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Bortezomib Overcomes Tumor Necrosis Factor-related Apoptosis-inducing Ligand Resistance in Hepatocellular Carcinoma Cells in Part through the Inhibition of the Phosphatidylinositol 3-Kinase/Akt Pathway*

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Hepatocellular carcinoma (HCC) is one of the most common and aggressive human malignancies. Recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-tumor agent. However, many HCC cells show resistance to TRAIL-induced apoptosis. In this study, we showed that bortezomib, a proteasome inhibitor, overcame TRAIL resistance in HCC cells, including Huh-7, Hep3B, and Sk-Hep1. The combination of bortezomib and TRAIL restored the sensitivity of HCC cells to TRAIL-induced apoptosis. Comparing the molecular change in HCC cells treated with these agents, we found that down-regulation of phospho-Akt (P-Akt) played a key role in mediating TRAIL sensitization of bortezomib. The first evidence was that bortezomib down-regulated P-Akt in a dose- and time-dependent manner in TRAIL-treated HCC cells. Second, LY294002, a PI3K inhibitor, also sensitized resistant HCC cells to TRAIL-induced apoptosis. Third, knocking down Akt1 by small interference RNA also enhanced TRAIL-induced apoptosis in Huh-7 cells. Finally, ectopic expression of mutant Akt (constitutive active) in HCC cells abolished TRAIL sensitization effect of bortezomib. Moreover, okadaic acid, a protein phosphatase 2A (PP2A) inhibitor, reversed down-regulation of P-Akt in bortezomib-treated cells, and PP2A knockdown by small interference RNA also reduced apoptosis induced by the combination of TRAIL and bortezomib, indicating that PP2A may be important in mediating the effect of bortezomib on TRAIL sensitization. Together, bortezomib overcame TRAIL resistance at clinically achievable concentrations in hepatocellular carcinoma cells, and this effect is mediated at least partly via inhibition of the PI3K/Akt pathway.

Hepatocellular carcinoma (HCC)[2] is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death. To date, surgery is still the only curative treatment but is only feasible in a small portion of patients (1). Drug treatment is the major therapy for patients with advanced stage disease. Unfortunately, the response rate to traditional chemotherapy for HCC patients is unsatisfactory (1). Novel pharmacological therapy is urgently needed for patients with advanced HCC. In this regard, the approval of sorafenib might open a new era of molecularly targeted therapy in the treatment of HCC patients.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein and a member of the TNF family, is a promising anti-tumor agent under clinical investigation (2). TRAIL functions by engaging its receptors expressed on the surface of target cells. Five receptors specific for TRAIL have been identified, including DR4/TRAIL-R1, DR5/TRAIL-R2, DcR1, DcR2, and osteoprotegerin. Among TRAIL receptors, only DR4 and DR5 contain an effective death domain that is essential to formation of death-inducing signaling complex (DISC), a critical step for TRAIL-induced apoptosis. Notably, the trimerization of the death domains recruits an adaptor molecule, Fas-associated protein with death domain (FADD), which subsequently recruits and activates caspase-8. In type I cells, activation of caspase-8 is sufficient to activate caspase-3 to induce apoptosis; however, in another type of cells (type II), the intrinsic mitochondrial pathway is essential for apoptosis characterized by cleavage of Bid and release of cytochrome *c* from mitochondria, which subsequently activates caspase-9 and caspase-3 (3).

Although TRAIL induces apoptosis in malignant cells but sparing normal cells, some tumor cells are resistant to TRAIL-induced apoptosis. Mechanisms responsible for the resistance include receptors and intracellular resistance. Although the cell surface expression of DR4 or DR5 is absolutely required for TRAIL-induced apoptosis, tumor cells expressing these death receptors are not always sensitive to TRAIL due to intracellular

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[2] The abbreviations used are: HCC, hepatocellular carcinoma; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FADD, Fas-associated protein with death domain; c-FLIP, cellular FLICE-inhibitory protein; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; DMEM, Dulbecco's modified Eagle's medium; DISC, death-inducing signaling complex; P-Akt, phospho-Akt; siRNA, small interfering RNA; PP2A, protein phosphatase 2A.

mechanisms. For example, the cellular FLICE-inhibitory protein (c-FLIP), a homologue to caspase-8 but without protease activity, has been linked to TRAIL resistance in several studies (4, 5). In addition, inactivation of Bax, a proapoptotic Bcl-2 family protein, resulted in resistance to TRAIL in MMR-deficient tumors (6, 7), and reintroduction of Bax into Bax-deficient cells restored TRAIL sensitivity (8), indicating that the Bcl-2 family plays a critical role in intracellular mechanisms for resistance of TRAIL.

Bortezomib, a proteasome inhibitor approved clinically for multiple myeloma and mantle cell lymphoma, has been investigated intensively for many types of cancer (9). Accumulating studies indicate that the combination of bortezomib and TRAIL overcomes the resistance to TRAIL in various types of cancer, including acute myeloid leukemia (4), lymphoma (10–13), prostate (14–17), colon (15, 18, 19), bladder (14, 16), renal cell carcinoma (20), thyroid (21), ovary (22), non-small cell lung (23, 24), sarcoma (25), and HCC (26, 27). Molecular targets responsible for the sensitizing effect of bortezomib on TRAIL-induced cell death include DR4 (14, 27), DR5 (14, 20, 22–23, 28), c-FLIP (4, 11, 21–23, 29), NF-κB (12, 24, 30), p21 (16, 21, 25), and p27 (25). In addition, Bcl-2 family also plays a role in the combinational effect of bortezomib and TRAIL, including Bcl-2 (10, 21), Bax (13, 22), Bak (27), Bcl-xL (21), Bik (18), and Bim (15).

Recently, we have reported that Akt signaling is a major molecular determinant in bortezomib-induced apoptosis in HCC cells (31). In this study, we demonstrated that bortezomib overcame TRAIL resistance in HCC cells through inhibition of the PI3K/Akt pathway.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Bortezomib (Velcade®) was provided by Millennium Pharmaceuticals. For *in vitro* studies, Bortezomib at various concentrations was dissolved in DMSO and then added to the cells in 5% fetal bovine serum-containing Dulbecco's modified Eagle's medium (DMEM). The final DMSO concentration was kept at 0.1% after the addition to medium. Recombinant TRAIL and Paclitaxel were purchased from Biomol (Plymouth Meeting, PA). LY294002 and okadaic acid were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies for immunoblotting, such as Akt1, Bad, Bak, Bax, Mcl-1, poly(ADP-ribose) polymerase (PARP), and PP2A-C, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other antibodies, such as Bcl-2, Bcl-xL, Bid, Bik, Bim, caspase-3, caspase-8, caspase-9, FADD, c-FLIP, and P-Akt (Ser^{473}), were from Cell Signaling (Danvers, MA). DR4 and DR5 were from Biolegend (flow cytometry) and Diaclone (Western blot).

Cell Culture—The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan; JCRB0403). The Sk-Hep-1, and Hep3B cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in a 37 °C humidified incubator under an atmosphere of 5% CO_2 in air.

Western Blot Analysis—Lysates of Huh-7, Sk-hep1, and Hep3B cells treated with bortezomib and/or TRAIL at the indicated concentrations for 24 h were prepared for immunoblotting of caspase-9, caspase-3, PARP, P-Akt, Akt, Bcl-2 family, DR4, DR5, FADD, c-FLIP, etc. Western blot analysis was performed as previously reported (32).

Apoptosis Analysis—The following three methods were used to assess drug-induced apoptotic cell death: the measurement of apoptotic cells by flow cytometry (sub-G_1) and Western blot analysis of caspase activations and PARP cleavage. HCC cells were treated with bortezomib and/or TRAIL at the indicated concentrations and time. The procedure was performed as described previously (32).

NF-κB Binding Activity—A Trans-AM NF-κB p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA) was used to determine the activity of NF-κB binding in bortezomib-treated HCC cells. The entire procedure was done in accordance with the manufacturer's manual. Briefly, HCC cells were exposed to bortezomib at the indicated doses for 4, 8, and 24 h, and cell lysates were prepared. NF-κB binding to related DNA fragments was determined by incubation with primary antibody, anti-p65, and quantified at 450 nm after incubation in anti-IgG-horseradish peroxidase conjugate as described previously (31).

