Hepatitis B virus X protein (HBx) is responsible for resistance to targeted therapies in hepatocellular carcinoma: ex vivo culture evidence

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Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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Statement of translational relevance

Proteasome inhibitors (PIs) such as bortezomib are used extensively in the clinical treatment of hematologic malignancies and exhibit antitumor effects against a variety of solid neoplasm, including hepatocellular carcinoma (HCC). Hepatitis B virus-related HCC (HBV-HCC) accounts for approximately 50% of all HCC cases. In this study, we demonstrate that the HBx protein causes resistance to bortezomib in HBV-related HCC, and this resistance can be antagonized by an inhibitor of MEK signaling, as well as the mechanism of resistance and synergistic anti-tumor effects using both ex vivo and in vivo models. Our ex vivo culture system allows for the assessment of the anti-tumor effect of drugs in a non-tumoral microenvironment. The combination of ex vivo and in vivo models facilitates the evaluation of drug resistance and efficacy, laying the foundation for a novel clinical trial.
Abstract

Purpose: Molecular targeted therapy is an important approach for advanced hepatocellular carcinoma (HCC). Hepatitis B virus-related HCC (HBV-HCC) accounts for approximately 50% of all HCC cases. Bortezomib, a proteasome inhibitor, is used extensively for the treatment of hematologic malignancies, but its application in HCC, particularly in HBV-HCC, has not been fully explored.

Experimental Design: The effects of bortezomib on HCC tissues were evaluated by TUNEL assays. The growth inhibitory activity was measured using cell viability assays, and apoptosis was measured using flow cytometry. The levels of HBx, P-Raf/Raf and P-Erk/Erk expression were measured by Western blot. The ability of the MEK inhibitor PD98059 to enhance the cell killing activity of bortezomib was evaluated using ex vivo and in vivo methods.

Results: The potency of bortezomib varied among HCC samples and cell lines, and HBV/HBx expression was associated with resistance to bortezomib. Bortezomib increased the levels of P-Raf and P-Erk in HBV/HBx-positive cells but not in HBV/HBx-negative HCC cells or in breast cancer or glioblastoma multiform cells. HBx was also upregulated after exposure to bortezomib, which was associated with the inhibition of proteasome activity. P-Erk upregulation mediated by bortezomib was effectively suppressed by the addition of the MEK inhibitor PD98059. Moreover, bortezomib and PD98059 synergistically inhibited HCC cell proliferation, as measured using both ex vivo and in vivo models.

Conclusions: Our studies demonstrate for the first time that HBx causes resistance to
bortezomib in HCC, and this resistance can be antagonized by a MEK signaling inhibitor, providing a novel therapeutic approach.
Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent type of tumor and the second most common cause of cancer-related death worldwide (1). Surgery is currently the best therapeutic choice, but it is limited to early-stage disease. The responses to traditional chemotherapy are quite poor for those patients with advanced-stage HCC (2). Thus, novel drugs and therapeutic strategies are urgently needed.

Human hepatitis B virus (HBV) infection is a global health problem, and HBV infection plays an important role in the pathogenesis of cirrhosis and HCC (3), with 350 million individuals chronically infected with HBV (4). Hepatitis B virus-related HCC (HBV-HCC) accounts for approximately 50% of all HCC cases (5). Several HBV factors, including the HBx gene, the pre-S2/S gene and the HBV spliced protein, have been implicated in the progression and prognosis of liver cancer (6). HBx, which is encoded by the fourth open reading frame of the HBV genome, is regularly detected in the tumors of patients with HBV-HCC (7, 8), and a few studies have demonstrated that both HBx RNA and protein expression are present in human HCC cells in the absence of HBV replication (9, 10). The activation of signaling cascades Ras/Raf/MAPK by HBx is important in HBV-associated pathogenesis (11, 12). Taken together, these findings demonstrate that HBx plays a key role in the molecular pathogenesis of HBV-HCC (13).

Bortezomib (Velcade/PS-341), a proteasome inhibitor, has been approved since 2003 by the USA Food and Drug Administration (FDA) for the treatment of refractory
multiple myeloma and mantle cell lymphoma (14). As such, most clinical trials of bortezomib have focused on hematological malignancies (15, 16). Recently, proteasome inhibitors (PIs) were noted to have antitumor effects against diverse solid neoplasms, including HCC (17). In hematologic malignancies, bortezomib stabilized IKB, resulting in decreased NF-κB activity (18, 19). Studies of HCC indicated that bortezomib downregulated the level of phospho-Akt (P-Akt) (20). Bortezomib was also shown to differentially affect the expression of E2F1, p21 and p27 (21) and to mediate a specific dual antitumor effect via NK cell antitumor reactivity (22). An international, multicenter phase II trial (23) and several preclinical studies (24-26) of bortezomib in HCC patients have been reported. However, to our knowledge, no studies have focused on the relationship between bortezomib and HBV-related HCC (HBV-HCC).

