nmol/L bortezomib with or without 50 $\mu\text{mol/L}$ Boc-D-fmk. Whole-cell lysates were analyzed by Western blotting for the indicated proteins. **D**, after exposure of Jurkat cells to 5 $\mu\text{mol/L}$ sorafenib and 5 nmol/L bortezomib for 16 h, whole-cell lysates were analyzed by Western blotting for the indicated proteins.



release from mitochondria as well as cleavage of procaspase-9, procaspase-3, and PARP in cells treated with the combination of sorafenib and bortezomib. However, ectopic expression of constitutively active Akt1 did not affect JNK1 phosphorylation, and surprisingly, it restored phosphorylated JNK2 levels. Phosphorylated JNK2 might be cleaved by activated caspase because pronounced apoptosis was present with sorafenib and bortezomib in clone 10. To test this hypothesis, a caspase inhibitor was added to K562 cells treated with sorafenib and bortezomib (Fig. 5D). At 6 hours, the combination of sorafenib/bortezomib activated both JNK1 and JNK2, but the activation of JNK2 was not apparent at 36 hours due to caspase-dependent cleavage, as the caspase inhibitor Boc-D-fmk restored phosphorylated JNK2 levels. Collectively, the results in Fig. 5 indicate that CA-Akt1 inhibits apoptosis but does not affect JNK activation after treatment with the sorafenib/bortezomib combination.

To further examine the contribution of signaling upstream of Akt, cells were exposed to the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 together with the combination of sorafenib and bortezomib. The results indicated that PI3K inhibition significantly enhanced apoptosis induced by bortezomib and sorafenib ($P < 0.05$; Fig. 5E) and support an important role for the PI3K/Akt pathway in mediating synergistic cytotoxicity.

JNK Contributes to Apoptosis Induction by Sorafenib/Bortezomib

Because the preceding results indicated that the combination of sorafenib/bortezomib activated the JNK pathway, we next assessed the role of this signal by exposing cells to the sorafenib/bortezomib combination in the presence or absence of the pharmacologic JNK inhibitor SP600125. Apoptosis was markedly attenuated by SP600125 ($P < 0.001$) using both morphologic and biochemical assays of apoptosis (Fig. 6A and B). The addition of the JNK inhibitor had no effect on the attenuation of phosphorylated Akt seen with the sorafenib/bortezomib combination. These data show that JNK signaling is also required for cytochrome *c* release and apoptosis induced by the combination of sorafenib and bortezomib.

Discussion

In this study, we describe synergistic cytotoxicity when cells are treated with the multikinase inhibitor sorafenib in combination with various proteasome inhibitors, including MG132 and bortezomib. Cytotoxic synergy was shown in a broad spectrum of solid tumor and leukemia cell lines as well as in primary glioma cell lines. Our data support a model (Fig. 6C) in which the combination of BAY 43-9006 and proteasome inhibitors synergistically modulates Akt and JNK signaling to activate apoptosis, with each

pathway being independently required for synergistic cytotoxicity. The model is supported by several findings. First, overexpression of an active form of Akt protects cells from apoptosis after treatment with sorafenib and bortezomib while having no effect on the induction of JNK phosphorylation. Second, Akt is primarily regulated by PI3K, and the PI3K inhibitor LY294002 enhanced the sensitivity of cells to the combination but did not modify JNK phosphorylation. Although the expression of a constitutively active form of Akt can protect against apoptosis induced by multiple agents, the relevance of Akt down-regulation to the synergism between sorafenib and bortezomib is supported by our results using LY294002. Finally, the JNK inhibitor SP600125 protected cells from apoptosis induced by the combination but did not affect reductions in Akt phosphorylation.

