

Down-regulation of Phospho-Akt Is a Major Molecular Determinant of Bortezomib-Induced Apoptosis in Hepatocellular Carcinoma Cells

Kuen-Feng Chen,¹ Pei-Yen Yeh,^{2,3} Kun-Huei Yeh,^{2,4} Yen-Shen Lu,^{2,3} Shang-Yi Huang,⁵ and Ann-Lii Cheng^{2,3,5,6}

Departments of ¹Medical Research and ²Oncology, ³Cancer Research Center, ⁴Graduate Institute of Clinical Medicine, and ⁵Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine; and ⁶Institute of Cancer Research, National Health Research Institute, Taipei, Taiwan

Abstract

Bortezomib, a proteasome inhibitor, has been clinically approved for the treatment of myeloma and lymphoma. Here, we report a differential effect of bortezomib on apoptosis in four hepatocellular carcinoma (HCC) cell lines and identify the major molecular event that determines sensitivity. Although bortezomib inhibited proteasome activity to a similar extent in all HCC cell lines, it showed differential effects on their viability: Huh-7 (IC₅₀ 196 nmol/L), Sk-Hep1 (IC₅₀ 180 nmol/L), Hep3B (IC₅₀ 112 nmol/L), and resistant PLC5 (IC₅₀ >1,000 nmol/L). Bortezomib caused cell cycle arrest at G₂-M phase in all HCC cells tested whereas apoptotic induction was found only in sensitive cells but not in PLC5 cells. No significant bortezomib-induced NF- κ B changes were noted in Huh-7 and PLC5. Bortezomib down-regulated phospho-Akt (P-Akt) in a dose- and time-dependent manner in all sensitive HCC cells whereas no alterations of P-Akt were found in PLC5. Down-regulation of Akt1 by small interference RNA overcame the apoptotic resistance to bortezomib in PLC5 cells, but a constitutively activated Akt1 protected Huh-7 cells from bortezomib-induced apoptosis. Furthermore, bortezomib showed suppression of tumor growth with down-regulation of P-Akt in Huh-7 tumors but not in PLC5 tumors. Down-regulation of P-Akt represents a major molecular event of bortezomib-induced apoptosis in HCC cell lines and may be a biomarker for predicting clinical response to HCC treatment. Targeting Akt signaling overcomes drug resistance to bortezomib in HCC cells, which provides a new approach for the combinational therapy of HCC. [Cancer Res 2008; 68(16):6698–707]

Introduction

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death. Risk factors for HCC include cirrhosis due to chronic hepatitis B virus (1) or chronic hepatitis C virus (1) infection or coinfection, aflatoxin exposure, alcoholic cirrhosis, male gender, cigarette smoking, and advanced age (2). Among these risk factors, chronic HBV and HCV infections are the two

most important for HCC development in the Far East including Taiwan (1). Notably, the importance of HBV in hepatocarcinogenesis has been confirmed by evidence indicating that vaccination for HBV decreases the incidence of HCC in children in Taiwan (3).

Surgery is currently the only curative treatment but it is only feasible in a minority of patients (~20–30%; ref. 4). For those patients who have advanced stage disease, poor liver function, or recurrent tumors after local treatments, systemic pharmacologic treatment is the final and main therapy. Unfortunately, the response rate to traditional chemotherapy for HCC patients is quite low and the outcome is also poor (4). Hence, new drugs with a higher therapeutic index are urgently needed for these patients. In this regard, molecular targeted agents offer hope for improving outcomes in the future as witnessed by recent approval of sorafenib (5).

A novel molecular agent, bortezomib (formally PS-341, or Velcade; Millennium Pharmaceuticals, Inc.), is a dipeptidyl boronic acid that reversibly inhibits the 20S proteasome. It has been approved clinically for the treatment of refractory multiple myeloma and mantle cell lymphoma worldwide (6, 7). Although a number of clinical trials of bortezomib in solid tumors have been conducted, the lack of significant clinical benefit in preliminary results of those trials (8–10) may suggest that the molecular targets of bortezomib in solid tumor are different from those reported in hematologic malignancies.

Among the known targets of bortezomib in myeloma and lymphoma, nuclear factor- κ B (NF- κ B) has been proposed as a major target of bortezomib as a proteasome inhibitor (7). NF- κ B plays a key role in cell proliferation, apoptosis, invasiveness, metastasis, tumorigenesis, and angiogenesis (11). Proteasome inhibitors such as bortezomib sequester NF- κ B in the cytoplasm and reduce its transcriptional activity through blocking of the degradation of its inhibitor I κ B (7). Numerous studies indicated that bortezomib inhibits antitumor activity against cancer cells through the mechanism of stabilizing I κ B and consequently decreasing the activity of NF- κ B (12, 13).

In addition, tumor suppressor p53 is also a substrate of the proteasome, and previous study has shown that expression of a dominant-negative p53 in Rat-1 fibroblasts decreased apoptosis induced by proteasome inhibitors (14). Moreover, Bcl-2 family protein is also the target of proteasome inhibitor. Bcl-2 family members play a critical role in the regulation of apoptosis and are substrates of the proteasome (15). Bortezomib has been shown to induce phosphorylation and cleavage of Bcl-2 in association with G₂-M phase cell cycle arrest (16). Other studies have also indicated that the accumulation of proapoptotic Bcl-2 proteins such as Bid,

Requests for reprints: Ann-Lii Cheng, Departments of Internal Medicine and Oncology, National Taiwan University Hospital, No 7, Chung-Shan South Road, Taipei, Taiwan. Phone: 886-2-23123456, ext. 7251; Fax: 886-2-23711174; E-mail: alcheng@ntu.edu.tw

©2008 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-0257

Bik, and Bim is associated with proteasome inhibitor-induced apoptosis (17). Moreover, evidence indicated that Bik and/or Bim may participate in the apoptotic effect of bortezomib.

In this study, we investigated the differential effects of bortezomib among various HCC cell lines and the extent of the inhibition of Akt signaling as a major molecular mechanism in determining bortezomib-induced apoptosis in HCC cells.

Materials and Methods

Reagents. Bortezomib (Velcade) was provided by Millennium Pharmaceuticals. LY294002 and rapamycin were purchased from Cayman Chemical and Calbiochem, respectively.

Cell culture. The Huh7 HCC cell line was obtained from the Health Science Research Resources Bank (JCRB0403). The Sk-Hep-1, Hep3B, and PLC5 cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 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% CO₂ in air.

Cell viability analysis. The effect of bortezomib on hepatoma cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 12 replicates. The procedure has previously been described (18).

Western blot analysis. Lysates of Huh-7, Sk-hep1, Hep3B, and PLC5 cells treated with bortezomib at the indicated concentrations for 24 h were prepared for immunoblotting of caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP), phospho-Akt (P-Akt), Akt, PTEN, phosphoinositide-dependent kinase 1 (PDK1), phosphatidylinositol 3-kinase (PI3K), phosphorylated mammalian target of rapamycin (mTOR), mTOR, Rictor, SIN1/MIP1, etc. Western blot analysis was done as previously reported (19). Antibodies for immunoblotting were purchased from Santa Cruz Biotechnology or Cell Signaling (18).

Flow cytometry for cell cycle analysis. HCC cells were treated with DMSO or bortezomib at the indicated concentrations for 24 h. The procedure has previously been described and cell cycle phase distributions were determined on a FACScan flow cytometer (Beckman-Coulter; ref. 19).

Apoptosis analysis. The following three methods were used to assess drug-induced apoptotic cell death: detection of DNA fragmentation with the Cell Death Detection ELISA kit (Roche Diagnostics), Western blot analysis of caspase activation and PARP cleavage, and measurement of apoptotic cells by flow cytometry (sub-G₁). The ELISA was done according to the manufacturer's instructions (19).