Detection of Surface TRAIL Receptors—The procedure for antibody staining and analysis by flow cytometry has been described previously. Briefly, HCC cells were exposed to bortezomib at 100 nM for 24 h and then incubated with DR4 or DR5 antibodies for 30 min. Antibodies and kit were purchased from Biolegend (San Diego, CA), and the procedure was done according to the manufacturer's instructions (23).

Gene Knockdown Using siRNA—Smart pool siRNAs, including control (D-001810-10), DR4 (L-008090-00), DR5 (L-004448-00), Akt1 (M-003000-02), and PP2A-C (L-003598-01), were all purchased from Dharmacon Inc. (Chicago, IL). The procedure has been described previously. Briefly, HCC cells were transfected with siRNA (the final concentration was 100 nM) in 6-well plates using the Dharma-FECT4 transfection reagent (Dharmacon) according to the instructions in the manufacturer's manual. After 48 h, the medium was replaced, and HCC cells were incubated for an additional 24 h with bortezomib and/or TRAIL for Western blot analysis and apoptosis analysis by flow cytometry, as described previously (31).

Huh-7 with Constitutive Active Akt1—Constitutive active (myristoylated) Akt1 construct was a gift from Dr. Tushar Patel (Ohio State University, Columbus, OH). Briefly, following transfections, cells were incubated in the presence of G418 at 0.78 mg/ml. After 8 weeks of selection, surviving colonies (*i.e.* those arising from stably transfected cells) were selected and individually amplified. Huh-7 cells with stable expression of constitutive Akt (Huh7-akt) were then treated with various doses of bortezomib for Western blot analysis as described previously (32).

PP2A Phosphatase Activity—The protein phosphatase activity of total cellular lysate was determined by measuring the generation of free phosphate from the threonine phosphopeptide using the malachite green-phosphate complex assay, as described by the manufacturer (Upstate Biotechnology, Inc.).

Cell lysates were prepared in a low detergent lysis buffer (1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 μg/ml leupeptin). The phosphatase assay was performed in a PP2A-specific reaction buffer (Upstate Biotechnology) using 750 μM phosphopeptide substrate. After 10 min of incubation at 30 °C, malachite dye was added, and free phosphate was measured by optical density at 650 nm. To avoid variability among different immunoprecipitated samples, the phosphatase activities were normalized with the amount of PP2A immunoprecipitated, as detected and quantified by immunoblot analysis of each treatment group.

Co-immunoprecipitation Assay—Cells were harvested and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, and protease inhibitor tablet). The cell lysates were centrifuged at 14,000 $\times$ *g* for 15 min, and the supernatants were recovered. Supernatants containing equal amounts of proteins were incubated with 2.5 mg of primary antibodies overnight at 4 °C. The immunoprecipitates were harvested by protein G PLUS-agarose beads (Santa Cruz Biotechnology). The beads were washed once with regular washing buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), twice with high salt washing buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), and another time with regular washing buffer. Immunoprecipitates were then eluted by 5-min boiling of the beads in SDS-PAGE sample buffer and characterized by Western blotting with appropriate antibodies (5).

Statistical Analysis—Comparisons of mean values were performed using the independent sample *t* test in SPSS for Windows version 11.5 software (SPSS, Inc., Chicago, IL) (32).

RESULTS

Bortezomib Enhances TRAIL-induced Apoptosis in Resistance HCC Cells—To investigate the anti-tumor effect of TRAIL on HCC cells, we first examined the apoptotic effect of TRAIL in a panel of three human HCC cell lines, including Huh-7, Sk-Hep1, and Hep3B, at the clinical relevant concentrations. Apoptotic cells (sub-G_1) were determined by flow cytometry after 24-h treatment. Our results showed that HCC cells were quite resistant to TRAIL, and TRAIL alone was unable to induce apoptosis in all three types of cells even at concentrations up to 1000 ng/ml. However, combing bortezomib at 50 nM with TRAIL reversed the resistance in three cell lines and induced significant apoptosis in a dose-dependent manner starting from TRAIL at a concentration of 100 ng/ml (Fig. 1*A*, *left*). We then examined the effect of TRAIL on various doses of bortezomib in three cell lines and found that TRAIL enhanced bortezomib-induced apoptosis in a dose-dependent manner. These results indicate that combinational effects of bortezomib and TRAIL on apoptosis exist in HCC cells (Fig. 1*A*, *right*).

To examine the apoptotic pathways, we used two types of approaches. First, we performed a dose escalation analysis of bortezomib on TRAIL-induced apoptosis in Huh-7 cells and found that the combination of bortezomib and TRAIL induced activation of caspase-8, followed by the cleavage of Bid and activation of caspase-9 and caspase-3 and PARP cleavage in a dose-dependent manner. Second, we examined combinational effects of TRAIL and bortezomib in two different periods of time in Sk-Hep1 cells. Our data showed that the combination of TRAIL at 100 ng/ml and bortezomib at 50 nM induced apoptosis in association with the cleavage of Bid and activation of caspase-9. These results indicate the importance of the intrinsic mitochondria pathway in TRAIL-induced apoptosis (Fig. 1*B*).

Bortezomib Does Not Affect NF-κB/Nuclear p65 DNA Binding Activity in HCC Cells—NF-κB has been proposed as a major target of bortezomib in myeloma and lymphoma (9). NF-κB plays a key role in cell proliferation, apoptosis, metastasis, invasiveness, angiogenesis, and metastasis (33). Bortezomib as a proteasome inhibitor sequestered NF-κB in the cytoplasm and reduced its transcriptional activity through inhibiting the reduction of its inhibitor IκB. Notably, NF-κB activation induced by TRAIL has been reported to be responsible for the resistance of TRAIL, and numerous reports have shown that bortezomib enhanced the anti-tumor activity of TRAIL through the inhibition of NF-κB activation in pancreatic cancer (30), non-small cell lung cancer (24), and non-Hodgkin lymphoma (12). In this study, we examined the NF-κB binding activity in HCC cells. Cells were exposed to TRAIL and/or bortezomib for the indicated time. As shown in Fig. 2, *A–C*, neither TRAIL nor bortezomib changed the NF-κB binding activity in three HCC cell lines, indicating that the NF-κB pathway was not a major target in mediating the effect of bortezomib on TRAIL-treated cells. Notably, bortezomib plus TRAIL reversed the degradation of IκB-α induced by tumor necrosis factor-α and subsequently inhibited NF-κB activation in Sk-Hep1 cells (Fig. 2*D*).

Effects of Bortezomib on TRAIL Receptors Are Not Essential in Mediating Its Effects on TRAIL-induced Apoptosis—To understand the effect of bortezomib on TRAIL-induced apoptosis, we next examined TRAIL receptors and TRAIL DISC proteins, including FADD and c-FLIP, in bortezomib-treated cells. HCC cells were exposed to bortezomib at 100 nM for 24 h and then harvested for analysis of surface expression of TRAIL receptors by immunofluorescent staining and subsequent flow cytometry. As shown in Fig. 3*A*, bortezomib up-regulated the surface expression of DR5 in three types of HCC cells. However, bortezomib did not affect the surface expression of DR4 in Huh-7 and Hep3B cells, whereas up-regulation of DR4 was observed in Sk-Hep1 exposed to bortezomib. We next examined the protein levels of DR4, DR5, FADD, and c-FLIP in HCC cells. Cells were exposed to bortezomib at various doses for 24 h, and analysis was done by Western blot. We found that bortezomib did not alter the expression of DR4 significantly; however, it up-regulated the expression of DR5 in all types of cells (Fig. 3*B*), which is consistent with previous findings on the surface expression of DR5 (Fig. 3*A*). In addition, TRAIL alone or in combination with bortezomib did not alter the protein levels of DR4 and DR5 significantly in Sk-Hep1 cells (see Fig. 7*A*). Moreover, FADD plays a key role in DISC formation and mediates TRAIL-induced apoptosis, and we found that the expression of FADD was not much changed in the presence of bortezomib. Notably, previous reports have suggested that c-FLIP may play an important role in mediating the effect of bortezomib on TRAIL-induced apoptosis. In this regard, we found that bort-