Sorafenib and proteasome inhibitors have not been previously shown to directly act on either Akt or JNK, but effects on these central pathways by either compound have been described. Thus, the precise mechanism by which sorafenib and proteasome inhibitors modulate these pathways requires further study. Nonetheless, it is important to emphasize that the effects on Akt and JNK are not

only byproducts of the cell death process. To the contrary, several observations place these effects upstream of cell death. First, the addition of a caspase inhibitor is unable to block the effects of the combination on either Akt or JNK. Second, enhancing Akt activation with a constitutively active form is able to rescue cells from the cytotoxic effects of the combination, yet has no effect on the increased levels of phosphorylated JNK. Likewise, inhibition of JNK signaling with SP600125 protects the cells from apoptosis induced by the combination but has no effect on inhibition of Akt. Each of these results supports a direct effect of the combination on Akt and JNK signaling rather than an indirect effect related to cytotoxicity.

Given the pleiotropic nature of both sorafenib and proteasome inhibitors, it may be difficult to identify the precise mechanism responsible for synergism of the combination. Sorafenib inhibits multiple receptor tyrosine kinases in addition to Raf, including VEGFRs and PDGFRs (1). Akt is a downstream effector of each of these families of receptors in several different cell lines (31–34). Interestingly, we find little inhibition of VEGFR-2 or PDGFR- β based on phosphorylation of the receptors by sorafenib but do see inhibition with the combination. Sorafenib potently