In this study, the effects of bortezomib were investigated in HBV/HBx-positive and HBV/HBx-negative HCC clinical samples and cell lines. We discovered that the upregulation of HBx is one of the mechanisms that mediate bortezomib resistance in HBV-HCC. We provide evidence that combining bortezomib with the MAPK signaling inhibitor PD98059 can overcome this resistance and suggest that this combination may be a novel approach for the treatment of HBV-HCC.

**Materials and methods**

**Reagents.** Bortezomib, GW5074 and PD98059 were purchased from Selleck Chemicals. All of these reagents were dissolved in dimethyl sulfoxide (DMSO) and
then added to cells in culture medium. The final DMSO concentration was 0.1% after addition to the medium.

**Ex vivo culture of clinical HCC samples.** Liver cancer tissue samples were obtained from surgical specimens of either liver segmental resections or hemihepatectomies. The procedures were approved by the ethical committee of the Sun Yat-Sen Memorial Hospital. The clinical characteristics of the tumors used in this study are detailed in Supplemental Table 1. Among the patients sampled, 10 exhibited HBV-related HCC, and 10 were HBV negative. An 8-mm core of tissue was dissected into 1-mm³ pieces and cultured in a static incubating system using 24-well plastic tissue culture plates containing 500 μL DMEM with 10% FBS and penicillin (100 U/mL)/streptomycin (100 mg/mL) at 37°C with saturated humidity and 5% CO₂. Following a resting phase on a rotating platform (60 rpm) for 2 hours, drug exposure was initiated. After treatment, the tissues were either formalin fixed and paraffin embedded for immunohistochemistry or lysed with RIPA buffer for Western blot (Supplemental Fig. 1A). HE and Ki-67 staining were used to assess the viability of the ex vivo cultured tissues (Supplemental Figs. 1B, C).

**Transferase-mediated dUTP nick end-labeling (TUNEL) assay.** The TUNEL assay was used to detect the apoptotic cells in tissues with the Cell Death Detection Kit (Roche, United States). Chamber slides were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 and then incubated with the TUNEL reaction mixture for 1 hour at 37°C. After the slides were washed with PBS, they were incubated with peroxidase-conjugated antibody for 30 min at 37°C and were
developed with the DAB system. The apoptosis index was calculated by counting the number of brown-stained nuclei, and the total number of cells measured by light microscopy represented at least 10 fields at ×40 magnification submitted to investigator-blinded counting. The results are expressed as a percentage of the number of apoptotic cells/total cells.

**Cell lines, stable infected cell lines, and plasmids.** The human HCC cell lines HepG2 (HBV-negative), Huh7 (HBV-negative), Sk-Hep1 (HBV-negative), PLC/PRF/5 and Hep3B (derived from HBV-infected liver) were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (CBTCCCAS). HepG2.2.15 (HepG2-derivative with integration of the HBV genome) (27, 28) was purchased from BioHermes. All cells were obtained in 2013 directly from either CBTCCCAS or BioHermes, which guaranteed cell line authenticity through short tandem repeat profiling and comparison to known cell line DNA profiles. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) under an atmosphere of 5% CO₂.

Three HCC cell lines (HepG2, Huh7, and Sk-Hep1) were selected to generate stable cell lines in this study. A retroviral packaging system was purchased from Clontech. Retroviral vector pMSCV-eGFP inserted with HBx-HA was generated according to the procedure described in the manufacturer’s instructions. These retroviruses were used to infect HepG2, Huh7, and Sk-Hep1 cells in the presence of 10 μg/mL Polybrene (Sigma-Aldrich) followed by selection with 1 μg/mL of puromycin (Calbiochem) for two weeks.
An expression plasmid containing Flag-tagged ubiquitin (Ub-Flag) for use in the in vivo ubiquitination assays was constructed as previously described (29). The pcDNA3.1-HBx-HA plasmid was obtained by inserting HBx-HA into pcDNA3.1.

**Analysis of cell viability and apoptosis.** The effects of bortezomib and PD98059 on hepatoma cell viability were assessed with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). In brief, 3,000 cells were seeded in 96-well culture plates and incubated overnight, followed by the addition of vehicle, bortezomib, PD98059 or a combination of bortezomib and PD98059 for 48 hours; then, they were incubated with CCK-8 reagent for 1 hour at 37°C. The staining intensity in the medium was measured at 450 nm. Cytotoxicity was expressed as a percentage of the control cells. The values were expressed as the means ± standard deviation (SD) of three separate experiments, each performed in triplicate.