Proteasome activity assay. A 20S Proteasome Activity Assay kit (Chemicon) was used to determine the inhibition of proteasome in drug-treated HCC cells. All the procedures were done according to the manufacturer's instructions (20).

NF-κB binding activity. The Trans-AM NF-κB p65 Transcription Factor Assay kit (Active Motif North America) 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 with anti-IgG-horseradish peroxidase conjugate as previously described (21).

Target validation. Two approaches were used for *in vitro* validation. First, the Akt1 small interference RNA (siRNA) duplex targeting the sequence 5'-UAAUGGCCCGUCCUUGUCUU-3' of human *Akt1* gene and control siRNA were purchased from Dharmacon, Inc. Briefly, PLC5 cells were transfected with siRNA (the final concentration is 100 nmol/L) in six-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 PLC5 cells were incubated for an additional 24 h with bortezomib for Western blot analysis and apoptosis analysis by flow cytometry as previously described. Second, Huh-7 cells were trans-

ected with the constitutive active Akt1 construct HA-PKB-T308D/S473D as previously described (19). Briefly, following transfections, cells were incubated in the presence of G418 at 0.78 mg/mL. After 8 wk 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 previously described (19).

Xenograft tumor growth. Male NCr athymic nude mice (5–7 wk of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The mice were group-housed under conditions of a constant 12-h photoperiod with *ad libitum* access to sterilized food and water. All experimental procedures using these mice were done in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of National Taiwan University. Each mouse was inoculated s.c. in the dorsal flank with 1×10^6 HCC cells suspended in 0.1 mL of serum-free medium containing 50% Matrigel (BD Biosciences). When tumors reached 200 to 300 mm³, mice received an i.p. injection of bortezomib at 1 mg/kg body weight twice weekly for the duration of the study. Controls received vehicle. Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: width² × length × 0.52. Body weight was measured weekly (19).

Western blot analysis of P-Akt status in HCC xenograft tumors. At sacrifice, tumors were harvested from mice, and a portion of each tumor was snap frozen in liquid nitrogen and stored at –80°C until analysis. Tumor tissue homogenates were prepared in SDS lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 2% SDS, and protease inhibitor mixture] as previously described (19). Western blot analysis was done.

Statistical analysis. Tumor growth data points are reported as mean tumor volume ± SE. Comparisons of mean values were done using the independent samples *t* test in SPSS for Windows 11.5 software (SPSS, Inc.; ref. 19).

Results

Differential effects of bortezomib on viability and apoptotic death in HCC cells. To investigate the antitumor effect of bortezomib on HCC cells, we first assessed the growth inhibitory effect of bortezomib in a panel of four human HCC cell lines, including Huh7, Sk-Hep1, Hep3B, and PLC5, at clinically relevant concentrations (according to data from Millennium, median concentration of *C*_{max} is 1,300 nmol/L). Cell viability was determined by MTT assay after 72 hours of treatment. As shown in Fig. 1A, bortezomib exhibited differential effects on viability of the four types of cells. Bortezomib caused a dose-dependent reduction in cell viability in Huh-7 (IC₅₀ 196 nmol/L), Sk-Hep1 (IC₅₀ 180 nmol/L), Hep3B (IC₅₀ 112 nmol/L), and PLC5 (IC₅₀ >1,000 nmol/L). PLC5 showed resistance to bortezomib on cell viability, which was significant at doses >100 nmol/L (*P* < 0.01, compared with all of the other three types of cells).

Evidence indicates that the growth inhibition was caused by cell cycle arrest and apoptosis induction. The activation of caspases and the cleavage of PARP were determined by Western blotting after 24 hours of treatment. Data illustrated in Fig. 1B indicate that bortezomib caused the activation of caspase-9 and caspase-3 and subsequently the cleavage of PARP in all sensitive cell lines in a dose-dependent manner, starting at a concentration of 50 to 100 nmol/L. However, no apoptotic evidence could be found in PLC5 cells at all tested concentrations. In addition, resistance to bortezomib-induced apoptosis in PLC5 was also confirmed by a DNA fragmentation ELISA assay. Bortezomib induced a dose-dependent induction of DNA fragmentation in Huh7 cells, starting at the concentration of 100 nmol/L after treatment for 24 hours, whereas the apoptotic induction was not found in bortezomib-treated PLC5 cells (Fig. 1C).

Differential effect of bortezomib on apoptosis in HCC cells may be unassociated with proteasome inhibition. To characterize the mechanism responsible for the differential apoptotic effects of bortezomib in HCC cells, we initially examined whether the differential effect of bortezomib on apoptosis is associated with the effects of bortezomib on the inhibition of proteasome.

Several lines of evidence suggest that the apoptotic effect of bortezomib may not be associated with proteasome inhibition. First, we examined the proteasome inhibitory effect of bortezomib in the four HCC cell lines. HCC cells were treated with different doses of bortezomib for 2 hours, according to methods used in a previous clinical study (8). Bortezomib showed a similar pattern and degree of proteasome inhibition on both sensitive and resistant cell lines without any significant differences, suggesting that the effect of bortezomib on proteasome inhibition is not

associated with its differential cellular effect on HCC cells (Fig. 2A).

According to a previous report (20), bortezomib causes cancer cell cycle arrest at G₂-M phase, which is associated with proteasome inhibition. As shown in Fig. 2B, bortezomib induced cell cycle arrest at G₂-M phase in a dose-dependent manner after treatment for 24 hours both in sensitive and resistant cells, which indicates that bortezomib is able to cause cell cycle arrest in PLC5 cells although it is unable to induce apoptosis. Given the fact that reports have linked the effects of bortezomib on cell cycle with proteasome inhibition (20–23), this result provides additional evidence to support the hypothesis that proteasome inhibition may not be associated with the differential effect of bortezomib on HCC cells.

Another molecular sequel of proteasome inhibition is the inhibition of NF- κ B signaling. Previous studies have shown that

