Bortezomib-Induced Unfolded Protein Response Increases Oncolytic HSV-1 Replication Resulting in Synergistic Antitumor Effects

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Abstract

Background: Bortezomib is an FDA-approved proteasome inhibitor, and oncolytic herpes simplex virus-1 (oHSV) is a promising therapeutic approach for cancer. We tested the impact of combining bortezomib with oHSV for antitumor efficacy.

Experimental Design: The synergistic interaction between oHSV and bortezomib was calculated using Chou–Talalay analysis. Viral replication was evaluated using plaque assay and immune fluorescence. Western blot assays were used to evaluate induction of estrogen receptor (ER) stress and unfolded protein response (UPR). Inhibitors targeting Hsp90 were utilized to investigate the mechanism of cell killing. Antitumor efficacy in vivo was evaluated using subcutaneous and intracranial tumor xenografts of glioma and head and neck cancer. Survival was analyzed by Kaplan–Meier curves and two-sided log-rank test.

Results: Combination treatment with bortezomib and oHSV (34.5ENVE), displayed strong synergistic interaction in ovarian cancer, head and neck cancer, glioma, and malignant peripheral nerve sheath tumor (MPNST) cells. Bortezomib treatment induced ER stress, evident by strong induction of Grp78, CHOP, PERK, and IRE1α (Western blot analysis) and the UPR (induction of hsp40, 70, and 90). Bortezomib treatment of cells at both sublethal and lethal doses increased viral replication (P < 0.001), but inhibition of Hsp90 ablated this response, reducing viral replication and synergistic cell killing. The combination of bortezomib and 34.5ENVE significantly enhanced antitumor efficacy in multiple different tumor models in vivo.

Conclusions: The dramatic synergy of bortezomib and 34.5ENVE is mediated by bortezomib-induced UPR and warrants future clinical testing in patients. Clin Cancer Res; 20(14); 3787–98. ©2014 AACR.

Introduction

Oncolytic herpes simplex virus-1 (oHSV) therapy utilizes viruses that are engineered to infect and replicate in cancer cells with minimal damage to non-neoplastic tissue. This therapy is currently being evaluated for safety and efficacy in multiple phase I, II, and III clinical trials (1). The results from a phase III testing of T-Vec (an oHSV developed by Amgen) have shown promising results in tumor shrinkage. Although the overall survival data have yet to be established, there is a significant need to optimize this promising therapy in vivo. Although second- and third-generation viruses are being created and tested in preclinical studies, drug-virus combinations can be rapidly translated to clinical trials to maximize efficacy and minimize toxicity (2). The proteasome is a cellular organelle that controls degradation and recycling of a wide variety of proteins that regulate diverse cellular functions, including cell-cycle progression, cell death, gene expression, signal transduction, metabolism, morphogenesis, differentiation, antigen presentation, and neuronal function. Inhibition of the proteasome...
Translational Relevance

This study describes synergy between proteasome inhibition and oncolytic herpes simplex virus-1 (oHSV). Induction of the cellular unfolded protein response (UPR) is usually associated with resistance to proteasome inhibition and other chemotherapies. Here, we found that proteasome inhibition-induced UPR sensitizes cells to oncolysis. Interestingly HSV-1 is known to exploit the proteasome during its replication cycle and proteasome inhibitors have been suggested as antiviral agents. In contrast, this study shows that bortezomib treatment increases viral replication, a finding additionally supported by the clinical observation of increased incidence of latent virus reactivation in patients treated with bortezomib. Because bortezomib is FDA approved, its combination with HSV can be rapidly translated in patients with multiple solid tumors. This study paves the way for a combination treatment strategy that can utilize suboptimal doses of bortezomib in conjunction with HSV to maximize efficacy with minimal toxicity.

can result in cellular aggregation of unfolded proteins which induce estrogen receptor (ER) stress and apoptosis. Cancer cells have increased metabolic demands and are thought to be constantly at the brink of ER stress. Thus, proteasome inhibition has been investigated as a potential way to target malignant cells.