After being incubated with the drugs for 48 hours, adherent cells were collected and washed once with PBS. The cells were stained with Annexin V (AV) and propidium iodide (PI) (BD Pharmingen TM, Heidelberg, Germany), and the percentage of Annexin V-positive cells was calculated by flow cytometry.

**Determination of synergistic activity.** The synergistic activity of bortezomib and MEK inhibitor was determined by the method described by Chou TC and Talalay P (30) using CalcuSyn software (Biosoft, Cambridge, United Kingdom). A combination index (CI) less than 1 was defined as synergy.

**Western blot analysis.** Cell cytosolic protein fractions were prepared using RIPA buffer (Beyotime Biotechnology, Haimen, China). For the Western blots,
antibodies against c-Raf, Phospho-c-Raf, Flag (Cell Signaling Technology), Erk, P-Erk, GAPDH, HA (Santa Cruz Biotechnology Inc. Santa Cruz, CA, US) and HBx (ab2741, Abcam) were used. Quantitative increases in protein phosphorylation were evaluated with a densitometry analysis of the ratio of phosphorylated protein/total protein of the treated cells. All Western blots were repeated 2-3 times, and the mean increases in the drug-treated groups versus non-treated cells are shown under the gels in the figures.

HBx knockdown using short interfering RNA. HBx RNA interference was achieved by specific small interfering RNA (siRNA) duplexes. Sense primers targeting HBx and a negative control were purchased from GenePharma Company (Shanghai, China). The sequences of the small interfering RNAs for HBx were as follows: si#1 GAGGCUGUAGGCAUAAAUU, si#2 GCACUUCGCUUCACCUCUG. siRNAs were transfected with the Lipofectamine 2000 transfection reagent (Invitrogen Corporation, Carlsbad, California) according to the manufacturer’s protocol. The knockdown efficiency was validated by Western blot analysis.

In vivo ubiquitination assay and cycloheximide chase assay. This procedure was performed as previously described (29). Briefly, HepG2 cells were transfected with the indicated plasmids for 24 hours and then treated with either 10 μmol/L MG132 or 150 nmol/L bortezomib for 6 hours prior to harvesting the cells. The cells were lysed in RIPA buffer with a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Calbiochem). The cell lysis solution was
immunoprecipitated using anti-HA agarose for 4 hours at 4°C. Polyubiquitinated HBx was detected using an anti-Flag antibody. For the cycloheximide (CHX) chase assay, cells were treated with 20 μg/mL CHX for 0, 0.5, 1, and 2 hours; lysed; and analyzed by Western blot.

**Xenograft tumor growth.** Animal experiments were approved by the Animal Research Committee of Sun Yat-Sen University and were performed in accordance with established guidelines. A total of 1×10⁶ cells were suspended in 200 μL serum-free DMEM and injected into the right flanks of 4-week-old BALB/c athymic nude mice. The mice received intraperitoneal injections of bortezomib (0.5 mg/kg, twice weekly), PD98059 (10 mg/kg, once daily), or a mixture of bortezomib and PD98059. The control mice received 200 μL of 0.1% DMSO. Once palpable tumors were observed, measurements of tumor volume were taken every 3 days using calipers. The tumor volume was calculated using the following formula: \( V = (\text{width}^2 \times \text{length}) \times 0.5 \) (31). Body weights were also recorded. Tumor samples from the mice were collected and analyzed by Western blot.

**Statistical analysis.** Data were expressed as the mean ± SD. Comparisons of the mean values were performed with SPSS 16.0 software using a 2-tailed Student \( t \) test or one-way ANOVA with Bonferroni post hoc corrections for multiple comparisons.

**RESULTS**

Influence of HBV/HBx after treatment with bortezomib using an *ex vivo* culture model and HCC cell lines.
To investigate the antitumor effect of bortezomib in HBV-related and non-HBV-related HCC, an ex vivo culture model was developed. Ten non-HBV-related HCC and ten HBV-related HCC tissues were treated with bortezomib at 250 nmol/L (data from Millennium stated that following the intravenous administration of 1.3 mg/m² doses, the C_max was 148 to 312 nmol/L). Apoptosis was measured with a TUNEL assay. Compared with HBV-negative HCC samples (36.86 ± 9.06), the apoptosis rate of HBV-related HCC samples (18.7 ± 6.46) was significantly decreased (p < 0.001) (Fig. 1A). Furthermore, the apoptosis and viability of HepG2 cells (HBV-negative) compared to HepG2.2.15 cells (HepG2-derivative with integration of the HBV genome) (27, 28) were examined. The apoptosis rates were 44.73 ± 5.25% and 29.37 ± 4.51%, respectively, after treatment with 150 nmol/L bortezomib for 48 hours (p < 0.01) (Fig. 1B). The half-maximal inhibitory concentration (IC50) of bortezomib against HepG2.2.15 cells was 383 nmol/L, which was two-fold greater than that against HepG2 cells (IC50, 166 nmol/L) (Fig. 1C). These data strongly suggested that HBV affected the sensitivity of HCC cells to bortezomib.