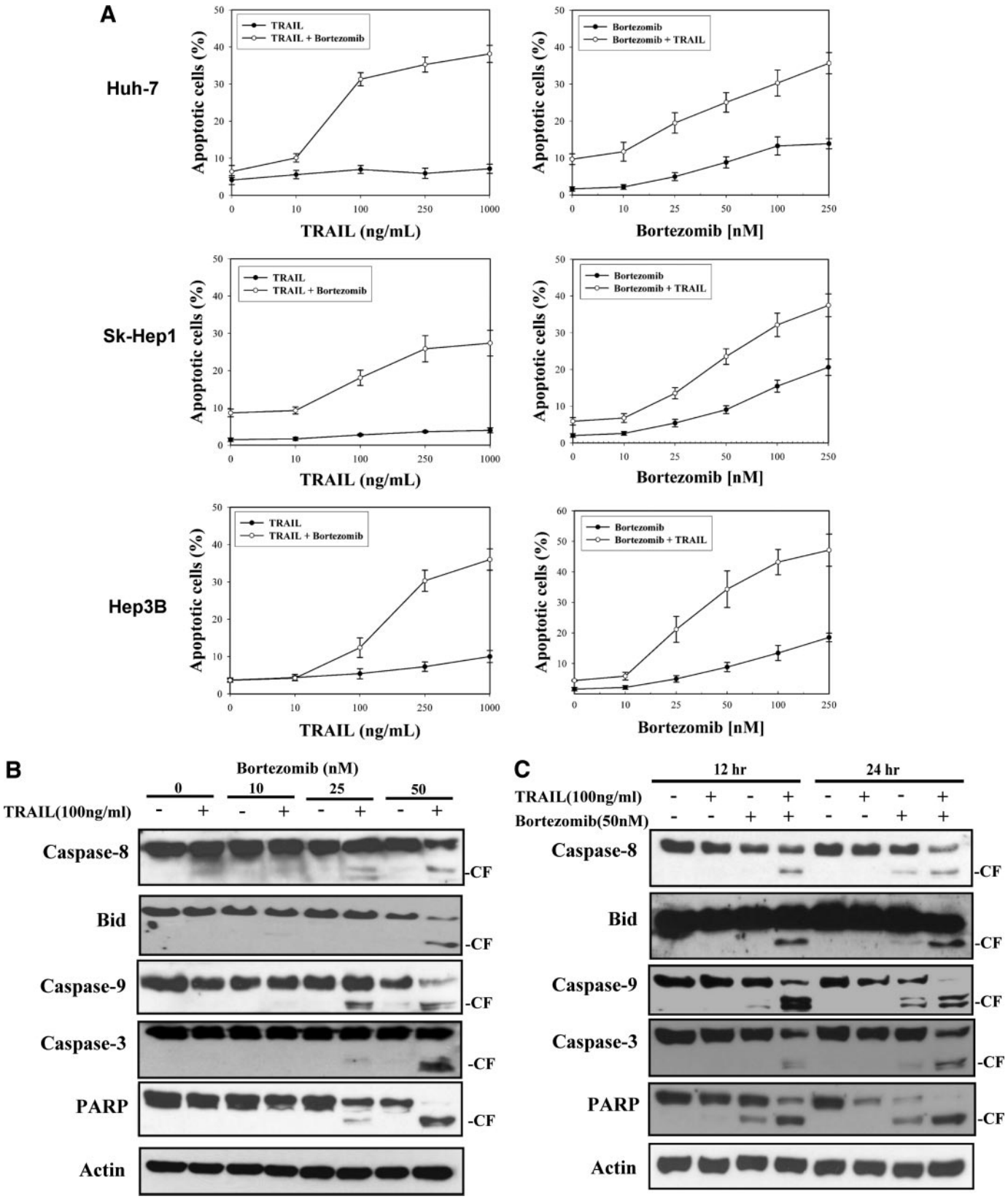


FIGURE 1. **Bortezomib enhances TRAIL-induced apoptosis in resistance HCC cells.** *A*, *left*, dose escalation effects of TRAIL with 50 nM bortezomib on apoptosis in three TRAIL-resistant HCC cells. *Right*, dose escalation effects of bortezomib with 100 ng/ml TRAIL. HCC cells were exposed to bortezomib and/or TRAIL at the indicated concentrations in DMEM with 5% fetal bovine serum in 6-well plates for 24 h, and apoptotic cells were assessed by flow cytometry. *Points*, mean; *bars*, S.D. ($n = 3$). *B*, dose-dependent effects of bortezomib on caspases and PARP in Huh-7. Huh-7 cells were exposed to TRAIL and/or bortezomib at the indicated doses in DMEM with 5% fetal bovine serum for 24 h. *C*, time-dependent effects of bortezomib in Sk-Hep1. Cells were exposed to TRAIL and/or bortezomib at the indicated doses in DMEM with 5% fetal bovine serum for 12 or 24 h. Cell lysates were prepare and analyzed for caspase-8, caspase-9, caspase-3, and PARP by Western blotting. Data are representative of three independent experiments. CF, cleaved form (activated form).