Bortezomib is a peptide-based, reversible proteasome inhibitor, which is currently FDA approved either as a single agent or in combination with other chemo-/radio-therapeutic agents for multiple myeloma. It is also used as a second-line treatment for ovarian and head and neck cancers and is currently under clinical evaluation for the treatment of several other cancer types. Recent evidence indicates that most patients do not respond to this drug when it is used as a single agent, and several strategies testing its efficacy in combination with other drugs are being pursued (3, 4).

The combination of bortezomib and HSV is intriguing because HSV-1 exploits the host proteasome during its life cycle (5, 6), but proteasome-mediated degradation of viral capsids in infected macrophages is also thought to be important for stimulating antiviral IFN responses in these cells (7). In addition, bortezomib treatment has also been shown to induce Epstein–Barr virus and Kaposi sarcoma virus lytic gene expression, suggesting that bortezomib treatment could also improve virus replication in vivo (8). These results suggest that bortezomib may have opposing effects on HSV efficacy.

In this study, we demonstrate for the first time that the induction of the unfolded protein response (UPR) after bortezomib treatment improved HSV replication and synergistically improved cancer cell killing in vitro and in vivo. These findings demonstrate that the synergistic interaction between HSV and bortezomib improves overall therapeu-
tic efficacy via augmenting the cancer cell killing, providing advanced rationale for combining these agents in a clinical trial. This is the first study to demonstrate the utility of combining a clinically relevant proteasome inhibitor to augment replication of HSV and to achieve synergistic cell killing.

Materials and Methods

Cell lines, viruses, antibodies, and molecular biology reagents

All cancer cell lines with the exception of ovarian cancer cells were cultured in DMEM (Gibco BRL) supplemented with 10% FBS (Gibco BRL). Ovarian cancer cell lines were maintained in RPMI medium (Gibco BRL) supplemented with 10% FBS. Human glioma cell lines (U251T3 and LN229), head and neck cancer cell lines (CAL27, UMSCC11A, and UMSCC74A), and ovarian cancer cell lines (PA1, A2780-CS, A2780-CR, and SKOV3) have been cultured in our laboratory and U251T3 cells were obtained as a tumorigenic clone of U251 cells by serial passage of these cells three times in mice. Normal human astrocyte (NHA), human umbilical vein endothelial cell (HUV EC), and hepatocyte cells were purchased from Sciencell Research Laboratories and cultured according to manufacturer's instruction. U87 AEGFR cell line expresses a truncated, constitutively active, mutant form of EGFRVIII. GBM169 are primary patient-derived neurosphere cultures maintained in neurobasal medium. Human ovarian cancer patient ascites cells were harvested from patient ascites under an Institutional Review Board-approved protocol. All cell culture media were supplemented with 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere at 5% CO2. The construction and generation of 34.5ENVE (viral ICP34.5 Expressed by Nestin promoter and Vst120 Expressing) and HSV1716 (a mutant HSV-1 deleted in both copies of RL1 gene which encodes the protein ICP34.5) has been previously described (9). Virus was propagated in Vero African green monkey kidney cells (ATCC) as previously described (10).

Cytotoxicity assay

The cytotoxicity of bortezomib and 34.5ENVE in each cell line was determined by a standard MIT assay (Roche) as manufacturer's instructions. To measure 50% effective dose (ED50) of each bortezomib or 34.5ENVE alone, cells were plated onto 96-well plates of approximately 50% confluence on day zero and then treated with bortezomib or PBS for 16 hours before drug washout and infection with 34.5ENVE or Hank’s Balanced Salt Solution (HBSS). Cell viability was measured 72 hours postinfection. To measure synergistic cell killing, cells were treated with bortezomib and 34.5ENVE (as detailed above) at serially diluted concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times of their ED50 in a constant ratio. Cell viability was measured 72 hours postinfection. All assays were performed in triplicate. For rescue assay with geldanamycin and 17-AAG, cells were
pretreated with either agent for 1 hour before bortezomib treatment.

**In vitro viral replication assay**

Cells were treated ± bortezomib (at indicated doses) for 16 hours, and following drug washout cells were infected with 34.5ENVE (at indicated doses) for 2 hours. Seventy-two hours postinfection, cells and supernatant were collected, and the number of infectious particles present in the resulting supernatant was determined as previously described (9).