As HBx plays a key role in the molecular pathogenesis of HBV-related HCC, HCC cell lines (HepG2, Sk-Hep1 and Huh7) were genetically modified to over-express HBx to evaluate the relationship between HBx and bortezomib. HBx protein expression was confirmed by Western blot. Bortezomib exhibited a selective inhibition of HCC cells depending on the expression of HBx (Fig. 1C). The IC50 of HepG2-HBx cells (IC50, 371 nmol/L) was two-fold greater than that of HepG2-Ctrl.
cells (IC50, 178 nmol/L). Similar results were noted with Sk-Hep1-HBx (IC50, 393 nmol/L) and Sk-Hep1-Ctrl (IC50, 192 nmol/L) cells, as well as with Huh7-HBx (IC50, 308 nmol/L) and Huh7-Ctrl (IC50, 146 nmol/L) cells. As HBx can influence cell proliferation (Supplemental Figs. 2A, B), the HepG2-Ctrl and HepG2-HBx cells were cultured in 10% FBS or 1% FBS (Supplemental Fig. 2C, upper panel) and then treated with different concentrations of bortezomib (Supplemental Fig. 2C, lower panel). The proliferation rates in 1% and 10% FBS exhibited obvious differences, whereas the ratio of inhibition of bortezomib was nearly the same, suggesting that bortezomib resistance may not be related to changes in the proliferation rate.

**HBx plays an important role in the resistance of HBV-HCC to bortezomib.**

Based on the above results, the role of HBx in mediating bortezomib resistance was investigated in HBV-HCC. HBx is known to induce a rapid cytoplasmic signaling cascade linking Ras, Raf, and MAP kinases, leading to transcription transactivation (11, 12). The protein levels of both HBx and members of the MAPK signaling pathway increased significantly in a dose- and time-dependent manner after treatment of HepG2.2.15 cells with bortezomib (Fig. 2A). The protein levels of total Raf and Erk did not change in these cells. Bortezomib also upregulated the level of HBx and MAPK activity in the PLC/PRF/5 and Hep3B cells, which were derived from HBV-infected livers containing an integrated hepatitis B virus genome (32-34) (Fig. 2A and Supplemental Fig. 3A). Additionally, HepG2-HBx, Sk-Hep1-HBx and Huh7-HBx cells were cultured with various doses of bortezomib, which also resulted
in the elevation of both HBx and the activated MAPK signal pathway (Fig. 2B and Supplemental Fig. 3B).

In vivid contrast, the HBV/HBx-negative HCC cells (HepG2, Huh7 and Sk-Hep1) (Fig. 3A), the non-HCC cells (breast cancer cell lines: MCF7 and MDA-MB-231; GBM cell lines: U138 and U87) (Fig. 3B) and the non-HBV-related HCC tissues (Fig. 3C) presented either decreased (HepG2, Huh7, Sk-Hep1, MCF7, U138, U87 cells and non-HBV-related HCC samples) or unchanged (MDA-MB-231 cells) levels of P-Raf and P-Erk after treatment with bortezomib.

Furthermore, small interfering RNA (siRNA) against HBx was used to reduce the level of HBx expression in the PLC/PRF/5 and Hep3B cells. The HBx siRNA silenced HBx, as confirmed by Western blot (Fig. 3D, left panel and Supplemental Fig 3C, upper panel). The downregulation of HBx in the cells considerably antagonized the bortezomib-mediated upregulation of P-Raf and P-Erk (Fig. 3D, right panel and Supplemental Fig 3C, lower panel). Taken together, the data support the concept that HBx is an important contributor to the resistance of HBV-HCC to bortezomib.

Bortezomib increased the levels of HBx via inhibition of the ubiquitin/proteasome pathway, and the elevated expression of HBx activated the MAPK pathway.

As HBx is rapidly degraded by the ubiquitin-proteasome pathway in HBV-infected cells (35), we hypothesized that bortezomib (a proteasome inhibitor) would increase the intracellular levels of HBx by inhibiting proteasome activity. To
test this hypothesis, an in vivo ubiquitination assay was performed. In the presence of either bortezomib or MG132 (a specific proteasome inhibitor), HBx protein was ubiquitinated (Fig. 4A). HBx was quickly degraded with a half-life of less than 1 hour, which contributed to the low intracellular levels of this protein in HBV-related HCC samples (Fig. 4B). Moreover, after treatment with MG132, the HBx protein accumulated in PLC/PRF/5 and Hep3B cells (Fig. 4C). Collectively, our results strongly suggested that bortezomib increased the levels of the HBx protein by inhibiting proteasome activity and that the elevated levels of HBx enhanced the phosphorylation (activation) of Raf and Erk. Indeed, when HBx was transiently expressed in HepG2 cells, upregulation of P-Raf and P-Erk was observed (Fig. 4D). Furthermore, HBx-expressing cell lines (Sk-Hep1, Huh7 and HepG2) exhibited elevated levels of P-Raf and P-Erk (Fig. 4E). HepG2.2.15 served as a positive control.