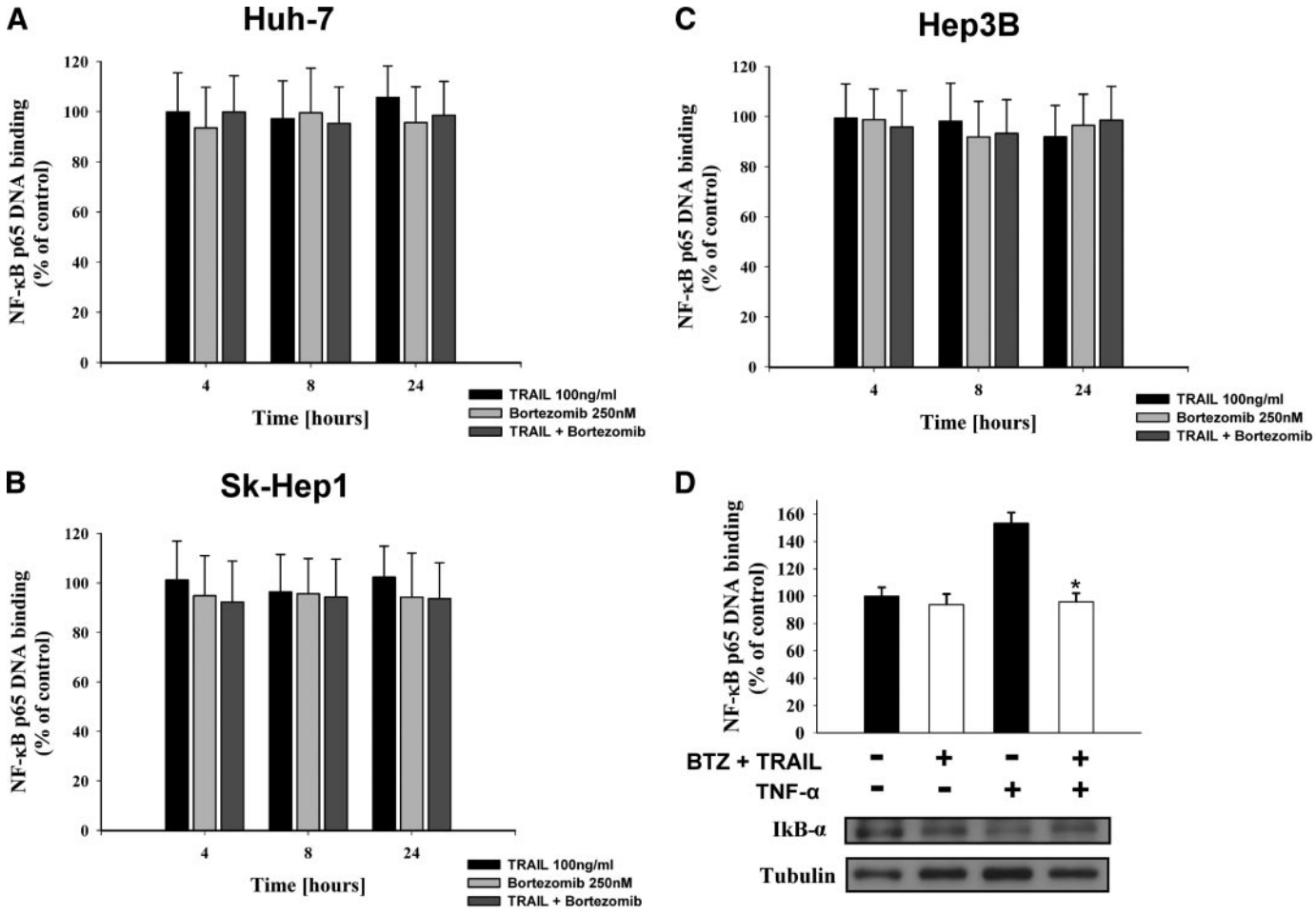
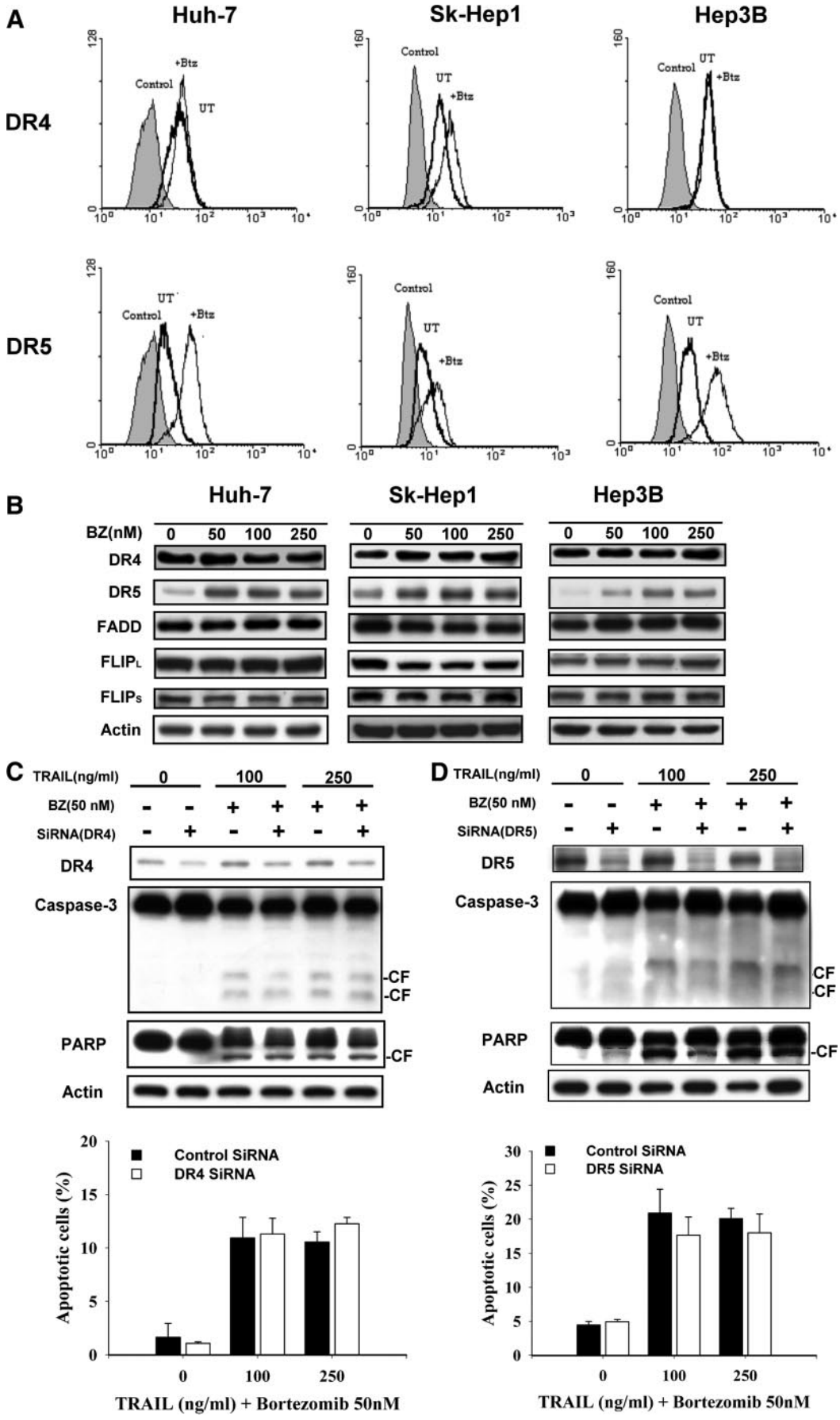


FIGURE 2. **Bortezomib does not affect NF-κB/nuclear p65 binding activity in HCC cells.** *A*, Huh-7. *B*, Sk-Hep1. *C*, Hep3B. Cells were treated with DMSO or bortezomib at the indicated doses for 4, 8, and 24 h. Nuclear extracts were prepared and assayed for p65 binding activity by an enzyme-linked immunosorbent assay kit. *Columns*, mean; *bars*, S.D. ($n = 3$). *D*, bortezomib abolished NF-κB activation induced by tumor necrosis factor α (*TNF-α*) in Sk-Hep1 cells. Cells were exposed to DMSO or 250 nM bortezomib with 100 ng/ml TRAIL for 1 h, and then 20 ng/ml tumor necrosis factor-α was added for 1 h. Nuclear extracts were prepared and assayed for p65 binding activity by an enzyme-linked immunosorbent assay kit. *Columns*, mean; *bars*, S.D. ($n = 3$). *, $p < 0.05$. Cytoplasmic extracts were prepared and assayed for IκB-α by Western blot.

ezomib did not alter protein levels of c-FLIP (two isoforms) in HCC cells (Fig. 3*B*).

To validate the role of TRAIL receptors on apoptosis induction of bortezomib and TRAIL, we next knocked down TRAIL receptors with small interference RNA (siRNA) in HCC cells. Given the fact that bortezomib only altered the surface expression of DR4 in Sk-Hep1 cells, Sk-Hep1 cells were transfected with control or DR4 siRNA for 48 h and then treated with DMSO or the combination of TRAIL and bortezomib for 24 h. Apoptosis analysis was done by Western blot and flow cytometry. Our data showed that silencing DR4 did not affect the apoptotic effect of TRAIL and bortezomib, indicating that DR4 was not essential in mediating the TRAIL-sensitizing effect of bortezomib (Fig. 3*C*). In addition, silencing of DR5 was performed in Huh-7 cells. The procedure was the same as we have used in Sk-Hep1 cells. As shown in Fig. 3*D*, knocking down DR5 did not alter the degree of apoptotic induction in Huh-7 cells treated with TRAIL and bortezomib, suggesting that DR5 did not play a key role in mediating the effect of bortezomib on TRAIL-induced apoptosis (Fig. 3*D*).

Down-regulation of P-Akt Is Associated with Sensitizing Effects of Bortezomib in HCC Cells—Our previous data suggested that the sensitizing effect of bortezomib was mediated through affecting neither NF-κB activity nor TRAIL-related signaling proteins (31). Given the fact that bortezomib sensitized TRAIL-induced apoptosis through the activation of the mitochondria apoptotic pathway (Fig. 1, *B* and *C*), we next investigated its related proteins, including Akt and the Bcl-2 family. Accordingly, we found that down-regulation of phospho-Akt was associated with the sensitizing effect of bortezomib on TRAIL-induced apoptosis. As shown in Fig. 4*A*, bortezomib down-regulated P-Akt (Ser^{473}) in all types of HCC cells in a dose-dependent manner, whereas protein levels of total Akt were not altered (Fig. 4*A*). To elucidate the possible role of bortezomib in TRAIL cytotoxicity, a time-dependent analysis of bortezomib and TRAIL co-treatment was performed in Hep3B cells. Western blotting analysis showed that TRAIL induced Akt activation, whereas bortezomib inhibited this activation to the same degree as bortezomib alone. Furthermore, down-regulation of P-Akt was associated with the cleavage of PARP as evidence of apopto-