**Western blot analysis and antibodies**

Cell lysates were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. In UL30 detection experiments, cell and nuclear fractions were separated by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Cat# 78835). Blocked membranes were then incubated with antibodies against Anti- LC3B, calnexin, PERK, IRE1α, PDI, Bip/GRP78, CHOP, Ero1-Lα, HSP40, HSP70, HSP90, and GAPDH (Abcam; each diluted 1:1,000); horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibody (each diluted 1:1,000; GE Healthcare); HRP-conjugated secondary goat anti-rabbit antibody (each diluted 1:1,000; Dako), and the immunoreactive bands were visualized using an enhanced chemiluminescence (GE Healthcare). UL30 antibody (diluted 1:100) was used purified rabbit IgG raised against recombinant *Escherichia coli*-expressed bacteriophage T7 gene 10 protein fused with the C-terminal portion of the HSV-1 pol gene (11).

**Animal surgery**

All mice experiments were housed and handled in accordance with the Subcommittee on Research Animal Care of the Ohio State University (Columbus, OH) guidelines and adhered to the NIH Guide for the Care and Use of Laboratory Animals. Female athymic nu/nu mice (Charles River Laboratories) were used for all in vivo experiments. Nude mice with subcutaneous tumors (100 mm³) were randomized to be treated with either intraperitoneal PBS or bortezomib (0.8 mg/kg) twice a week. For intracranial implantation, mice were randomized to receive PBS or tumor cells as described (9). Three days following cell implantation, anesthetized nude mice were implanted with bortezomib (0.8 mg/kg) twice a week. For intracranial tumor studies, anesthetized nude mice were randomized at the indicated time points or when they showed signs of morbidity (hunched posture and weight loss). For subcutaneous studies, tumor volume was calculated on the basis of tumor length and width using the following formula: volume = 0.5 L W² as described (9). For histologic analysis, subcutaneous CAL27 tumors in nude mice were treated with/without bortezomib (0.8 mg/kg) twice a week. Seven days later, virus was intratumorally injected, and then injected again 2 days later. Three days after the second virus injection, tumors were harvested from each treatment group. Representative sections were stained with hematoxylin and eosin (H&E) and anti-HSV-1 antibody (Dako, 1:200 dilution) and then examined by microscopy.

**Statistical analysis**

To compare two independent treatments for continuous endpoints, the Student t test was used. When multiple pairwise comparisons are made, one-way ANOVA was used. Log-rank test was used to compare survival curves for survival data. When median survival was not observed for two or more groups in an experiment, survival probability on the last day of observation was calculated for each group. *P* values were adjusted for multiple comparisons by Holm's procedure. A *P* value 0.05 or less is considered significant. For all synergy analysis, cancer cells were seeded into a 96-well plate, and were treated with vehicle or bortezomib for 18 hours followed by drug washout, and then virus infection. Three days after infection, cell viability was measured using a standard MTT assay (Roche Applied Sciences). For synergy analysis, dose–response curves and ED₅₀ values were determined for each individual treatment (drug or virus). Fixed ratios of ED₅₀ of drug and virus were then used to treat the cells, either with dual treatment or with individual treatments (as controls). CompuSyn software program algorithm assessed the combination index (CI). Combined dose–response curves were fitted to Chou–Talalay algorithm assessed the combination index (CI). Combined dose–response curves were fitted to Chou–Talalay lines, which are derived from the law of mass action. CI < 1 indicates synergistic interaction, whereas CI > 1 is antagonistic and CI = 1 is considered additive (12).

**Results**

**Interaction of bortezomib treatment with oHSV**

The sensitivity of head and neck squamous cell carcinomas, ovarian cancer, malignant peripheral nerve sheath tumors (MPNST), and glioma to bortezomib or 34.5ENVE alone in vitro was evaluated. Cells were treated with indicated doses of bortezomib and/or oHSV and cell death was measured by a standard MTT assay. Figure 1A shows that combination treatment increased cell killing compared with the predicted additive effects (dotted line), suggesting synergistic cell killing.