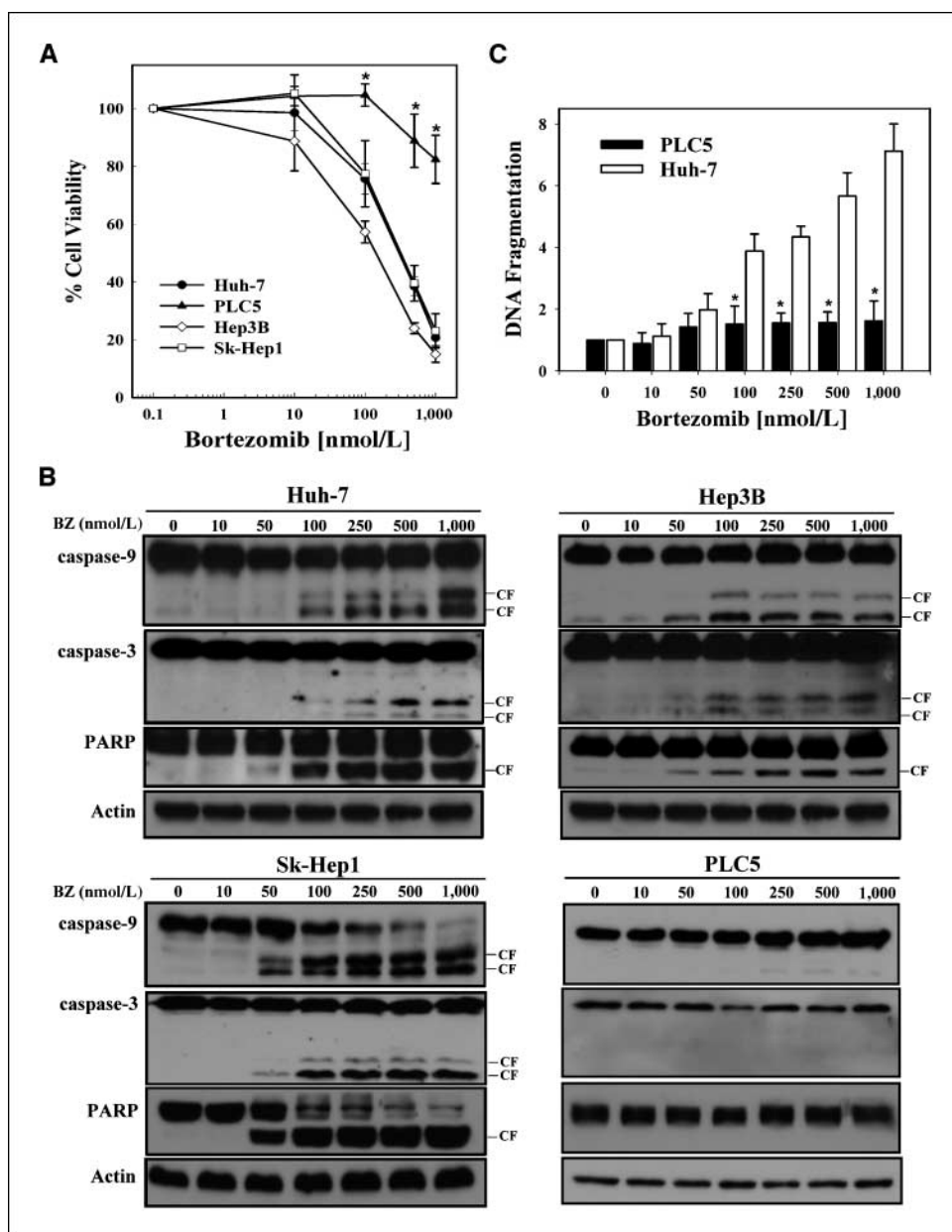
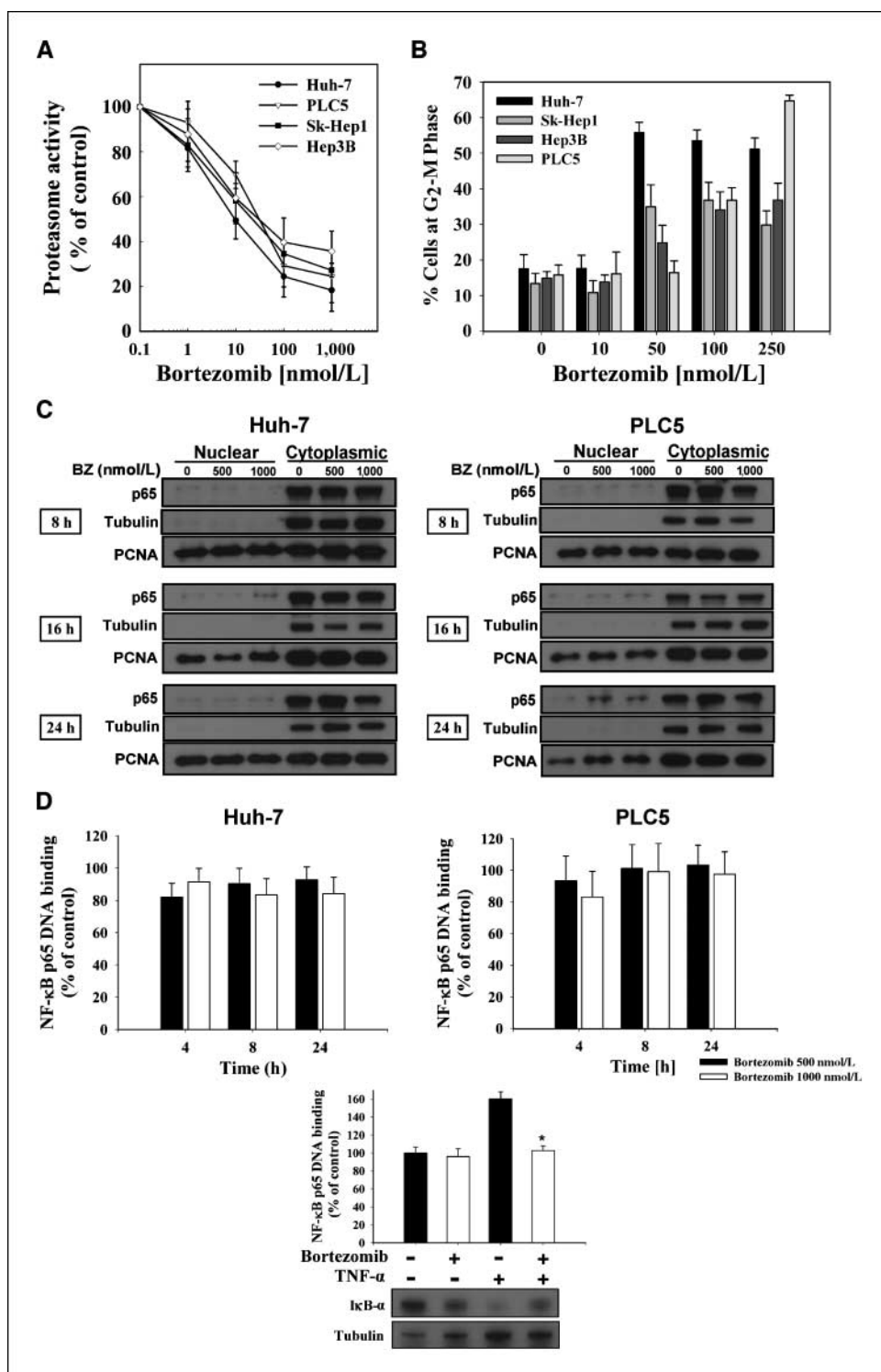


Figure 1. Differential effects of bortezomib on apoptotic death in HCC cells. **A**, dose-dependent effects of bortezomib on cell viability in four human HCC cell lines. HCC cells were exposed to bortezomib at the indicated concentrations in DMEM with 5% FBS in 96-well plates for 72 h and cell viability was assessed by MTT assay. *Points*, mean ($n = 6$); *bars*, SD. *, $P < 0.01$. **B**, analysis of caspases and PARP. HCC cells were exposed to bortezomib (BZ) at the indicated doses in DMEM with 5% FBS for 24 h. Cell lysates were prepared and assayed for caspase-9, caspase-3, and PARP by Western blotting. Representative of three independent experiments. *CF*, cleaved form (activated form). **C**, analysis of DNA fragmentation. HCC cells were treated with bortezomib for 24 h. DNA fragmentation was quantitatively measured with a cell death ELISA kit. *Columns*, mean ($n = 3$); *bars*, SD. *, $P < 0.01$, Huh-7 versus PLC5.