**PD98059 inhibited the MAPK pathway and reduced cell growth synergistically with bortezomib.**

To repress the activation of the MAPK pathway induced by bortezomib in HBx-positive cells, a MAPK pathway inhibitor was combined with bortezomib. The Raf-1 kinase inhibitor GW5074 was not effective in HepG2 cells, and it even slightly increased the levels of P-Raf in PLC/PRF/5 and Huh7 cells (Fig. 5A, upper panel). The MEK inhibitor PD98059 effectively suppressed P-Erk (Fig. 5A, lower panel) and suppressed the upregulation of P-Erk by bortezomib in HBV/HBx-HCC cells (Fig. 5B).
To investigate whether bortezomib plus PD98059 acted synergistically, CalcuSyn software was used to determine the type of interaction that occurred between these agents. In three cell lines, the MEK inhibitor PD98059 had little effect on cell viability when used alone (versus control untreated cells). Compared to each alone, the combination of bortezomib and PD98059 significantly increased the efficacy of treatment (Fig. 5C) [Supplemental Table 2 presents the combination index (CI) observed after treatment with the two drugs and indicates their synergy]. The viability of PLC/PRF/5 after treatment with bortezomib is shown in Supplemental Fig. 4.

Normalized isobolograms for drug combinations applied at varying concentration ratios were also constructed for the three cell lines treated with bortezomib and PD98059 (Fig. 5D). All combination data points were plotted in the synergistic region. Overall, these data indicated that bortezomib and PD98059 interacted synergistically.

**Effect of bortezomib and PD98059 on ex vivo and in vivo models.**

To confirm the synergistic effect of bortezomib and PD98059, their *ex vivo* and *in vivo* activities were examined. The expression of HBx and the phosphorylation of Raf and Erk were increased in all of the HBV-positive samples after treatment with bortezomib, and the addition of PD98059 decreased the levels of P-Erk (Fig. 6A and Supplemental Fig. 5A). The percentage of apoptotic cells as measured by TUNEL assays was 4.8% ± 2.56% in the control group, 19.82% ± 5.43% in the bortezomib group, 8.60% ± 3.12% in the PD98059 group and 41.06% ± 7.56% in the bortezomib and PD98059 group (Fig. 6B). We also investigated the effect of bortezomib and
PD98059 in HBV-negative HCC samples (Supplemental Fig. 5B). No obvious synergistic effects were observed between bortezomib and PD98059 compared with either drug alone.

Tumor growth in PLC/PRF/5 xenografts was significantly inhibited by treatment with bortezomib plus PD98059 versus either drug alone (Figs. 6C, D). Tumor size in the co-treatment group was only one-fourth of that of the control group at the end of the study. Treatment with PD98059 had no significant effect on PLC/PRF/5 tumor growth, and bortezomib alone showed only modest effects. Animal body weight was similar in each treatment group (Supplemental Fig. 6), suggesting no additive toxicity of the two agents. Consistent with our in vitro data, the levels of HBx, P-Raf and P-Erk were significantly upregulated in mice treated with bortezomib alone, and the levels of P-Erk were downregulated in the mice treated with both bortezomib and PD98059 (Fig. 6E). Taken together, the in vitro, ex vivo and in vivo studies demonstrated that the combination of bortezomib and PD98059 had synergistic anti-HCC tumor effects.

Discussion

The overall survival rate for most advanced HCC patients is poor. Chemotherapy for HCC is limited in part because of drug resistance. Bortezomib is clinically effective in hematologic malignancies (14, 36). Recently, proteasome inhibitors exhibited antitumor effects against diverse solid neoplasms, including tumors of the breast, lung, prostate and liver; additionally, laboratory findings have revealed that
combining bortezomib with other drugs may enhance the treatment of HCC (17, 23-26). HBV infection is a major etiologic cause of HCC, with more than 60% of cases in Asia and Africa and at least 20% of HCC cases in Europe, Japan and the USA associated with chronic infection with HBV (5). Research focused on the mechanism of resistance to bortezomib in HBV-HCC is fundamental for the efficient utilization of these drugs for HCC; in addition, these studies provide a roadmap to circumvent drug resistance to other therapeutic agents.