To further evaluate synergistic interaction between bortezomib and oHSV, we measured sensitivity of each cell line to bortezomib and oHSV. Briefly, cells were treated with bortezomib at concentrations ranging from 0.1 to 500 nmol/L for 16 hours, followed by drug washout. To test sensitivity to 34.5ENVE, cells were infected with different doses of oHSV ranging from 0.001 to 0.5 multiplicity of infection (MOI) defined as virus pfu/cell. Seventy-two hours following bortezomib washout or 34.5ENVE infection, cell viability was measured by a standard MTT assay. The ED₅₀ of bortezomib and 34.5ENVE was each defined as the dosage yielding 50% cell viability at 72 hours following...
Figure 1. Effect of bortezomib and oHSV on cancer cell killing. A, treatment of ovarian cancer, head and neck cancer, and glioma cells with bortezomib and oHSV showed more than additive cell killing. Briefly, the indicated cells were treated with sublethal doses of bortezomib or PBS for 16 hours, followed by washout drug and treatment with sublethal dosed of 34.5ENVE or PBS. Seventy-two hours later from 34.5ENVE infection, cell viability was measured via MTT assay. (Continued on the following page.)
treatment as compared with untreated controls. Supplementary Table S1 shows the sensitivity of each cell line to bortezomib and 34.5ENVE. To study the interaction between bortezomib and 34.5ENVE, cells were treated with bortezomib for 16 hours, followed by drug washout and treatment with 34.5ENVE at concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times of their ED$_{50}$ in a constant ratio. Cell viability was measured 72 hours after virus infection. The results were analyzed using the median-effect method of Chou–Talalay (COMPU Syn; ref. 12). Figure 1B shows combination index plots of Chou–Talalay. The plots show synergistic cell killing between bortezomib and 34.5ENVE (combination) in PA1, A2780-CS (cisplatin sensitive), SKOV3, and A2780-CR (cisplatin resistant) ovarian cancer cells CAL27, UMSCC11A, and UMSCC74A, head and neck squamous cell carcinomas and in U251T3, LN229, and U87ΔEGFR glioma cells (Fig. 1B). In a separate experiment, similar synergistic cell killing was observed when clinical grade oHSV1716 was used in combination with bortezomib to treat MPNST cells (Supplementary Fig. S1; CI values less than 1 at all fractions affected). To evaluate the effect of bortezomib on oHSV safety, we compared the cytotoxic ability of oHSV toward NHA, HUVEC, and hepatocyte cells treated with/without ED$_{50}$ concentrations of bortezomib (Fig. 1C). In all three normal cells tested, no additive (dotted line) or synergy was observed between bortezomib and 34.5ENVE. These data support the conclusion that the combination of bortezomib and oHSV significantly increased sensitivity to cell death for a wide variety of solid tumor cells but not for normal cells.

**Impact of bortezomib on oHSV replication**

To investigate the impact of bortezomib on 34.5ENVE replication, U251T3 glioma cells were treated with sublethal and ED$_{50}$ doses of bortezomib (2 and 12 nmol/L) followed by 34.5ENVE infection (Fig. 2A and B). Fluorescent microscopy was used to image GFP-positive (infected) cells, 48 hours postinfection. Figure 2A shows increased GFP-positive infected cells in both concentrations of bortezomib, indicating that bortezomib treatment increased viral replication in vitro. In addition, quantification of viral titers showed a significant increase in viral titer in the both sublethal and ED$_{50}$ doses of bortezomib (Fig. 2B–C). Quantification of viral titers in cells treated with or without bortezomib revealed a significant increase in viral replication in the multiple cell lines treated with sublethal doses of bortezomib in all cells tested: ovarian: SKOV3 cells: 20.3-fold, head and neck cancer cells: CAL27: 1.8-fold, UMSCC74A: 1.8-fold, and glioma: U87ΔEGFR: 8.4-fold (Fig. 2C and supplementary Table S2). Collectively, these data demonstrate that bortezomib increased viral replication.