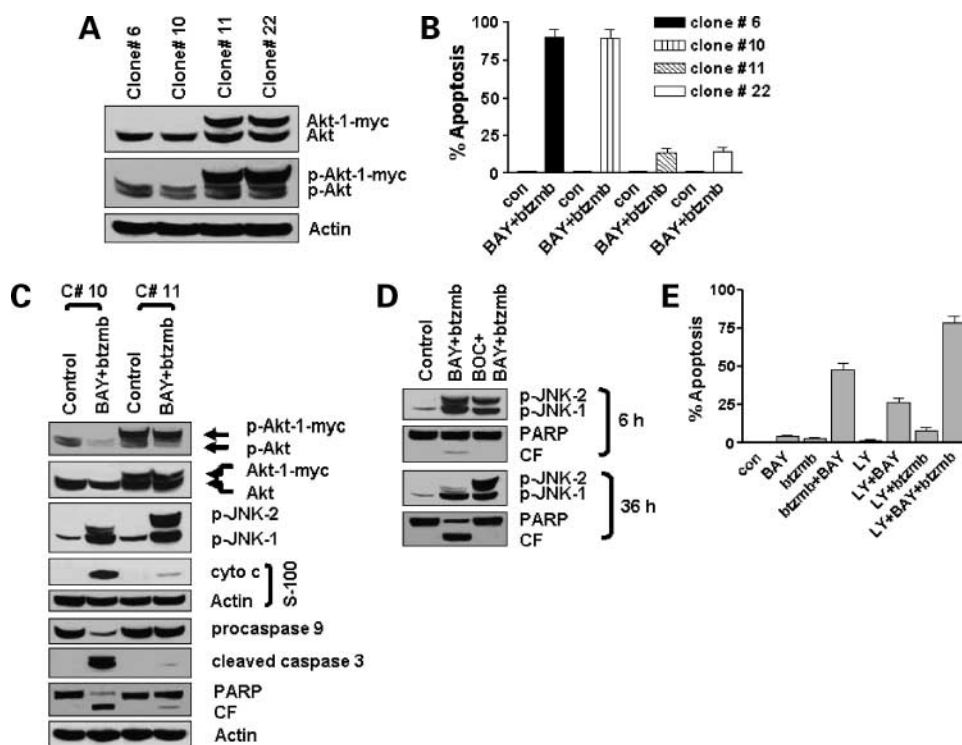


Figure 5. Enhanced Akt activity protects cells against apoptosis induced by the combination of sorafenib and bortezomib. **A**, whole-cell lysates from K562 clones stably transfected with constitutively active Akt1 (*CA-Akt1-myc*) were analyzed by Western blot analysis for expression of recombinant protein. **B**, after clones 6, 10, 11, and 22 were treated with 2.5 $\mu\text{mol/L}$ sorafenib + 6.0 nmol/L bortezomib for 48 h, the percentage of apoptotic cells was determined by DAPI staining. **C**, after exposure to 2.5 $\mu\text{mol/L}$ sorafenib and 6 nmol/L bortezomib in K562 clones 10 and 11 for 36 h, Western blot analysis of phosphorylated Akt, Akt, phosphorylated glycogen synthase kinase-3, phosphorylated JNK, procaspase-9, procaspase-3, and PARP from whole-cell lysates was done. In addition, Western blot analysis of cytochrome *c* from S-100 fraction was also done. **D**, after K562 cells were exposed to 2.5 $\mu\text{mol/L}$ sorafenib and 6 nmol/L bortezomib for 6 or 36 h, Western blot analysis of phosphorylated JNK and PARP from whole-cell lysates was done. **E**, K562 cells were treated with 2.5 $\mu\text{mol/L}$ sorafenib and 6 nmol/L bortezomib in the presence or absence of 25 $\mu\text{mol/L}$ LY294002 (LY) or 0.5 $\mu\text{mol/L}$ wortmannin for 24 h as indicated, and the percentage of apoptotic cells was determined by DAPI staining.

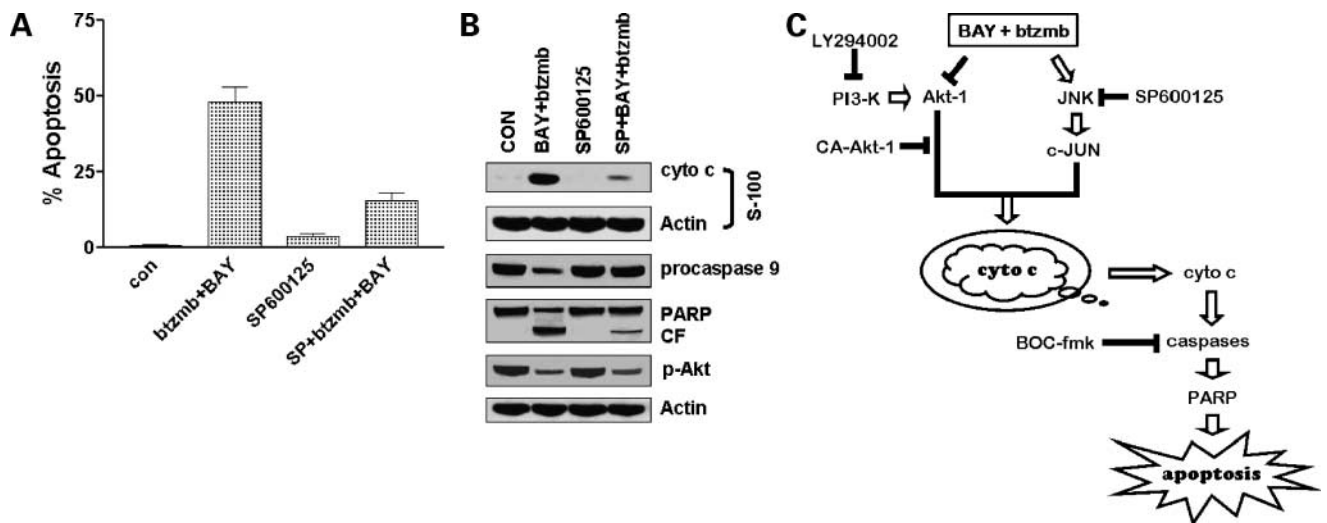


Figure 6. JNK activation is important for apoptosis induction by sorafenib and bortezomib. **A**, K562 cells were treated with 2.5 $\mu\text{mol/L}$ sorafenib and 6.0 nmol/L bortezomib in the presence or absence of 30 $\mu\text{mol/L}$ SP600125 (SP) for 24 h, and the percentage of apoptotic cells was determined by DAPI staining. **B**, K562 cells were treated as in (A) and analyzed by Western blot analysis for expression of procaspase-9, Akt, phosphorylated Akt, and PARP from whole-cell lysates. Western blot analysis of cytochrome *c* from S-100 fraction was also done. **C**, the mechanism of synergy from combining sorafenib and bortezomib seems to involve inhibition of Akt activity and activation of JNK. Modulation of both of these pathways results in cytochrome *c* release from mitochondria and activation of caspases, ultimately leading to apoptotic cell death. Based on our data, it is unclear whether the drug combination directly targets Akt or exerts its inhibitory effects on PI3K. Blocking either pathway, Akt via a constitutively active form of Akt or JNK via the inhibitor SP600125, protects the cells from apoptosis induced by sorafenib and bortezomib. In addition, the PI3K inhibitor LY294002 sensitizes the cells to the combination.