This study demonstrated for the first time an association between HBx and bortezomib resistance in HBV-related HCC via the upregulation of the Ras/Raf/MAPK pathway. Bortezomib combined with a MEK inhibitor (PD98059) resulted in a synergistic anti-tumor effect against HBV-related liver cancer as measured by using in vitro, ex vivo and in vivo models. Ex vivo culture of primary human tissues is widely applied in many research fields, and this strategy provides a bridge between in vitro and in vivo experiments. In our manuscript, tissue was dissected into 1-mm³ pieces; this approach has been used in cancer tissues because it maintains cell viability in both benign and malignant tissues over culture periods of at least 1 week (37, 38). To ensure adequate gas-nutrient-waste-drug exchange in all cells of the liver slices, other methods have also been applied. Ex vivo tissue slices with a thickness of 200-300 µm have also been used, which can maximize tissue consistency and perfusion (39).

HBV plays a crucial role in HCC development. The key viral protein HBx is a multifunctional regulator that modulates transcription, signal transduction, cell cycle
progression, apoptosis, protein degradation and genetic stability through interactions with host factors (40, 41). We noted that HBV caused resistance to bortezomib at clinically achievable concentrations in our *ex vivo* model, and this phenomenon was reconfirmed using HepG2 and HepG2.2.15 cells *in vitro*. Furthermore, HCC cell lines stably transfected with HBx (HepG2-HBx, Huh7-HBx and Sk-Hep1-HBx) exhibited similar resistance to bortezomib. HBx activates several pathways, including the RAS/RAF/MAPK pathway (11, 40, 42), the JAK/STAT pathway (43), and the Wnt/beta-catenin pathway (44). The MAPK pathway is activated directly by HBx, as the protein also enhances both cell proliferation and survival. We showed that bortezomib enhanced both the level of HBx and the phosphorylation of Raf and Erk in HepG2.2.15, PLC/PRF/5 and Hep3B cells (each exhibited integration of the HBV genome) and in HBx-expressing cell lines (genetically modified). In contrast, this cellular pathway was not stimulated by bortezomib either in HBV-negative cell lines (HepG2, Huh7 and Sk-Hep1) or in PLC/PRF/5 and Hep3B cells after silencing their HBx protein (HBx-siRNA). Taken together, these results strongly suggest that HBx plays a crucial role in the resistance to bortezomib in HBV-HCC.

The effect of proteasome inhibition on several protein kinase pathways is variable depending on the tissue type and pathology. The RAS/RAF/MAPK pathway can respond to a wide variety of stress stimuli (45). Proteasome inhibition can increase the phosphorylation of Erk in kidney cancer cells (46) and decrease its levels in human mast cells (47), glioma cells (48), NIH 3T3 murine fibroblasts and HT-1080 human fibrosarcoma cells (49). We examined the activity of the Ras/Raf/MAPK
pathway after bortezomib treatment in HBV-negative HCC and non-HCC (breast cancer and GBM) cell lines. The phosphorylation levels of Raf and Erk either decreased or did not change. These findings support the concept that the upregulation of the MAPK pathway by bortezomib was a consequence of the upregulation of HBx. When a MEK inhibitor (PD98059) was added to bortezomib, the activation of Erk was effectively inhibited. We observed a similar phenomenon when studying the HBV-HCC ex vivo samples. In contrast, the Raf1 kinase inhibitor (GW5074) did not inhibit the phosphorylation of Raf and even slightly enhanced the phosphorylation of this protein in the HCC cell lines.

Bortezomib is the first clinically approved proteasome inhibitor. We demonstrated for the first time that a MEK inhibitor (PD98059) and bortezomib synergistically inhibited cell growth in HBV/HBx-related HCC. Several other drugs that are either approved or in clinical trials target Raf, MEK and/or Erk. For example, sorafenib, an inhibitor of Raf kinase and P-Erk in HCC (50), may have a similar effect to that of PD98059 against HBV-HCC when combined with bortezomib. In summary, our data demonstrate that HBx is involved in the drug resistance of bortezomib by upregulating the Ras/Raf/MAPK pathway in HBV-related HCC. Furthermore, the MEK inhibitor PD98059 can block this resistance. Bortezomib combined with a MEK inhibitor results in a synergistic anti-tumor effect against HBV-related liver cancer. Our study lays the foundation for a novel clinical trial in HBV-related HCC.