**Efficacy of combination treatment in patient-derived primary tumor cells ex vivo**

Next, we tested synergistic interaction of bortezomib and 34.5ENVE in ovarian cancer patient ascites derived tumor cells and glioblastoma (GBM) patient-derived primary GBM neurospheres (GBM169) ex vivo (Fig. 2D). Briefly, cells were treated with bortezomib for 16 hours, followed by drug washout and treatment with 34.5ENVE at concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times of their ED$_{50}$ in a constant ratio. Cell viability was measured 72 hours after virus infection. The results were analyzed using the median-effect method of Chou–Talalay (COMPU Syn). Synergistic cell killing was obvious in both patient-derived ascites and primary GBM neurosphere cells (Fig. 2D). Patient-derived primary GBM neurosphere cells (GBM169) pretreated with or without bortezomib were labeled with red cell tracker and treated with 34.5ENVE. Fluorescent microscopy images showed increased GFP-positive infected cells in bortezomib-treated cells (Fig. 2E). Quantification of viral titers in cells treated with or without bortezomib revealed a significant increase in viral replication in both patient ascites and primary GBM neurospheres: Ovarian patient ascites: 4.3-fold and GBM169: 1.9-folds increase over oHSV alone (Fig. 2F).

**ER stress and unfolded protein response in cells treated with bortezomib and oHSV**

Bortezomib is reversible proteasome inhibitor that blocks the chymotrypsin-like activity of the proteasome resulting in the accumulation of unfolded proteins. Accumulation of unfolded proteins induces ER stress leading to caspase-dependent apoptosis (13–15). Treatment of both glioma and head and neck cancer cells with bortezomib showed induction of proteins indicative of ER stress in a dose-dependent manner (Fig. 3A and Supplementary Fig. S2A). In contrast, oHSV is known to disarm ER stress during infection (16). To test the impact of combination treatment of oHSV and bortezomib on ER stress, we measured changes in levels of cellular proteins that are induced during ER stress. Increased expression of PERK, Calnexin, IRE1a, CHOP, Er1a-La, and GRP78 proteins was observed in oHSV-infected and uninfected glioma and head and neck cancer cells after bortezomib treatment (Fig. 3B).

Along with ER stress, unfolded proteins also lead to the induction of an UPR, which constitutes increased expression of HSP and cellular chaperone proteins. Both glioma

(Continued.) Data shown are % cell death ± SD of cells treated with bortezomib/PBS for 16 hours before media washout alone or with oHSV infection. B, Chou–Talalay analysis of combining bortezomib with 34.5ENVE in ovarian cancer, head & neck cancer, and glioma cells. Briefly, cells were treated with bortezomib for 16 hours, followed by drug washout and treatment with 34.5ENVE at concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 times of their ED$_{50}$ in a constant ratio. Data shown as fraction affected (fa) versus CI plots. CI < 1 indicate synergy, CI = 1 indicate additive, and CI > 1 indicate an antagonistic interaction. C, normal human astrocytes (NHA), HUVEC, and hepatocyte cells were plated and treated with/without ED$_{50}$ concentration of bortezomib for 16 hours, followed by drug washout and treatment with 34.5ENVE. 72 hours following 34.5ENVE infection, cell viability was measured by MTT assay. Data points represent the mean ± SD for each group.
Figure 2. Effect of bortezomib treatment on viral replication. U251T3 cells were treated with 2 or 12 nmol/L bortezomib for 16 hours before drug washout and 34.5 ENVE infection (MOI = 0.002 with 2 nmol/L bortezomib and 0.01 for 12 nmol/L bortezomib) and evaluated for virus replication. A, bright field (Top) and fluorescence microscopic images (bottom) of GFP-positive infected cells 48 hours postinfection demonstrate increased virus replication following bortezomib treatment at both sublethal (2 nmol/L) and ED50 (12 nmol/L) concentrations relative to no bortezomib-treated cells (magnification, × 100). (Continued on the following page.)
and head and neck cancer cells treated with bortezomib showed a dose-dependent induction of HSP proteins (Fig. 3C and Supplementary Fig. S2C). Consistent with this, we observed induction of HSPs (HSP40, 70, and 90α) in oHSV-infected and uninfected glioma and head and neck cancer cells pretreated with bortezomib (Fig. 3D and Supplementary Fig. S2D). Hsp90α induction was observed at both low and high concentrations of bortezomib treatment of glioma and head and neck cancer cells. HSP90α has been previously shown to be important for localization of HSV polymerase to the nucleus (17), thus we hypothesized that bortezomib-induced HSP90α induction could be critical for the increased viral replication and cell killing following combination treatment. To test this hypothesis, we examined the impact of geldanamycin, an HSP90 inhibitor, on combination treatment-induced tumor cell cytopathy. Figure 4A shows combination index plots of Chou–Talalay of glioma cells treated with bortezomib and 34.5ENVE in the presence or absence of geldanamycin. Treatment of glioma cells with geldanamycin reduced synergistic cell killing interactions between 34.5ENVE and bortezomib (Fig. 4A). The synergistic interaction of the combination treatment was also reduced in cells treated with 17-AAG, a less toxic alternative HSP90 inhibitor (Fig. 4B), supporting the significance of HSP90 in oHSV and bortezomib synergistic cell killing. Consistent with the importance of HSP90, both 17-AAG and geldanamycin treatment resulted in a loss of the increased viral replication achieved by bortezomib treatment (Fig. 4C). HSP90 is thought to increase HSV polymerase localization to the nucleus. To directly test whether bortezomib treatment affected nuclear localization of HSV polymerase (UL30), we compared the cytosolic and nuclear fractions (C.F and N.F, respectively) of oHSV-infected cells treated without/with bortezomib at the indicated concentrations (Fig. 4D). Increased nuclear localization of UL30 was observed in bortezomib-treated cells. Collectively, these results demonstrate that the induction of HSP90 by bortezomib is essential for the synergy with 34.5ENVE treatment.