Figure 2. Evidence suggesting that the differential effect of bortezomib on apoptosis in HCC cells is not associated with proteasome inhibition. **A**, a similar dose-dependent effect of bortezomib on proteasome inhibition was found in four HCC cell lines. HCC cells were exposed to bortezomib at the indicated doses for 2 h before measurement of proteasome activity. **B**, bortezomib induced the accumulation of all HCC cell lines in G₂. HCC cells were exposed to bortezomib at the indicated doses for 24 h. Cell cycle was analyzed by flow cytometry. Columns, mean (*n* = 3); bars, SD. **C**, protein levels of NF- κ B/nuclear p65 were not altered by bortezomib in either sensitive (Huh-7) or resistant (PLC5) cells. Cells were exposed to bortezomib at the indicated doses for 8, 16, and 24 h. Nuclear extracts were prepared and assayed for p65 protein levels by Western blot. **D**, top, NF- κ B/nuclear p65 binding activity was not changed after bortezomib treatment in both types of cells. Cells were treated with bortezomib at the indicated doses for 4, 8, and 24 h. Nuclear extracts were prepared and assayed for p65 binding activity by ELISA kit. Columns, mean (*n* = 3); bars, SD. *, *P* < 0.05. Bottom, bortezomib abolished NF- κ B activation induced by TNF- α in PLC5 cells. Cells were exposed to DMSO or 500 nmol/L bortezomib for 1 h and then treated with 20 ng/mL TNF- α for 1 h. Nuclear extracts were prepared and assayed for p65 binding activity by ELISA kit. Columns, mean (*n* = 3); bars, SD. *, *P* < 0.05. Cytoplasmic extracts were prepared and assay for I κ B- α by Western blot.



bortezomib inhibits NF- κ B signaling by preventing degradation of I κ B- α , whereas nuclear translocation and accumulation of NF- κ B subunit p65 is critical in activating NF- κ B signaling. Here, we analyzed protein levels of nuclear p65 and NF- κ B binding activity in both sensitive Huh-7 and resistant PLC5 cells treated with bortezomib. As shown in Fig. 2C, nuclear expression levels of p65 protein were quite low in both sensitive Huh-7 and resistant PLC5

cells. The time-dependent analysis indicates that bortezomib did not cause significant alterations in the expressions levels of nuclear p65 in both sensitive and resistant cells. In addition, bortezomib did not show a significant inhibitory effect on NF- κ B binding activity in both cell lines at three time points within 24 hours (Fig. 2D, top). Notably, bortezomib reversed the degradation of I κ B- α induced by tumor necrosis factor α (TNF- α) and

subsequently inhibited NF- κ B activation in PLC5 cells (Fig. 2D, bottom). Together, these results indicate that the NF- κ B pathway may not play a key role in mediating the differential effect of bortezomib on apoptosis in the tested HCC cells.

Down-regulation of Akt signaling determines apoptosis induction. Our results indicate that bortezomib may mediate apoptosis in HCC cells through a nonproteasome mechanism. In addition, bortezomib induced a caspase-dependent apoptosis in association with the activation of caspase-9, suggesting that the intrinsic mitochondria pathway plays an important role in mediating the effect of bortezomib on apoptosis (Fig. 1B). Accordingly, we identified that down-regulation of P-Akt at Ser⁴⁷³ was the major molecular determinant of bortezomib-induced apoptosis in HCC cells. As shown in Fig. 3A, bortezomib induced down-regulation of P-Akt at Ser⁴⁷³ in all sensitive HCC cells in a dose-dependent manner, but not in resistant PLC5 cells. Furthermore, down-regulation of P-Akt was associated with apoptosis induction characterized by the activation of caspase-9 and PARP cleavage in sensitive Huh-7 cells in a time-dependent manner (Fig. 3B). In light of the effects of bortezomib on protein turnover, we analyzed the expression levels of upstream PI3K signaling proteins, which might affect P-Akt level in Huh7 cells. As shown in Fig. 3C, levels of PI3K pathway proteins, including p85 (regulatory subunit of PI3K), PTEN, PDK1, and P-Akt at Thr³⁰⁸, were not altered significantly after treatment with bortezomib for 24 hours in sensitive Huh7 cells, although a moderate decrease of p110 in cells at high doses of bortezomib was noted. Recent studies indicated that a mTOR complex 2 (mTORC2 or Rictor-mTOR) is responsible for the activation of P-Akt at Ser⁴⁷³ (24, 25). Our data showed that the protein levels of mTORC2, including phospho-mTOR, mTOR, Rictor, and SIN1/MIP1, were not altered significantly in both Huh-7 and PLC5 cells after exposure to bortezomib for 24 hours (Fig. 3D).

In addition, we analyzed other signaling proteins that may regulate mitochondria-related apoptotic pathway in both sensitive Huh-7 and resistant PLC5 cells. Protein levels of proapoptotic proteins such as Bax, Bak, Bad, and Bik were not significantly changed in both Huh-7 and PLC5 cells. Cleaved Bim was slightly increased after treatment with bortezomib in Huh-7 cells but not in PLC5 cells, providing additional evidence of the differential apoptotic effects of bortezomib in HCC cells. For antiapoptotic proteins, we compared protein levels of Bcl-2, Bcl-xL, Mcl-1, and X-linked inhibitor of apoptosis between Huh-7 and PLC5 cells and found no major alterations in bortezomib-treated HCC cells (data not shown).

Target validation. Three approaches were carried out to validate the inhibition of Akt signaling as a major molecular target responsible for the *in vitro* apoptotic effect of bortezomib in HCC cells. First, we knocked down protein expression of Akt1 in resistant PLC5 by using siRNA. Down-regulation of Akt1 sensitized PLC5 cells to bortezomib-induced apoptosis in a dose-dependent manner, and this effect was associated with the activation of caspase-3, PARP cleavage, and increased number of apoptotic cells (Fig. 4A).

Next, Huh7-Akt, which stably expressed constitutive active Akt1, was generated to examine the apoptotic effect of bortezomib. As shown in Fig. 4B, Huh7-Akt showed relative resistance to bortezomib-induced apoptosis with statistical significance at concentrations >100 nmol/L ($P < 0.05$).

Additionally, we examined whether inhibition of PI3K, which is upstream of Akt, may alter the proapoptotic effect of

bortezomib in HCC cells. Our data showed that combination of LY294002, a PI3K inhibitor, with bortezomib overcame the resistance of bortezomib in PLC5 cells in association with down-regulation of P-Akt (Ser⁴⁷³), indicating that inhibition of the PI3K pathway is a critical step to the proapoptotic effect of bortezomib in resistant HCC cells (Fig. 4C). We found that inhibition of PI3K alone was not sufficient to induce apoptosis in PLC5 cells, suggesting that bortezomib may have effects other than regulation of P-Akt in resistant cells.

Finally, we found that rapamycin, an inhibitor of mTOR, down-regulated phospho-S6 ribosomal protein (Fig. 4D) but was unable to enhance apoptosis in bortezomib-treated PLC5 cells. However, given the fact that mTORC2 is rapamycin insensitive (25), the possibility that mTORC2 may still play a role in this scenario cannot be excluded, although protein levels of the complex were not affected (Fig. 3D).

***In vivo* effect of bortezomib in HCC xenograft tumor.** To confirm whether the differential antitumor effect of bortezomib in HCC cell lines has potentially relevant clinical implications, we assessed the *in vivo* effect of bortezomib on the growth of HCC xenograft tumors. Tumor-bearing mice were treated with vehicle or bortezomib i.p. at the clinically relevant dose of 1.0 mg/kg twice a week for the duration of the study. All animals tolerated the treatments well without observable signs of toxicity and were characterized by stable body weights throughout the course of study. No gross pathologic abnormalities were noted at necropsy.