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Figure Legends

Figure 1. Influence of HBV/HBx after treatment with bortezomib using an ex vivo culture model and HCC cell lines. (A) Left, representative photomicrographs of TUNEL assays to measure apoptosis in HBV-related HCC (HBV+) and non-HBV-related HCC (HBV-) after treatment for 48 hours with DMSO (Ctrl) or bortezomib (BZ) at 250 nmol/L. Scale bars = 100 μm. Right, the analysis of apoptotic cells in HCC samples. The data are presented as the apoptosis rate ± SD (n = 10 per experimental group). Two-way ANOVA with Bonferroni post hoc correction is shown; ***, p < 0.001. (B) Left, analysis of apoptosis in HepG2 cells and HepG2.2.15 (integration of the HBV genome) cells cultured with DMSO (Ctrl) or bortezomib (BZ, 150 nmol/L) for 48 hours; the cells were analyzed by flow cytometry. Right, the data are presented as the percentage of Annexin-V positive cells ± SD (n = 3). Two-way ANOVA with Bonferroni post hoc correction is shown; ***, p < 0.001; **, p < 0.01. (C) Dose-dependent effects of bortezomib on the viability of HepG2 cells versus HepG2.2.15 cells, HepG2-Ctrl cells versus HepG2-HBx cells, Sk-Hep1-Ctrl cells versus Sk-Hep1-HBx cells and Huh7-Ctrl cells versus Huh7-HBx cells. The cells were cultured with bortezomib at the indicated concentrations for 48 hours. The data are expressed as the percentage of control cells; mean ± SD of three separate experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01, using Student’s t test.

Figure 2. HBx is associated with bortezomib resistance in HBV/HBx-related
HCC. (A) Dose- and time-dependent changes in the levels of HBx, phospho-Raf (P-Raf)/total Raf (Raf) and phospho-Erk (P-Erk)/total Erk (Erk) in HepG2.2.15 cells and PLC/PRF/5 cells. Left panels, cells were treated with bortezomib (BZ) at the indicated concentrations for 24 hours; right panels, cells were exposed to bortezomib (100 nmol/L) for 0, 6 and 12 hours. (B) Changes in the levels of HBx, P-Raf/Raf and P-Erk/Erk in HepG2-HBx and Sk-Hep1-HBx cells after being cultured with various concentrations of bortezomib for 24 hours. Fold increase, calculated as described in “Materials and Methods”, represents the mean of three independent experiments.

Figure 3. Validation of HBx-related bortezomib resistance. (A) Changes in the levels of P-Raf and P-Erk in HepG2, Huh7 and Sk-Hep1 cells after being cultured with various concentrations of bortezomib (BZ) for 24 hours (n = 3). (B) Breast cancer cells (MCF, MDA-MB-231) and GBM cells (U138, U87) treated with various concentrations of bortezomib for 24 hours (n = 3). (C) Western blot analysis of three non-HBV-related HCC samples after being treated with vehicle or bortezomib (250 nmol/L) for 48 hours (n = 2). (D) Effect of bortezomib on PLC/PRF/5 cells after the downregulation of HBx by siRNA. Left panel, siRNA-mediated knockdown of HBx was verified by Western blot (NC, negative control; siRNA #1, #2 are HBx specific siRNA duplexes). Right panel, knockdown of HBx by siRNA #2 for 48 hours followed by treatment with bortezomib for 24 hours (n = 3).

Figure 4. HBx levels increased via inhibition of the ubiquitin/proteasome
pathway, and the elevated expression of HBx activated the MAPK pathway. (A) HepG2 cells were co-transfected with the indicated plasmids for 24 hours and then treated with vehicle, MG132 (10 μmol/L) or bortezomib (BZ, 150 nmol/L) for 6 hours. The cell lysates were either incubated with HA agarose and then analyzed by Western blot or directly analyzed by Western blot (representative of two experiments). Ub-Flag, Flag-tagged ubiquitin. (B) Sk-Hep1-HBx and HepG2-HBx cells were treated with cycloheximide for 0, 0.5, 1 or 2 hours followed by Western blot for HBx (representative of three experiments). (C) PLC/PRF/5 and Hep3B cells were treated with MG132 for 0, 3, 6 or 12 hours. Cell lysates were analyzed by Western blot (representative of two experiments). (D) HepG2 cells were transfected with 0, 0.5 or 1 μg HBx expression construct (pcDNA3.1-HBx-HA). After 24 hours, the levels of P-Raf/Raf and P-Erk/Erk were examined by Western blot (n = 3). (E) The levels of HBx (HBx-HA), P-Raf/Raf and P-Erk/Erk were measured by Western blot in HBx-expressing cell lines (Sk-Hep1, Huh7 and HepG2) and HepG2.2.15 cells (n = 2).