**Combination treatment increased therapeutic efficacy in vivo**

We next examined the therapeutic efficacy of bortezomib in subcutaneous U251T3 glioma model (Fig. 5A). Mice bearing the U251T3 glioma tumor xenograft displayed tumor growth inhibition of 91.7% in response to combination treatment. Moreover, at day 23 after treatment, six out of eight tumors treated with bortezomib plus 34.5ENVE had completely regressed.

Combination treatment also improved survival of mice bearing intracranial GBM169 tumors (Fig. 5B). All mice treated with PBS, bortezomib alone, or 34.5ENVE alone died with a median survival of 31 days (bortezomib alone: HR of survival = 1.18, $P = 0.927$; 34.5ENVE alone: HR of survival = 0.89, $P = 0.797$). Whereas, by day 100 following treatment, 70% (median survival > 100 days; HR of survival = 0.11, $P < 0.001$) of the animals in the bortezomib combined with 34.5ENVE group were still viable (Fig. 5B).

Similar tumor growth suppression was observed in a combination with 34.5ENVE in the CAL27 head and neck xenograft model established in nude mice (Fig. 5C and D). Untreated tumors grew rapidly, leading to a tumor volume of 1,407.36 mm³ by day 33 (95% confidence interval = 1,142.36 to 1,672.36 mm³), whereas mean tumor volumes of bortezomib, 34.5ENVE, and combination treated mice were 813.76 mm³ (difference with PBS = 566.93 mm³, $P = 0.004$), 281.46 mm³ (difference with PBS = −107.33 mm³, $P = 0.001$), and 116.46 mm³ (difference with PBS = −128.90 mm³, $P < 0.001$), respectively (Fig. 5C). These data correlate to 34.6, 84.6, and 91.7% tumor growth inhibition, respectively, compared with control PBS-treated mice. In addition, combination treatment resulted in increased survival of mice (Fig. 5D). Together these results highlight the translational significance of combining bortezomib treatment with oHSV therapy for both glioma and head and neck cancers.

Combination treatment increased viral replication and necrotic cell death in vivo

The apparent enhanced antitumor efficacy and survival benefit resulting from combinatorial treatment was further investigated by immunohistological examination of CAL27 subcutaneous tumor xenografts 3 days after virus treatment. As shown in Fig. 6, there was significantly increased HSV staining in tumors treated with combination treatment as compared with tumors treated with oHSV alone. A large portion of tumor sections derived from tumors treated with...
bortezomib and oHSV were necrotic, as evident with H&E staining (Fig. 6).