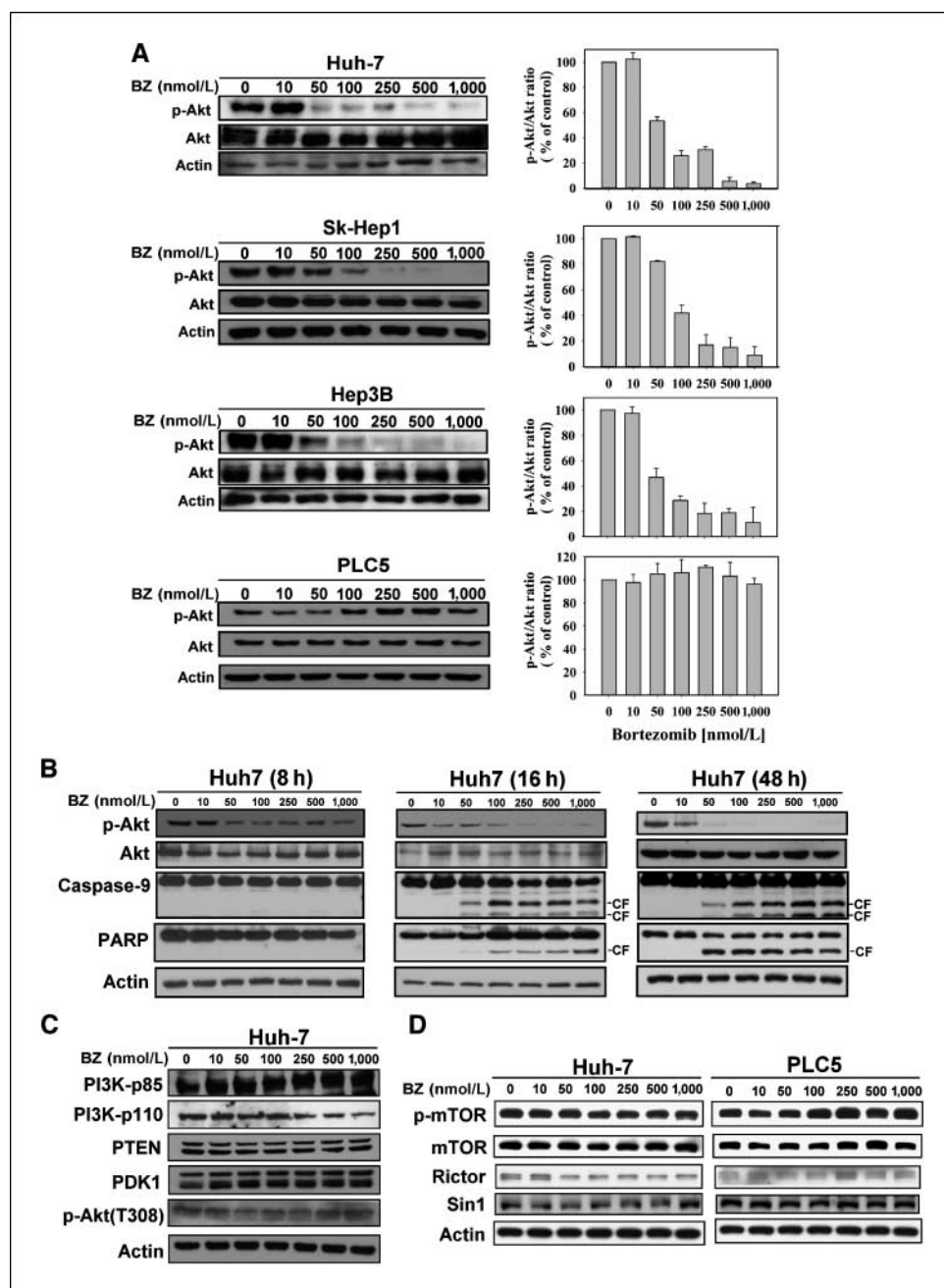
As shown in Fig. 5A, treatment with bortezomib had a significant inhibitory effect on Huh-7 tumor after 2 weeks of treatment ($P < 0.05$) and tumor size in the treatment group was only 50% that of control at the end of study. To correlate biological response with the mechanism of action identified *in vitro*, the effect of bortezomib on P-Akt in Huh tumor was examined by Western immunoblotting. Figure 5A shows Western blots of Akt and P-Akt in the homogenates of three representative Huh-7 tumors. Overall, a significant decrease in P-Akt was noted in Huh7 tumors treated with bortezomib.

In contrast, treatment with bortezomib did not show a significant inhibitory effect in PLC5 tumor. Mice bearing PLC5 tumor were treated with the same dose and schedule as mice with Huh-7 tumor. As shown in Fig. 5B, bortezomib treatment was unable to significantly reduce the size of PLC5 tumor. In line with the data on tumor growth, bortezomib showed a marginal effect on P-Akt in PLC5 tumor.

Discussion

Although bortezomib has clinically proven activity in hematologic malignancies such as multiple myeloma and mantle cell lymphoma, development toward clinical use of bortezomib in solid tumor has been slow and the results have not been promising (8–10, 26). The findings suggest that the antitumor mechanism of bortezomib may be different from that in blood malignancies. This study provides new information about the apoptotic effects of bortezomib in HCC cells. In all four HCC cell lines studied, bortezomib at clinically relevant doses induced apoptosis in a concentration-dependent manner in most of the HCC cell lines tested but not in resistant PLC5 cells. Bortezomib-induced apoptosis is associated with the activation of caspase-9, caspase-3, and PARP cleavage, suggesting that a mitochondria signaling pathway is important here, which is consistent with previous reports (27, 28). Our findings are also consistent with differential

Figure 3. Differential effects of bortezomib on the P-Akt status of four HCC cell lines. *A, left*, Western blot analysis of Akt (Akt1) and P-Akt (Ser⁴⁷³) levels in HCC cells treated with bortezomib at the indicated doses for 24 h. Representative of three independent experiments. *Right*, immunoblots were scanned by a UVP BioSpectrum AC image system and quantitated using VisionWork LS software to determine the ratio of the level of P-Akt to Akt. *Columns*, mean ($n = 3$); *bars*, SD. *B*, time- and dose-dependent analysis of P-Akt level and apoptotic death in Huh-7 cells. Huh-7 cells were exposed to bortezomib at the indicated concentrations for 8, 16, and 24 h. Cell lysates were prepared and assayed for P-Akt (Ser⁴⁷³), Akt, caspase-9, and PARP. *C*, protein levels of the PI3K signaling pathway in bortezomib-treated Huh-7 cells. Huh-7 cells were exposed to bortezomib at the indicated doses for 24 h. Cell lysates were prepared and assayed for individual protein levels by Western blot. *D*, effects of bortezomib on protein levels of mTORC2, including P-mTOR (Ser²⁴⁴⁸), mTOR, Rictor, and Sin1, in Huh-7 and PLC5 cells. Cells were exposed to bortezomib at the indicated doses for 24 h. Cell lysates were assayed by Western blot.



responses to bortezomib, which have been shown recently in many types of cancer such as lung (29), prostate (30), breast (20), and B-cell lymphoma (21, 31).

In this study, we show that whereas bortezomib inhibits proteasome activity in both Huh7 and PLC5 cells, their sensitivities to bortezomib are strikingly different. These results might indicate that the differential effect of bortezomib on apoptosis in HCC cells is not associated with proteasome inhibition activity, consistent with a previous study on breast cancer (20). However, the possibility that other uncertain defects of proteasome downstream exist in PLC5 cells could not be ruled out. Although a large body of the underlying mechanism accounting for the resistance of PLC5 cells to bortezomib remains to be uncovered, further studies are important for the application of bortezomib.

Numerous studies have indicated that bortezomib-induced cell cycle arrest and apoptosis through inhibition of the NF- κ B signaling pathway is a sequel of proteasome inhibition (7, 12, 32). However, in the present study, bortezomib did not show significant activity on the NF- κ B signaling pathway, including the DNA binding activity and nuclear translocation of p65 in both sensitive Huh-7 and PLC5 cells. This result supports that the apoptotic effect of bortezomib on HCC cells is independent of the inhibition of NF- κ B activation. Other studies have also shown similar NF- κ B-independent phenomena in various types of tumors (20, 21, 28, 33).

Besides NF- κ B, tumor suppressor p53 has been shown to be a target of bortezomib, and bortezomib was more sensitive in lung cells with functional p53 (29). The four HCC cell lines used in this

study vary with respect to their p53 functional status. Huh7 cells express a codon 220-mutant p53, and PLC5 cells express a codon 139/161- and codon 249-mutated p53, whereas Hep3B is a p53-null cell line. Sk-hep1 has homozygous deletion of p14^{ARF} in the p53 pathway (34). Whereas all four HCC lines bear different types of p53 mutation, unexpectedly, bortezomib still induced differential apoptotic activities among them. It might be important to ask whether different types of p53 mutation are associated with the sensitivity of HCC cells to bortezomib, particularly, about the fact that p53 protein is frequently mutated in HCC. Recently reports show that mutant p53 might not only lose its normal biological

functions but acquire some uncertain oncogenic functions, a process of "gain of function," which might be involved in tumor progression and drug resistance of tumor cells (35). Therefore, it is very possible that different kinds of p53 mutation in HCC cells might be an important factor in cellular response to bortezomib. Further studies on the manipulation of *p53* gene, with wild-type or different types of mutation, in HCC cells are expected to provide a clearer picture of the relationship between p53 status and the sensitivity of tumor cells toward bortezomib.