inhibits both of these receptors *in vitro* and in cell-based assays (1). The reasons we do not see inhibition are unclear and may represent cell line-specific effects. Although no direct links between proteasome inhibitors and inhibition of VEGF or PDGFRs have been reported, several studies have noted decreased VEGF expression following treatment with proteasome inhibitors (35, 36). Proteasome inhibitors have also been shown to decrease phosphorylated Akt levels in a process thought to be related to accumulation of the inhibitor PTEN (37) or inhibition of upstream pathways (38). We did not see decreased Akt phosphorylation with bortezomib alone at synergistic concentrations, which may be due to either cell line-specific effects or dose differences. Combining proteasome inhibitors with other agents, such as the farnesyl transferase inhibitor lonafarnib or the cyclin-dependent kinase inhibitor flavopiridol, also down-regulates phosphorylated Akt (39, 40). Therefore, Akt seems to play a central role in mediating the cytotoxicity of proteasome inhibitors in combination with a diverse group of therapeutics. Further work will be needed to clarify the interaction between sorafenib and proteasome inhibitors in relation to effects on Akt.

Similarly, sorafenib and proteasome inhibitors do not have direct effects yet reported on JNK, but existing evidence supports a link between these agents and JNK. The JNK isoforms are downstream components of one of the major mitogen-activated protein kinase cascades and are primarily involved in proapoptotic signaling. JNK is activated by a variety of signals, including tumor necrosis factor- α and reactive oxygen species, and can also be

suppressed by alternative signaling pathways, such as nuclear factor- κB (41). One of the major targets of proteasome inhibitors is suppression of nuclear factor- κB activity by blocking the degradation of I κB , thus preventing nuclear translocation and activation of gene transcription by nuclear factor- κB (9). Blocking nuclear factor- κB activity would be expected to induce JNK signaling. JNK signaling may also be stimulated by proteasome inhibitors via the production of reactive oxygen species and endoplasmic reticulum stress, which has been shown by several groups, but the precise mechanism is not well understood (42–45). Interruption of cytoprotective mechanisms to reactive oxygen species and endoplasmic reticulum stress by sorafenib may be an important component of the synergism we describe between sorafenib and bortezomib. Downstream targets of sorafenib, including Akt and ERK, have well-known cytoprotective functions. Although we do not see significant JNK activation by bortezomib alone at synergistic concentrations, we do find at higher doses that bortezomib activates JNK and stimulates apoptosis, an effect blocked by SP600125 (data not shown). Sorafenib inhibits multiple receptors, including members of the VEGFR family. VEGF activates JNK in human umbilical vascular endothelial cells (46) in a pathway that likely involves *cdc42* and VEGFR-2 (47, 48). Given the pleiotropic nature of sorafenib and proteasome inhibitors, it is likely that multiple pathways are contributing to the synergistic activation of JNK that we report in response to combined treatment. Further work will be needed to clarify the precise mechanism.

Both sorafenib and bortezomib have proven clinical activity based on initial clinical trials. Our results show synergistic cytotoxicity of the combination in multiple established tumor cell lines of varying histologies. We also show synergism in short-term cultures of glioma cells, a model system that is likely to be more clinically relevant than cell lines maintained in cell culture for long periods. The mechanism of synergism involves modulation of both Akt and JNK signaling cascades. These results support the hypothesis that disruption of multiple signals important for tumor cell survival and proliferation will be more effective than targeting a single pathway. Clearly, these data support further preclinical and/or clinical evaluation of this combination.

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Mol Cancer Ther 2006;5:2378-2387.

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