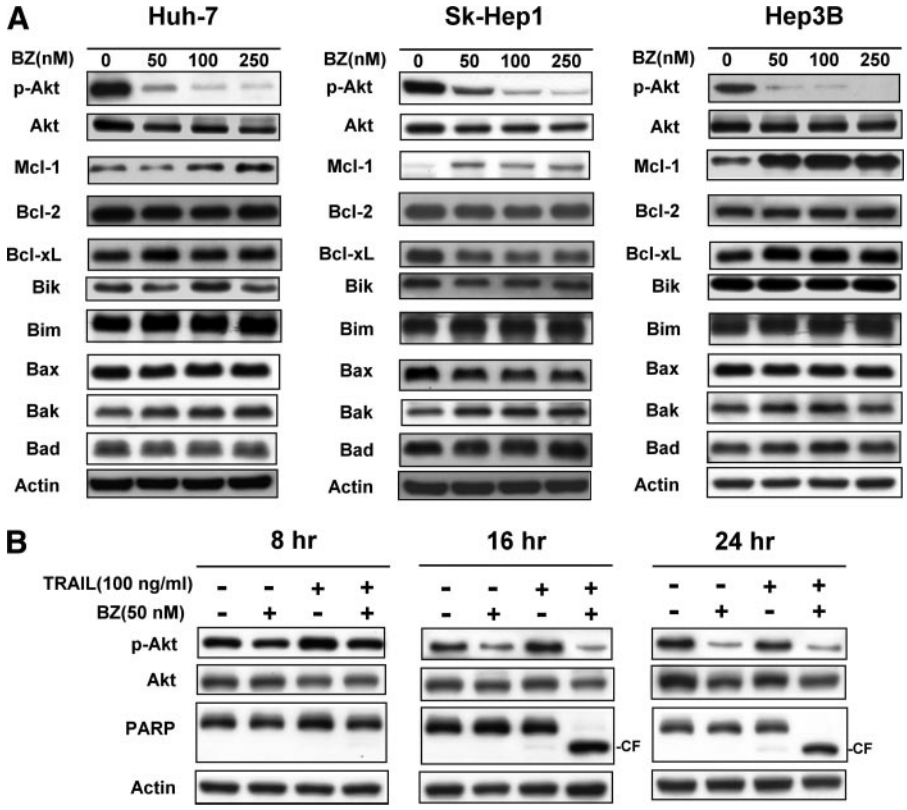


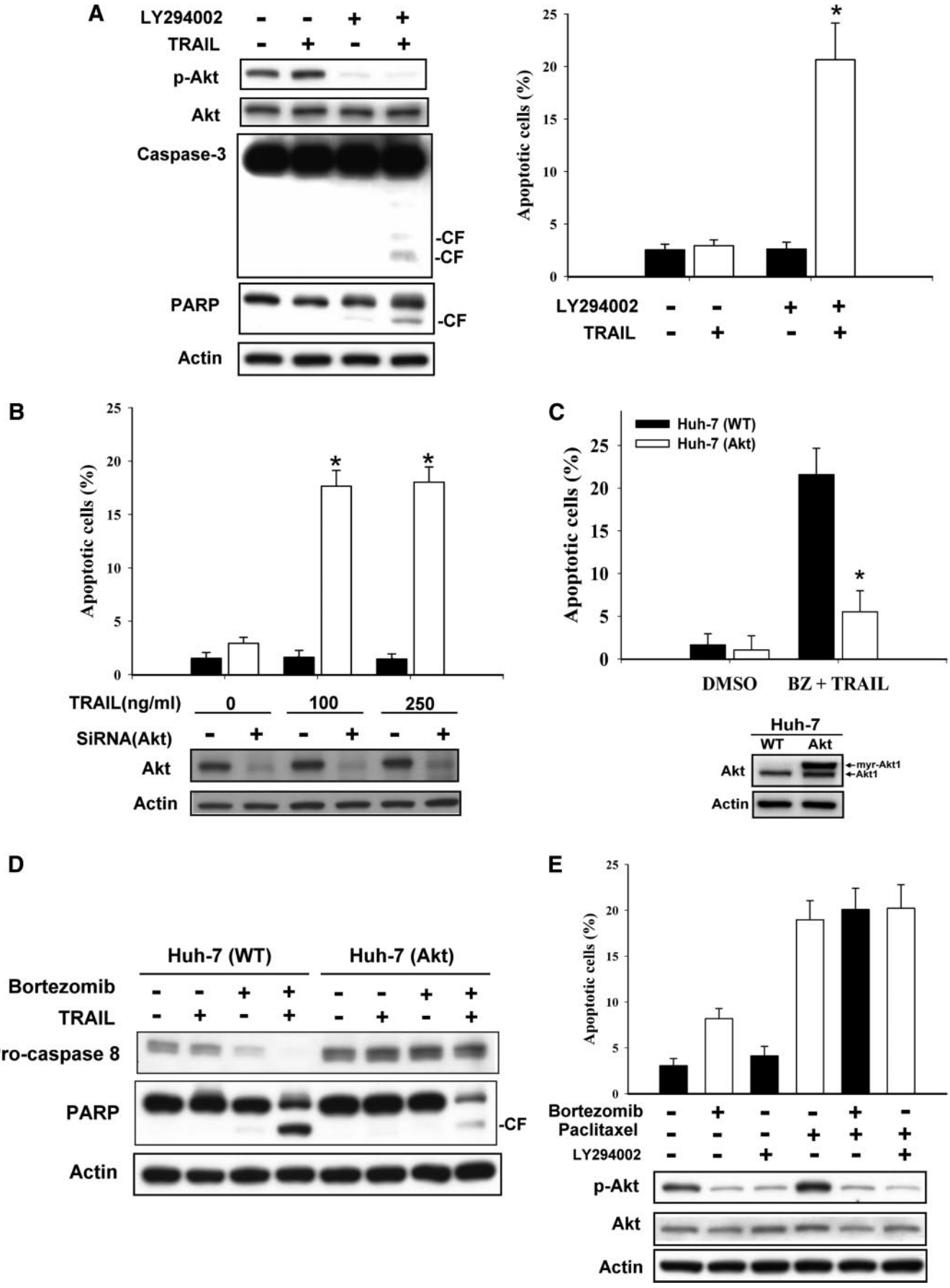
FIGURE 4. **Down-regulation of P-Akt is associated with sensitizing effects of bortezomib in HCC cells.** *A*, dose-dependent effects of bortezomib on protein levels of P-Akt, Akt, and Bcl-2 family in HCC cells. HCC cells treated with DMSO or bortezomib at the indicated doses for 24 h and cell lysates were prepared for Western blot. Data are representative of three independent experiments. *B*, time-dependent analysis of P-Akt level and apoptotic death in the combination of bortezomib and TRAIL. Hep3B cells were exposed to DMSO or bortezomib plus TRAIL at the indicated concentrations for 24 h. Cell lysates were prepared and assayed for P-Akt (serine 473), Akt, and PARP. *CF*, cleaved form (activated form).

sis induction in cells exposed to bortezomib and TRAIL for 16 and 24 h (Fig. 4*B*).

Regarding the Bcl-2 family, protein levels of Bcl-2 and Bcl-xL were not changed significantly in cells exposed to bortezomib. Up-regulation of Mcl-1 was found in cells treated with bortezomib, which has been reported previously as the effects of bortezomib (34). However, this effect may be not essential in TRAIL sensitization, since it would inhibit apoptotic induction of bortezomib and TRAIL. In addition, Bcl-2 and Bcl-xL were not changed significantly in cells treated with bortezomib. In comparison with P-Akt, protein levels of proapoptotic proteins, including Bik, Bim, Bax, Bak, and Bad, were not altered significantly in HCC cells after the treatment with bortezomib (Fig. 4*A*).