Examination of mitochondria-related apoptotic proteins revealed no significant alterations between sensitive Huh-7 and

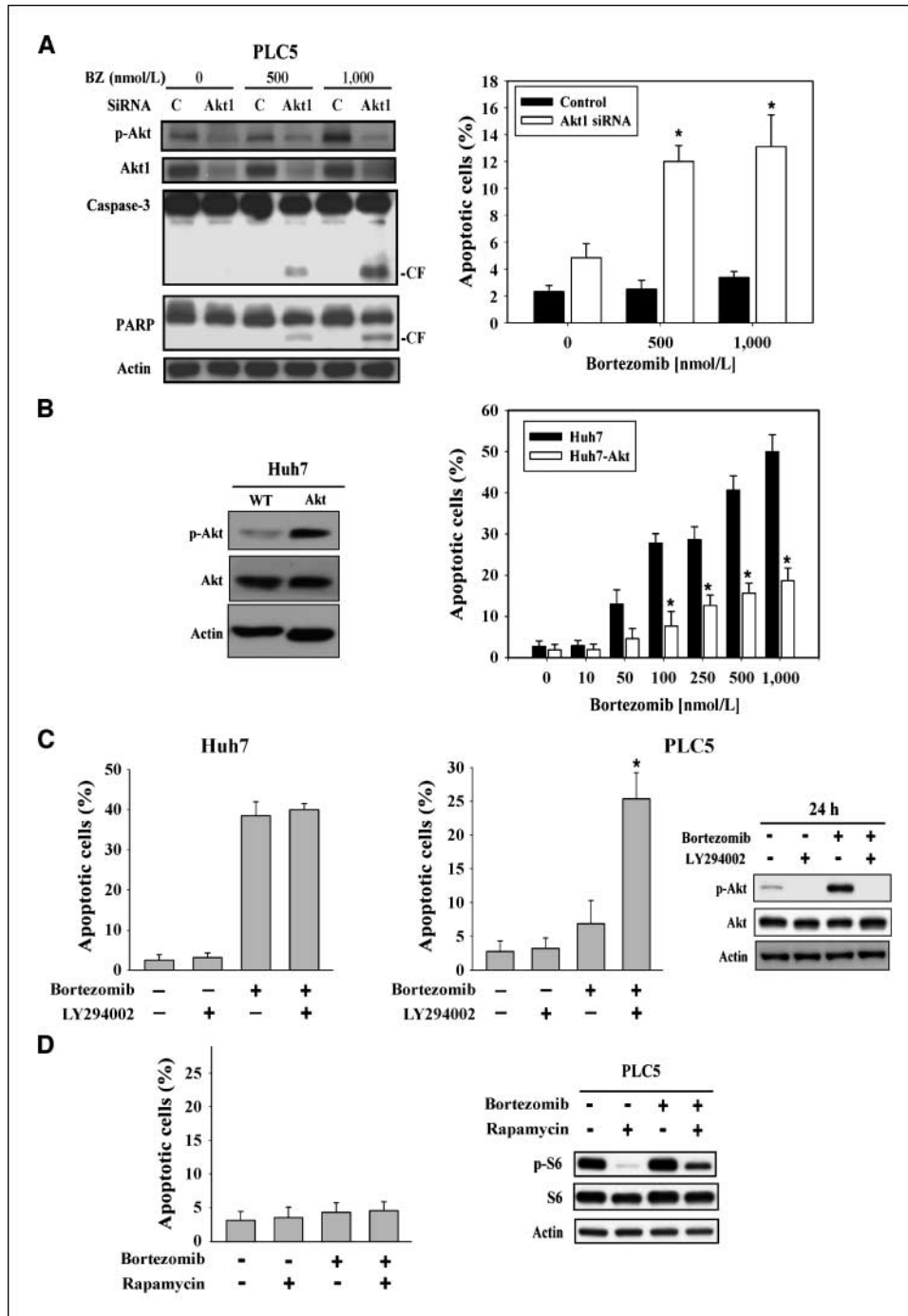
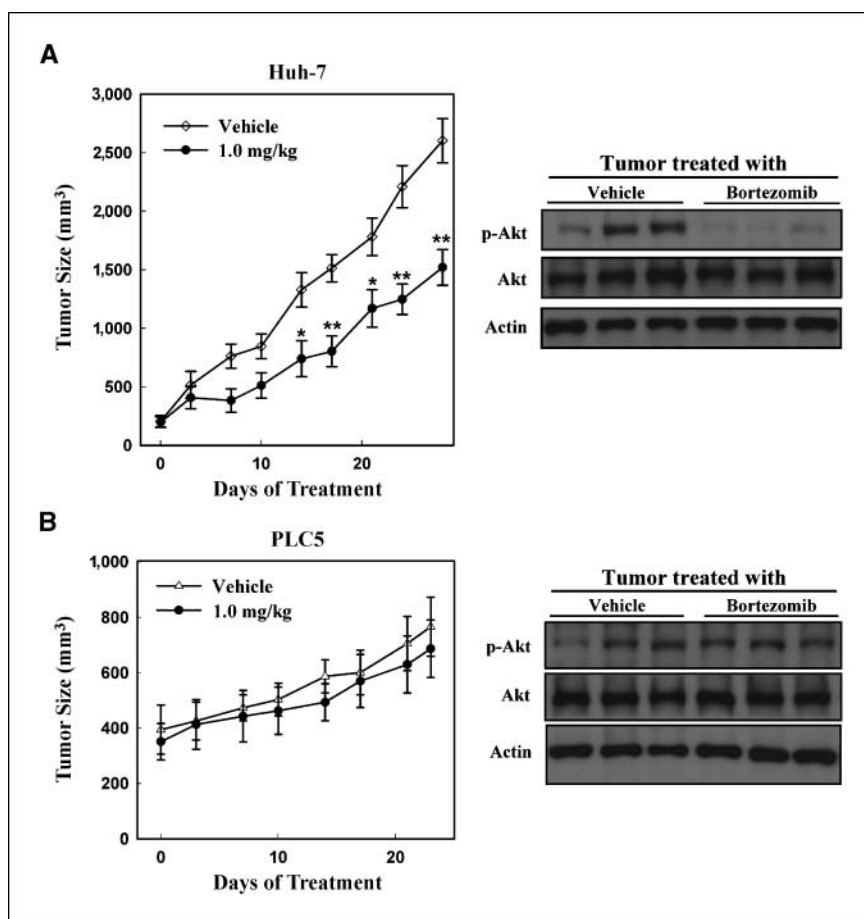