Figure 5. An inhibitor of the MAPK pathway synergistically reduced HCC cell growth when applied in combination with bortezomib. (A) Upper panels, PLC/PRF/5, HepG2 and Huh7 cells were treated with GW5074 (Raf-1 kinase inhibitor) at the indicated concentrations for 24 hours. Lower panels, the same cells were treated with PD98059 (a MEK inhibitor). The cell lysates were analyzed by Western blot (n = 3). (B) PLC/PRF/5, HepG2-HBx and Huh7-HBx cells were treated with bortezomib and/or PD98059 for 24 hours, the cells were lysed, and Western
 blotting was performed (n = 3). (C) PLC/PRF/5, HepG2-HBx and Huh7-HBx cells were incubated with the indicated concentrations of bortezomib and/or PD98059 for 48 hours. The data are expressed as the percentage of control cells; mean ± SD of three separate experiments, each performed in triplicate. *, p < 0.05; **, p < 0.01 versus each agent alone. (D) Normalized isobolograms of the combination of bortezomib and PD98059 in PLC/PRF/5, HepG2-HBx and Huh7-HBx cells. The cells were treated with the indicated concentrations of bortezomib in combination with PD98059 at 5, 10 and 20 μmol/L. The diagonal line is the additive line. Data points below the additive line represent a synergistic effect. The concentration ratios (bortezomib:PD98059 ratio) are indicated below each isobologram. The data were analyzed with the CalcuSyn program.

**Figure 6. Effect of bortezomib and PD98059 on ex vivo and in vivo models.** (A) Tumor samples (HBV+ #15, 16, 17, 18, 19, 20) were treated with vehicle, bortezomib (BZ), and PD98059 (PD) either alone or combined at the indicated concentrations for 48 hours and then analyzed by Western blot (n = 3). (B) Left, representative photomicrographs of TUNEL assays of HBV-related HCC samples after treatment with vehicle (①), bortezomib (②), or PD98059 (③) alone or combined (④) for 48 hours. Scale bars = 100 μm. Right, quantitative analysis of apoptotic cells. The data are presented as the apoptosis rate ± SD (n = 6 per experimental group). One-way ANOVA with Bonferroni post hoc correction is shown; ***, p < 0.001; **, p < 0.01. (C) PLC/PRF/5 HCC cells (1×10⁶) were injected into the right flank of BALB/c
athymic nude mice. The mice were treated with intraperitoneal injections of vehicle, bortezomib (0.5 mg/kg, twice weekly), PD98059 (10 mg/kg/d) or bortezomib and PD98059 (n = 5 per experimental group). The photographs show mice bearing subcutaneous tumors from each group (upper panel) or the dissected tumors (lower panel). (D) The tumor volumes were measured and recorded every 3 days, and tumor growth curves were created for each group. The values are the means ± SD. *, p < 0.05; **, p < 0.01, ***, p < 0.001 using Student’s t test. (E) Western blot of HBx, P-Raf/Raf and P-Erk/Erk in PLC/PRF/5 tumors. (F) Schematic representation of bortezomib resistance in HBV-HCC and the rationale for the combined therapy with a MEK inhibitor (PD98059, etc.).
Figure 1

A

HBV-

HBV+

HE Ctrl BZ BZ

B

HepG2 HepG2.2.15

Ctrl BZ Ctrl BZ

Annexin-V

C

HepG2 HepG2.2.15

% Cell viability

Bortezomib (nmol/L)

% Cell viability

Bortezomib (nmol/L)

% Cell viability

Bortezomib (nmol/L)

% Cell viability

Bortezomib (nmol/L)

% Cell viability

Bortezomib (nmol/L)
Figure 3

A

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Figure 4

A

Ub-Flag
+ + + + +
HBx-HA
- + + + +
MG 132
- - + - +
BZ
- - - - +

IP: HA

IB: Flag

IB: HA

GAPDH

B

CHX (20 μg/ml)

Time (hr) 0 0.5 1 2

Sk-Hep1-HBx

HBx-HA

GAPDH

HepG2-HBx

HBx-HA

GAPDH

C

MG 132 (10 μM)

Time (hr) 0 3 6 12

PLC/PRF/5

Hep3B

HBx

GAPDH

D

pDNA3.1-HBx-HA (μg) 0 0.5 1

P-Raf

Raf

fold increase 1 2.2 4.3

P-Erk

Erk

fold increase 1 2.5 3.3

HBx-HA

GAPDH

E

Sk-Hep1

Ctrl HBx Ctrl HBx Ctrl HBx

P-Raf

Raf

fold increase 1 4.1 1 3.8 1 3.3 2.1

P-Erk

Erk

fold increase 1 2.3 1 3.6 1 3.8 2.4

HBx

HBx-HA

GAPDH
Hepatitis B virus X protein (HBx) is responsible for resistance to targeted therapies in hepatocellular carcinoma: ex vivo culture evidence

Dong Yin, Pinbo Huang, Baoxiong Zhuang, et al.

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