Target Validation—Three approaches were carried out to validate the inhibition of Akt signaling responsible for the TRAIL-sensitizing effect of bortezomib on apoptosis in HCC cells. First, we employed LY294002, a well known PI3K inhibitor, to examine whether the PI3K/Akt signaling pathway plays a key role in mediating the apoptotic effect of TRAIL on HCC cells. Our data showed that the combination of LY294002 with TRAIL overcame the resistance of TRAIL in Huh-7 cells in association with down-regulation of P-Akt (Ser^{473}) and resulted in activation of caspase-3 and PARP cleavage, indicating that inhibition of the PI3K/Akt signaling pathway was critical to the combinational effect of bortezomib and TRAIL on apoptosis (Fig. 5*A*). Notably, inhibition of PI3K alone was not sufficient to induce apoptosis in Huh-7 cells, suggesting that effects of TRAIL are still essential to this synergy. Next, we knocked down protein expression of Akt1 by using siRNA. Huh-7 cells were transfected with either control or Akt1 siRNA for 48 h and then exposed to DMSO or TRAIL at the indicated doses for another 24 h. Our data showed that silencing Akt1 sensitized Huh-7 cells to TRAIL-induced apoptosis significantly ($p < 0.05$) (Fig. 5*B*). Finally, we generated Huh-7 (Akt) cells with stably expressed constitutive active Akt1 (myristoylated) to investigate the TRAIL-sensitizing effect of bortezomib. As shown in Fig. 5*C*, constitutive Akt1 abolished the TRAIL-sensitizing effect of bortezomib on apoptosis with statistical significance ($p < 0.05$). Moreover, our data showed that up-regulation of Akt signals diminished the activation of caspase-8 and subsequent PARP cleavage in cells treated with bortezomib and TRAIL, indicating that the Akt signaling pathway plays an important role in mediating the sensitizing effect of bortezomib to TRAIL in HCC (Fig. 5*D*). In addition, we further examined whether bortezomib-induced Akt inhibition may sensitize the cells to other agents that do not work through TRAIL receptors. As shown in Fig. 5*E*, paclitaxel, an anti-microtubule chemotherapeutic drug, induced notable apoptosis in Sk-Hep1 cells. However, although both bortezomib and LY294002 down-regulated P-Akt in HCC cells, combination of either one of these drugs with paclitaxel did not

FIGURE 3. **Effects of bortezomib on TRAIL receptors are not essential in mediating its effects on TRAIL-induced apoptosis.** *A*, cell surface distributions of DR4 and DR5 in the presence of bortezomib. HCC cells were exposed to bortezomib at 100 nM for 24 h and then harvested for analysis of cell surface DR4 and DR5 by immunofluorescent staining and subsequent flow cytometry. *Filled gray peaks*, cells stained with a matched control phycoerythrin-conjugated IgG isotype antibody; *open peaks*, cells stained with phycoerythrin-conjugated anti-DR4 or DR5 antibody. *UT*, untreated. *Btz*, bortezomib. *B*, dose-dependent analysis of protein levels of TRAIL receptors and related components in HCC cells. HCC cells were exposed to bortezomib at the indicated concentrations for 24 h. Cell lysates were prepared and assayed for DR4, DR5, FADD, and c-FLIP by Western blot. *C* and *D*, apoptosis analysis of DR4 and DR5 in HCC cells. HCC cells were transfected with control siRNA or DR4 siRNA (Sk-hep1) or DR5 siRNA (Huh-7) for 48 h and then treated with DMSO or bortezomib plus TRAIL for 24 h. Cell lysates were prepared and assayed for TRAIL receptors, caspase-3, and PARP. *CF*, cleaved form (activated form). For analysis of apoptotic cells (sub-G_1), cells were analyzed by flow cytometry. *Columns*, mean; *bars*, S.D. ($n = 3$).



significantly enhance cell apoptosis. These data suggested that the mechanism by which bortezomib sensitizes HCC cells to TRAIL-induced cell death may not apply to other TRAIL-unrelated agents.

Inhibition of PP2A Reverses Effects of Bortezomib on P-Akt and TRAIL-induced Apoptosis—To answer the question of how bortezomib down-regulates phospho-Akt in HCC cells, we have examined the expression levels of upstream PI3K signaling proteins, including p85, p110, PTEN, PDK1, and mTORC2 (mammalian target of complex 2) in our previous study and found that these proteins were not altered significantly after treatment with bortezomib. Evidence suggests that the level of Akt phosphorylation is determined by the balance between increasing force (kinase) and decreasing force (phosphatases) (35). Therefore, we investigate the role of protein phosphatase in the effect of bortezomib on P-Akt. Our data showed that okadaic acid, a protein phosphatase 2A inhibitor, reversed the down-regulation of P-Akt in Sk-Hep1 cells after the treatment with bortezomib in a time-dependent manner (Fig. 6*A*). Notably, bortezomib did not affect the expression level of PP2A in HCC cells, suggesting that it might increase the function of PP2A through an effect other than protein turnover caused by proteasome inhibition.

Moreover, we validated the role of PP2A in the effect of bortezomib on TRAIL sensitization by silencing PP2A with siRNA. Sk-Hep1 cells were transfected with control or PP2A siRNA for 48 h and then were exposed to DMSO or the combination of bortezomib and TRAIL for 24 h. Our data showed that knockdown of PP2A decreased the effect of bortezomib on TRAIL-induced apoptosis, indicating that PP2A may play a role in mediating the effect of bortezomib on TRAIL sensitization (Fig. 6*B*).

In addition, our data indicated that bortezomib significantly increased the phosphatase activity of PP2A in HCC cells (Fig. 6*C*). We further demonstrated that forskolin, a PP2A agonist, reduced P-Akt and sensitized HCC cells to TRAIL-induced apoptosis (Fig. 6, *C* and *D*), suggesting that bortezomib might affect P-Akt through up-regulation of PP2A activity. However, bortezomib did not affect the expression of PP2A complex, including subunit A, B56γ, and C. To examine the protein-protein interaction between PP2A and Akt, we performed the co-immunoprecipitation analysis. Our data showed that bortezomib did not alter the dynamic interactions between Akt and PP2A, suggesting that bortezomib may not direct PP2A to Akt in HCC cells.

Cellular FLIP Is Not Associated with the Sensitizing Effect of Bortezomib on TRAIL-induced Apoptosis—Three approaches have been used to investigate the role of c-FLIP in mediating the effect of bortezomib on TRAIL-induced apoptosis. First, we found that bortezomib did not alter the protein expressions of c-FLIP in HCC cells (Fig. 7*A*). Our data also showed that treatment with bortezomib and/or TRAIL did not alter the amount of c-FLIP in TRAIL DISC (Fig. 7*B*). Additionally, knocking down c-FLIP by siRNA did not alter significantly the sensitizing effect of bortezomib on TRAIL-induced apoptosis in HCC, indicating that c-FLIP is not associated with the TRAIL-sensitizing effect of bortezomib in HCC. However, down-regulation of c-FLIP did sensitize cells to TRAIL (Fig. 7*C*), suggesting that c-FLIP may still play a role in mediating TRAIL resistance of HCC.