Figure 4. *In vitro* target validation. **A**, down-regulation of Akt (Akt1) by siRNA overcomes the resistance to bortezomib in PLC5 cells. *Left*, protein levels of P-Akt (Ser⁴⁷³), Akt, caspase-3, and PARP. *Right*, analysis of apoptotic cells. *Columns*, mean ($n = 3$); *bars*, SD. *, $P < 0.01$. Cells were transfected with either control siRNA or Akt1 siRNA for 48 h and then were exposed to bortezomib for 24 h. To determine protein expressions, cell lysates were prepared and assayed by Western blotting. For analysis of apoptotic cells (sub-G₁), cells were analyzed by flow cytometry. **B**, protective effect of constitutive Akt on bortezomib-induced apoptosis in Huh7 cells. *Left*, protein levels of P-Akt (Ser⁴⁷³) and Akt (Akt1) in both wild-type and Huh7-Akt cells. *WT*, wild-type Huh7. *Right*, analysis of apoptotic cells. *Columns*, mean ($n = 6$); *bars*, SD. *, $P < 0.05$. Huh7 cells were transfected with constitutive Akt1 and were selected for 8 wk by G-418. Analysis of apoptotic cells was done by flow cytometry after cells were exposed to the indicated concentrations of bortezomib for 24 h. **C**, apoptotic effects of LY294002, a PI3K inhibitor, in Huh-7 and PLC5 cells. Analysis of apoptotic cells. *Right*, protein levels of P-Akt (Ser⁴⁷³) and Akt (Akt1) in PLC5 cells. *Columns*, mean ($n = 6$); *bars*, SD. *, $P < 0.05$. Cells were treated with 500 nmol/L bortezomib and/or 25 μ mol/L LY294002 for 24 h. **D**, apoptotic effects of rapamycin, an inhibitor of mTOR, in PLC5 cells. *Left*, analysis of apoptotic cells. *Right*, protein levels of P-S6 (Ser^{235/236}) and S6 (S6 ribosomal protein) in PLC5 cells. *Columns*, mean ($n = 6$); *bars*, SD. Cells were treated with 500 nmol/L bortezomib and/or 100 nmol/L rapamycin for 24 h.

Figure 5. *In vivo* effect of bortezomib on Huh-7 and PLC5 xenograft nude mice. **A**, bortezomib shows antitumor effect on Huh-7 tumors. *Left*, points, mean ($n = 6$); bars, SE. *, $P < 0.05$; **, $P < 0.01$. *Right*, Western blot analysis of P-Akt and Akt in Huh-7 tumors. **B**, bortezomib did not show a significant antitumor effect on PLC5 tumors. *Left*, points, mean ($n = 6$); bars, SE. *Right*, Western blot analysis of P-Akt and Akt in PLC5 tumor. Each mouse was inoculated s.c. in the dorsal flank with 1×10^6 Huh-7 or PLC5 cells suspended in Matrigel. When the tumor reached a volume of $\sim 200 \text{ mm}^3$, mice received treatments of vehicle or 1.0 mg/kg bortezomib i.p. twice weekly for the duration of the study. For analysis of Akt and P-Akt levels, the homogenates of three representative tumors treated with vehicle or bortezomib for 24 d were analyzed by Western blot.



resistant PLC5 cells, whereas Bax degradation and Bik accumulation have previously been shown in bortezomib-treated cancer cells (36). A mild decrease of Bcl-2 protein level in Huh-7 was observed in cells treated with high doses of bortezomib in this study. However, this reduction was not dose dependent and a similar effect was not observed in Hep3B and Sk-Hep1 (data not shown), indicating that this effect on Bcl-2 is not a major event for all HCC cells. In addition, a recent study showed that Mcl-1 is a key target for bortezomib-induced apoptosis in myeloma cells (37). However, our results show that bortezomib increased Mcl-1 levels in both sensitive and resistant cells, suggesting that alterations of Mcl-1 should not be a major event involved in apoptosis induction.

In the present study, down-regulation of P-Akt was shown to occur in a time- and dose-dependent manner after bortezomib treatment of all sensitive HCC cells. Several studies have reported that bortezomib applied solely in breast cancer cells (20, 38), having synergy with sorafenib in various types of cancer cells (39), or acting as a radiosensitizer in head and neck cancer cells can also alter Akt activity (40). Importantly, the PI3K/Akt signaling pathway plays a major role in carcinogenesis and drug resistance in numerous types of cancer including HCC (41). 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 (42, 43). Notably, the activation of PI3K signaling including PTEN mutation has been observed in 40% to 50% of HCC tumors (2, 43), indicating the importance of Akt signaling in HCC.

With respect to the mechanism by which bortezomib affects the level of P-Akt, our results showed that bortezomib reduced P-Akt, but not total Akt, in drug-treated cells, which suggests at least two putative mechanisms. First, bortezomib may down-regulate P-Akt by decreasing upstream kinase signaling, such as PDK1 and PI3K, or increasing the activity of phosphatase, PTEN. In this regard, our results show that protein levels of these upstream proteins do not change significantly after bortezomib treatment although kinase activities were not examined in this study. Second, bortezomib may increase the activity of protein phosphatases, which can reduce the phosphorylation status of P-Akt. Recent studies have shown that PHLPP is a specific protein phosphatase for P-Akt at Ser⁴⁷³ (44, 45). We found that bortezomib did not alter the expression levels of PHLPP in either Huh-7 or PLC5 cells (data not shown), suggesting that PHLPP is not a target for down-regulating P-Akt in bortezomib-treated HCC cells. Furthermore, inhibitors of other nonspecific phosphatases, such as protein phosphatase 1 and protein phosphatase 2A, seem to be able to restore down-regulation of P-Akt in bortezomib-treated Huh-7 cells (our preliminary data), indicating that bortezomib might reduce P-Akt in association with the activation of protein phosphatases. This result may be supported by a recent study that indicated that the proteasome inhibitor MG-132 down-regulates endothelial nitric oxide synthase and Akt phosphorylation by inducing intracellular protein phosphatase 2A translocation (46). Further study of related mechanisms of bortezomib on protein phosphatases is needed. The underlying

mechanism of the constitutive activation of Akt in bortezomib-treated PLC5 cells remains unknown. One possible explanation is that the protein phosphatases for Akt dephosphorylation are defective in PLC5 cells, although it remains to be identified. On the other hand, a distinct compensatory survival pathway that activates Akt might be induced by bortezomib in PLC5 cells. This hypothesis is supported by several recent studies on mTOR inhibitors. For example, RAD001, a mTOR inhibitor, induces Akt phosphorylation at Ser⁴⁷³ by activating the insulin-like growth factor I receptor signaling pathway (47, 48).

Combinational therapy for molecular targeted therapy has been a common approach to improve responsiveness in cancer therapy (5, 49, 50). This study revealed that targeting the Akt signaling pathway overcomes drug resistance to bortezomib in PLC5 cells. This may provide a promising strategy for designing clinical trials of bortezomib in patients with HCC. In addition, evidence indicates that the major hindrance of molecular targeted therapy in solid tumors, especially HCC, is very low response rate and high costs (5, 50). However, patients are more tolerant to targeted therapies compared with traditional chemotherapeutic agents

(5, 49). Hence, the ability to select patients with a high response rate by detecting drug-related biomarkers is important to improve clinical efficacy. In this regard, this study has shown that P-Akt status is a good biomarker for predicting the response of HCC tumors to bortezomib, which may be useful in future clinical investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/22/2008; revised 5/21/2008; accepted 6/3/2008.

Grant support: Grants NTUH95A11 and NTUH96A11 from National Taiwan University; grant NSC95-2314-B-002-072 from National Science Council, Taiwan, ROC; grant NTU95R0066-BM-1-02 from National Taiwan University; and grants DOH94-TD-B-111-001 and DOH95-TD-B-111-001 from Department of Health, Executive Yuan, ROC.

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.

We thank Prof. Pei-Jer Chen for supporting this study and revising the manuscript.

References

- Kao JH, Chen DS. Changing disease burden of hepatocellular carcinoma in the Far East and Southeast Asia. *Liver Int* 2005;25:696-703.
- El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;132:2557-76.
- Chang MH, Chen CJ, Lai MS, et al. Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med* 1997;336:1855-9.
- Schwartz M, Roayaie S, Konstadoulakis M. Strategies for the management of hepatocellular carcinoma. *Nat Clin Pract Oncol* 2007;4:424-32.
- Pang RW, Poon RT. From molecular biology to targeted therapies for hepatocellular carcinoma: the future is now. *Oncology* 2007;72 Suppl 1:30-44.
- Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* 2004;4:349-60.
- Voorhees PM, Orłowski RZ. The proteasome and proteasome inhibitors in cancer therapy. *Annu Rev Pharmacol Toxicol* 2006;46:189-213.
- Papandreou CN, Daliani DD, Nix D, et al. Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer. *J Clin Oncol* 2004;22:2108-21.
- Shah MH, Young D, Kindler HL, et al. Phase II study of the proteasome inhibitor bortezomib (PS-341) in patients with metastatic neuroendocrine tumors. *Clin Cancer Res* 2004;10:6111-8.
- Davis NB, Taber DA, Ansari RH, et al. Phase II trial of PS-341 in patients with renal cell cancer: a University of Chicago phase II consortium study. *J Clin Oncol* 2004;22:115-9.
- Orłowski RZ, Baldwin AS, Jr. NF- κ B as a therapeutic target in cancer. *Trends Mol Med* 2002;8:385-9.
- Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 2001;61:3071-6.
- LeBlanc R, Catley LP, Hideshima T, et al. Proteasome inhibitor PS-341 inhibits human myeloma cell growth *in vivo* and prolongs survival in a murine model. *Cancer Res* 2002;62:4996-5000.
- Lopes UG, Erhardt P, Yao R, Cooper GM. p53-dependent induction of apoptosis by proteasome inhibitors. *J Biol Chem* 1997;272:12893-6.
- Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 2003;22:8590-607.
- Ling YH, Liebes L, Ng B, et al. PS-341, a novel proteasome inhibitor, induces Bcl-2 phosphorylation and cleavage in association with G₂-M phase arrest and apoptosis. *Mol Cancer Ther* 2002;1:841-9.
- Breitschopf K, Zeiher AM, Dimmeler S. Ubiquitin-mediated degradation of the proapoptotic active form of bid. A functional consequence on apoptosis induction. *J Biol Chem* 2000;275:21648-52.
- Shiau CW, Yang CC, Kulp SK, et al. Thiazolidinediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPAR γ . *Cancer Res* 2005;65:1561-9.
- Kulp SK, Yang YT, Hung CC, et al. 3-phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. *Cancer Res* 2004;64:1444-51.
- Codony-Servat J, Tapia MA, Bosch M, et al. Differential cellular and molecular effects of bortezomib, a proteasome inhibitor, in human breast cancer cells. *Mol Cancer Ther* 2006;5:665-75.
- Strauss SJ, Higginbottom K, Juliger S, et al. The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. *Cancer Res* 2007;67:2783-90.
- Brignole C, Marimpietri D, Pastorino F, et al. Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis. *J Natl Cancer Inst* 2006;98:1142-57.
- Zheng B, Georgakis GV, Li Y, et al. Induction of cell cycle arrest and apoptosis by the proteasome inhibitor PS-341 in Hodgkin disease cell lines is independent of inhibitor of nuclear factor- κ B mutations or activation of the CD30, CD40, and RANK receptors. *Clin Cancer Res* 2004;10:3207-15.
- Sarbasov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307:1098-101.
- Jacinto E, Facchinetti V, Liu D, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 2006;127:125-37.
- Maki RG, Kraft AS, Scheu K, et al. A multicenter phase II study of bortezomib in recurrent or metastatic sarcomas. *Cancer* 2005;103:1431-8.
- Voortman J, Checinska A, Giaccone G, Rodriguez JA, Krut FAE. Bortezomib, but not cisplatin, induces mitochondria-dependent apoptosis accompanied by up-regulation of noxa in the non-small cell lung cancer cell line NCI-H460. *Mol Cancer Ther* 2007;6:1046-53.
- Zhu H, Zhang L, Dong F, et al. Bik/NBK accumulation correlates with apoptosis-induction by bortezomib (PS-341, Velcade) and other proteasome inhibitors. *Oncogene* 2005;24:4993-9.
- Ling Y-H, Liebes L, Jiang J-D, et al. Mechanisms of proteasome inhibitor PS-341-induced G₂-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clin Cancer Res* 2003;9:1145-54.
- Williams S, Pettaway C, Song R, Papandreou C, Logothetis C, McConkey DJ. Differential effects of the proteasome inhibitor bortezomib on apoptosis and angiogenesis in human prostate tumor xenografts. *Mol Cancer Ther* 2003;2:835-43.
- Shringarpure R, Catley L, Bhole D, et al. Gene expression analysis of B-lymphoma cells resistant and sensitive to bortezomib. *Br J Haematol* 2006;134:145-56.
- Sunwoo JB, Chen Z, Dong G, et al. Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor- κ B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clin Cancer Res* 2001;7:1419-28.
- Kashkar H, Deggerich A, Seeger J-M, et al. NF- κ B-independent down-regulation of XIAP by bortezomib sensitizes HL B cells against cytotoxic drugs. *Blood* 2007;109:3982-8.
- Erdal E, Ozturk N, Cagatay T, Eksioğlu-Demiralp E, Ozturk M. Lithium-mediated down-regulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells. *Int J Cancer* 2005;115:903-10.
- Strano S, Dell'Orso S, Di Agostino S, Fontemaggi G, Sacchi A, Blandino G. Mutant p53: an oncogenic transcription factor. *Oncogene* 2007;26:2212-9.
- Li B, Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. *Proc Natl Acad Sci U S A* 2000;97:3850-5.
- Podar K, Gouill SL, Zhang J, et al. A pivotal role for Mcl-1 in Bortezomib-induced apoptosis. *Oncogene* 2007;27:721-31.
- Shi YY, Small GW, Orłowski RZ. Proteasome inhibitors induce a p38 mitogen-activated protein kinase (MAPK)-dependent anti-apoptotic program involving MAPK phosphatase-1 and Akt in models of breast cancer. *Breast Cancer Res Treat* 2006;100:33-47.
- Yu C, Friday BB, Lai JP, et al. 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. *Mol Cancer Ther* 2006;5:2378-87.
40. Weber CN, Cerniglia GJ, Maity A, Gupta AK. Bortezomib sensitizes human head and neck carcinoma cells SQ20B to radiation. *Cancer Biol Ther* 2007;6:156-9.
41. Cully M, You H, Levine AJ, Mak TW. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 2006;6:184-92.
42. Nakanishi K, Sakamoto M, Yamasaki S, Todo S, Hirohashi S. Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis in hepatocellular carcinoma. *Cancer* 2005;103:307-12.
43. Hu TH, Huang CC, Lin PR, et al. Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. *Cancer* 2003;97:1929-40.
44. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell* 2005;18:13-24.
45. Brognard J, Sierceki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Mol Cell* 2007;25:917-31.
46. Wei Q, Xia Y. Proteasome inhibition down-regulates endothelial nitric-oxide synthase phosphorylation and function. *J Biol Chem* 2006;281:21652-9.
47. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006;66:1500-8.
48. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 2007;26:1932-40.
49. Avila MA, Berasain C, Sangro B, Prieto J. New therapies for hepatocellular carcinoma. *Oncogene* 2006;25:3866-84.
50. Abou-Alfa GK. Hepatocellular carcinoma: molecular biology and therapy. *Semin Oncol* 2006;33:S79-83.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Down-regulation of Phospho-Akt Is a Major Molecular Determinant of Bortezomib-Induced Apoptosis in Hepatocellular Carcinoma Cells

Kuen-Feng Chen, Pei-Yen Yeh, Kun-Huei Yeh, et al.

Cancer Res 2008;68:6698-6707.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/68/16/6698>

Cited articles This article cites 50 articles, 24 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/68/16/6698.full.html#ref-list-1>

Citing articles This article has been cited by 19 HighWire-hosted articles. Access the articles at:
</content/68/16/6698.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.