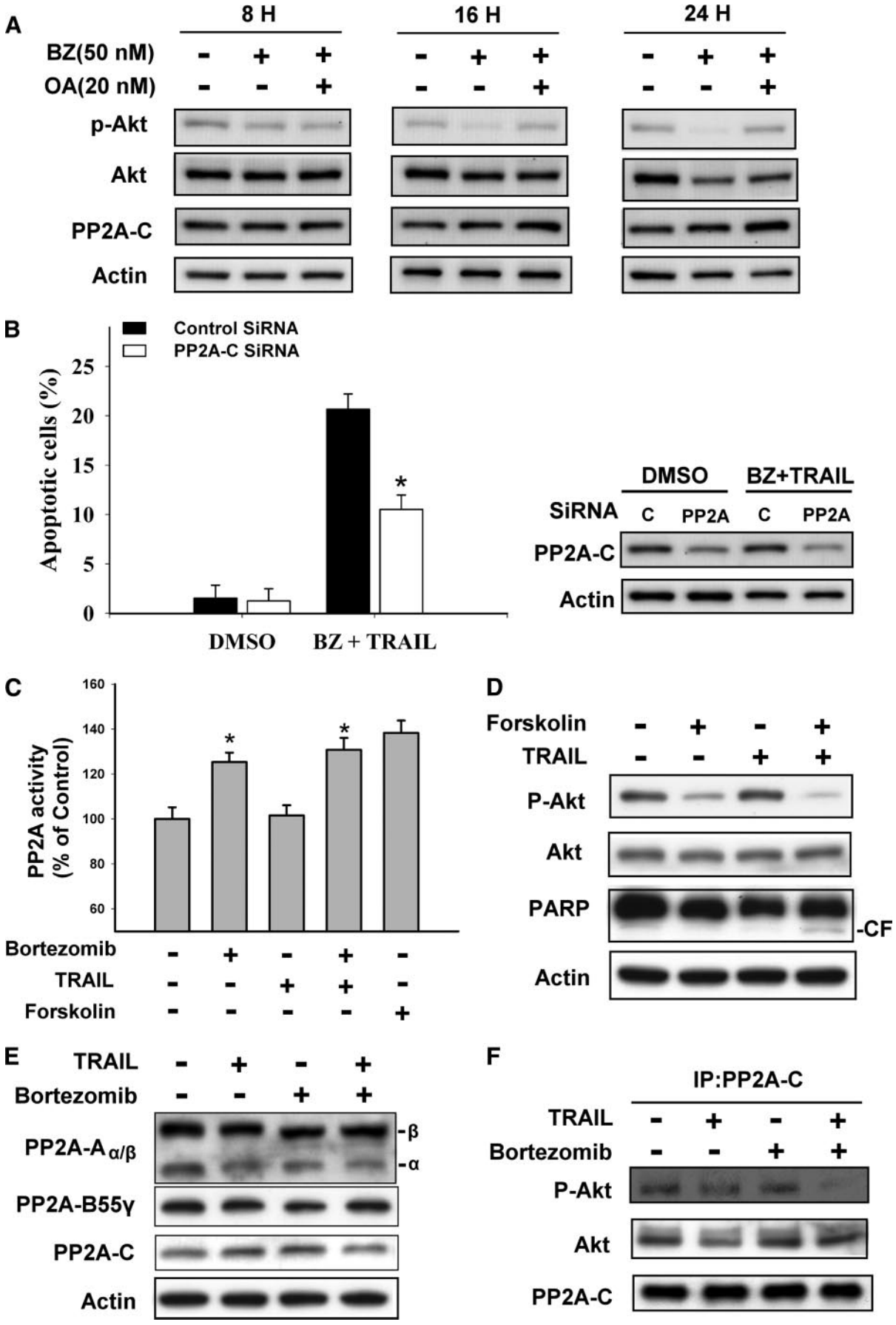
DISCUSSION

Although the TRAIL-sensitizing effect of bortezomib has been confirmed in numerous types of cancer since 2003 (4), the molecular mechanisms responsible for this synergy seem quite complicated and specific to cancer types. Here, our report provides new information about the effects of bortezomib on TRAIL sensitization in HCC cells. In all three HCC cell lines studied, TRAIL at clinically relevant levels is unable to induce apoptosis. However, the combination of bortezomib and TRAIL induces apoptosis, which is associated with the activation of caspase-8, Bid cleavage, activation of caspase-9, activation of caspase-3, and PARP cleavage, indicating that the intrinsic apoptotic pathway may be essential in this synergy.

Trauzold *et al.* (36) has shown that TRAIL and its death receptors can engage the nonapoptotic signaling pathway resulting in activation of NF-κB in pancreatic cancer cells. In addition, many studies have also indicated that bortezomib induced apoptosis through inhibition of the NF-κB signaling pathway (9, 37, 38). However, in the present study, the treatment of TRAIL or bortezomib did not alter the DNA binding activity of NF-κB significantly in our HCC cells, indicating that the effect of bortezomib is independent of the inhibition of NF-κB activation, which is consistent with a previous report in HCC (26). Similar NF-κB-independent phenomena have also been reported in other types of cancer (39–42).

Up-regulation of TRAIL receptors has been suggested as a mechanism to explain the TRAIL-sensitizing effect of bortezomib in cancer cells (14, 20, 22, 23, 27, 28). A recent study also showed that bortezomib up-regulated death receptors in Hep3B cells, but the validation of receptor was not shown in this study (27). We have observed the similar effect of bortezomib on the expression of receptors; however, knockdown of receptors by siRNA did not affect the magnitude of apoptosis induced by the combination of bortezomib and TRAIL, indicating that receptors may not be the major target, although the expression level increased after bortezomib treatment.

FIGURE 5. ***In vitro* target validation.** *A*, sensitization of TRAIL-induced apoptosis by LY294002, a PI3K inhibitor, in Huh-7 cells. *Left*, protein levels of P-Akt (Ser^{473}), Akt (Akt1), caspase-3, and PARP. *CF*, cleaved form (activated form). *Right*, analysis of apoptotic cells. Cells were treated with TRAIL (100 ng/ml) and/or LY294002 (25 μM) for 24 h. Cell lysates were prepared for Western blot, and apoptotic cells were analyzed by flow cytometry. *Columns*, mean; *bars*, S.D. ($n = 6$). *, $p < 0.01$. *B*, down-regulation of Akt (Akt1) by siRNA overcomes the resistance to TRAIL in Huh-7 cells. Cells were transfected with either control siRNA or Akt1 siRNA for 48 h and then were exposed to bortezomib for 24 h. For analysis of apoptotic cells (sub-G_1), cells were analyzed by flow cytometry. *Columns*, mean; *bars*, S.D. ($n = 3$). *, $p < 0.01$. *C* and *D*, protective effects of constitutive Akt1 on apoptosis induced by the combination of TRAIL and bortezomib in Huh-7 cells. *WT*, wild type. *Myr-Akt1*, myristoylated Akt1. *Columns*, mean; *bars*, S.D. ($n = 3$). *, $p < 0.05$. Huh-7 cells were transfected with constitutive Akt 1 and were selected for 8 weeks by G418. Analysis of apoptotic cells was performed after cells were exposed to the combination of bortezomib (50 nM) and TRAIL (100 ng/ml) for 24 h. *E*, bortezomib does not enhance paclitaxel-induced apoptosis in Sk-Hep1 cells. *Top*, analysis of apoptotic cells. *Bottom*, protein levels of P-Akt (Ser^{473}), Akt (*Akt1*). Cells were exposed to bortezomib (50 nM) or LY294002 (25 μM) or paclitaxel (100 nM) or in combinations for 24 h.



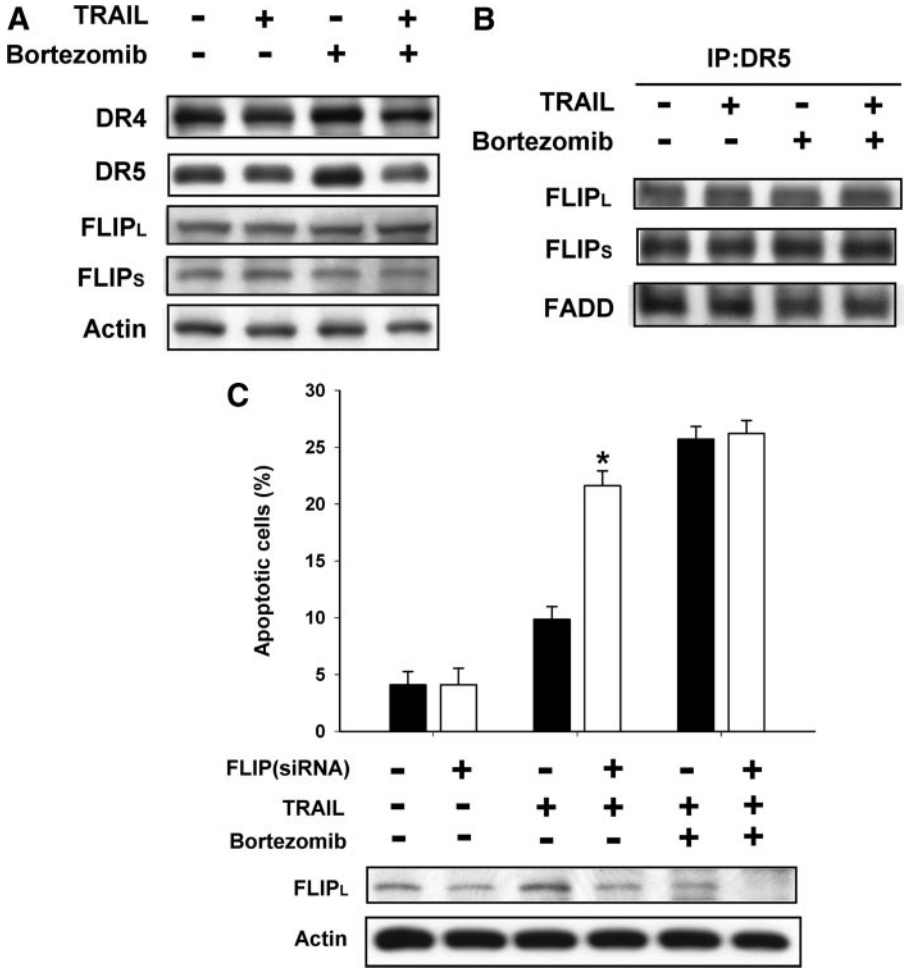


FIGURE 7. **Cellular FLIP is not associated with the sensitizing effect of bortezomib on TRAIL-induced apoptosis.** *A*, protein levels of c-FLIP, DR4, and DR5 in Sk-Hep1 cells. Cells were exposed to bortezomib at the indicated concentrations for 24 h. Cell lysates were prepared and assayed for DR4, DR5, FADD, and c-FLIP by Western blot. *B*, protein levels of c-FILP in DISC. Sk-Hep1 cells were exposed to 100 ng/ml TRAIL and/or 250 nM bortezomib, and then cells were lysed 2 h later, and DR5 was immunoprecipitated. Immune complexes and lysates were analyzed by SDS-PAGE using antibodies specific for FLIP and FADD. *C*, effects of silencing c-FLIP by siRNA on TRAIL-induced apoptosis in Sk-Hep1 cells. *Columns*, mean. Cells were transfected with control siRNA or $FILP_L$ siRNA for 48 h, and then cells were treated with TRAIL (100 ng/ml) or TRAIL plus 250 nM bortezomib for an additional 24 h. Cells were prepared for Western blot, and apoptotic cells were analyzed by flow cytometry.

In the present study, we showed that TRAIL increased the Akt activation. It is very possible that the activation of Akt provides a survival signal against TRAIL insults in HCC cells. Our previous study demonstrated that bortezomib inhibits Akt activation in HCC cells (31). In this study, we showed that bortezomib suppressed TRAIL-induced Akt activation to a level as low as for bortezomib-treated alone. Therefore, it might be an underlying mechanism to explain how bortezomib enhances the cytotoxic effect of TRAIL on HCC cells. However, Akt might not be the only determinant in the cytotoxicity of bortezomib/TRAIL co-treatment, since 50 nM bortezomib alone induced less apoptotic cell death than bortezomib and TRAIL co-treatment. Notably, other studies have also reported Akt signaling changes in cells exposed to bortezomib (20, 38, 39, 40). In light of the key role of the PI3K/Akt signaling pathway in governing intrinsic apoptosis, the effect of bortezomib on P-Akt should be crucial in affecting the TRAIL pathway, especially for type II cancer cells. Furthermore, the PI3K/Akt pathway plays a major role in drug resistance in various types of cancer, including HCC (43). Several studies have also shown that tumors with the activation of PI3K/Akt signaling become more aggressive and are associated with poor prognosis in patients with HCC (44, 45). Notably, the activation of PI3K signaling, including PTEN mutation, has been observed in 40–50% of HCC tumors (45, 46), indicating the importance of Akt signaling in HCC.

Further, our data indicated that although okadaic acid, an inhibitor of PP2A, reversed P-Akt reduction, the protein level of PP2A did not change in bortezomib-treated cells (Fig. 6*A*). A possible explanation is that bortezomib might direct PP2A targeting Akt instead of increasing the protein level of PP2A through proteasome inhibition. Indeed, it has been reported (47) that bortezomib increases PP2A and Akt physical interaction. On the other hand, silencing of PP2A by siRNA did not completely reverse the effects of bortezomib and TRAIL on HCC cells (Fig. 6*B*), suggesting that, in addition to PP2A, other pathways might be involved in bortezomib- and TRAIL-induced apoptotic cell death.

Previously, Ganten *et al.* (26) has shown that cellular FLIP was up-regulated in the TRAIL DISC by MG-132 pretreatment

FIGURE 6. **Inhibition of PP2A reverses effects of bortezomib on P-Akt and TRAIL-induced apoptosis.** *A*, okadaic acid, a PP2A inhibitor, abolishes down-regulation of P-Akt in bortezomib-treated cells. *B*, silencing of PP2A-C by siRNA reduces effects of bortezomib on TRAIL-induced apoptosis in HCC cells. *Left*, analysis of apoptotic cells. *Columns*, mean; *bars*, S.D. ($n = 3$). *, $p < 0.05$. *Right*, protein levels of PP2A. Sk-Hep1 cells were transfected with control siRNA or PP2A-C siRNA for 48 h and then treated with DMSO or the combination of bortezomib (50 nM) and TRAIL (100 ng/ml) for 24 h. Cells were prepared for Western blot, and apoptotic cells were analyzed by flow cytometry. *C*, analysis of PP2A activity in Sk-Hep1 cells. *Columns*, mean; *bars*, S.D. ($n = 3$). *, $p < 0.05$. Cells were treated with DMSO or 250 nM bortezomib and/or 100 ng/ml TRAIL for 24 h or forskolin at 40 μM for 24 h. Cell lysates were prepared for detecting PP2A activity as described under "Experimental Procedures." *D*, forskolin, a PP2A agonist, down-regulates P-Akt and enhances the effect of TRAIL on apoptosis in Sk-Hep1 cells. Cells were exposed to DMSO or 250 nM TRAIL and/or 40 μM forskolin for 24 h. Cell lysates were prepared and assayed for P-Akt, Akt, caspase-3, and PARP by Western blot. *CF*, cleaved form (activated form). *E*, effects of bortezomib and/or TRAIL on protein levels of PP2A complex. Sk-Hep1 cells were exposed to DMSO or 250 nM bortezomib and/or 100 ng/ml TRAIL for 24 h. *F*, effects of bortezomib and/or TRAIL on PP2A/Akt interactions in Sk-Hep1 cells. Cells were treated with 100 ng/ml TRAIL and/or 250 nM bortezomib for 24 h, and cell lysates were immunoprecipitated with anti-PP2A-C antibodies. The immunoprecipitates were probed with P-Akt, Akt, and PP2A-C by Western blot as described under "Experimental Procedures."

in HCC. However, our data showed that bortezomib did not alter the amount of c-FLIP in DISC. MG132 and bortezomib are both proteasome inhibitors, but their chemical structures and biological activities are actually quite different (9). It is reasonable that bortezomib and MG-132 may sensitize cells to TRAIL-induced cell death via different mechanisms. Notably, Ganten *et al.* (26) employed MG132, instead of bortezomib, to perform most of the mechanistic study, and they thus did not demonstrate any data for bortezomib on c-FLIP or DISC. In addition, our data showed that the effect of bortezomib on TRAIL-induced apoptosis is not associated with NF-κB, a major target of proteasome inhibition (Fig. 2), suggesting that the effect of bortezomib on TRAIL is independent of proteasome inhibition, consistent with our previous report (31). Importantly, down-regulation of c-FLIP by siRNA did not affect the effect of bortezomib on TRAIL-induced cell death (Fig. 7*C*), indicating that c-FLIP may not be relevant to the effect of bortezomib on TRAIL.

A recent study showed that bortezomib sensitized solid tumor cells to apoptosis induced by MD5–1, a mouse anti-DR5 antibody, and prevented lung metastasis through the extrinsic pathway, indicating that the effect of bortezomib on TRAIL may be applied more extensively than modulating intrinsic resistance mechanisms (48). Monoclonal antibodies targeting DR4 or DR5, which has a longer half-life in humans and does not bind TRAIL decoy receptors, may be more effective than soluble recombinant TRAIL in clinical use. Several antibodies, including mapatumumab (anti-DR4), lexatumumab (anti-DR5), and AMG 655 (anti-DR5), have been investigated in clinical trials (3). A phase II study of Mapatumumab in combination with bortezomib and bortezomib alone in patients with refractory multiple myeloma is under way in the United States, indicating that the synergy of bortezomib and TRAIL pathway is very important in the treatment of cancer (National Institutes of Health study NCT00315757).

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Bortezomib Overcomes Tumor Necrosis Factor-related Apoptosis-inducing Ligand Resistance in Hepatocellular Carcinoma Cells in Part through the Inhibition of the Phosphatidylinositol 3-Kinase/Akt Pathway

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