Discussion

oHSV therapy has shown promise in preclinical models and several clinical studies testing its efficacy in patients are ongoing and interim analysis of a large-phase three trial for a GMCSF expressing oncolytic HSV has revealed significant improvement in durable response rates (18). Proteasome inhibition has recently emerged as a promising target in cancer therapy, but its efficacy in conjunction with oHSV has not been previously investigated. Here, we report the first study to show that proteasome inhibition with bortezomib can be combined with oHSV to effectively target various solid tumors. Such combinatorial strategies hold great promise as cancer treatments because they can enhance efficacy while minimizing toxicity (19). We utilized Chou–Talalay analysis and found that bortezomib interacted synergistically with oHSV in vitro in killing various solid cancer cells, including ovarian, head and neck, MPNST, and glioma. Importantly, combination treatment resulted in a greater than 2- to 8-fold decrease in the dosage of either bortezomib or oHSV necessary for similar cell killing.

HSV-1 encoded ICP0 is an E3 ubiquitin ligase that utilizes cellular machinery to selectively degrade host proteins that enhance the innate antiviral immune response (20, 21). The proteasome has three distinct proteolytic activities: chymotrypsin-like, trypsin-like, and caspase-like activities. Specifically, the chymotrypsin-like activity of the proteasome has also been shown to be important for virus entry (22). In addition, dysregulation of cellular ubiquitin-proteasome system has been shown to augment antiviral activity against HSV-1 and HSV-2 viruses (23). These results suggest that it may be counterintuitive to utilize bortezomib to increase the efficacy of oHSV. However, although ICP0-mediated modification leads to degradation of cellular antiviral proteins (21), its own proteasomal degradation by SIAH-1, a cellular ubiquitin ligase, has also been shown to inhibit viral infection (24). Clinically, increased incidence of herpes zoster, Varicella zoster virus, and hepatitis B virus reactivation has also been noted in patients treated with bortezomib (25–28). Interestingly an oncolytic adenovirus was shown to synergize with bortezomib by increasing cellular apoptosis in vitro and by its immunomodulatory effects in vivo (29). Bortezomib treatment has also been found to increase apoptosis of EBV-positive transformed B cells suggesting that it may be a novel strategy for the treatment of EBV-
associated lymphomas (30). Although our data show no evidence of increased apoptosis, we have not examined the contribution of immunomodulatory effects on virus propagation and antitumor immune responses generation in vivo. Bortezomib-induced activation of UPR has also been shown to induce cellular transcription factors that can activate EBV viral promoters and promote a lytic switch. Direct activation of EBV lytic cycle by bortezomib has also been reported in a variety of tumor cells. This effect is thought to be due to the induction of C/EBPβ, a cellular transcription factor that can then initiate the activation of EBV lytic gene expression (31). Although bortezomib has been shown to increase EBV replication, other studies have also found that bortezomib inhibits VSV via activation of NF-κB, resulting in an antiviral state that ultimately inhibits VSV propagation (32). Consistent with these reports, treatment of myeloma cells with bortezomib was found to inhibit VSV replication and show less than additive cell killing in vitro (33). Interestingly, the authors found that despite the antagonistic results in vitro bortezomib and VSV treatment improved antitumor efficacy in vivo. These seemingly contradictory studies make the combination of

**Figure 4.** Effect of bortezomib induced HSP90 on virus replication and synergistic cell killing. A, Chou-Talalay analysis of combining bortezomib with 34.5ENVE in ovarian cancer, head and neck cancer, and glioma cells treated with geldanamycin. Briefly, geldanamycin (1 μmol/L) was added before bortezomib treatment (1 hour) and synergy between bortezomib and oHSV measured as described earlier. Data are presented as fraction affected (fa) versus CI plots of cells with or without geldanamycin treatment. B, effect of 17-AAG on bortezomib and oHSV interaction in cell killing. Briefly, 17-AAG (0.1 μmol/L) was added before bortezomib treatment (1 hour) and synergy between bortezomib and oHSV measured as described earlier. The interaction between bortezomib and oHSV on cell killing was analyzed by the median-effect method of Chou-Talalay analysis. Data are presented as fraction affected (fa) versus CI plots of cells treated with or without 17-AAG. C, effect of 17-AAG and geldanamycin on increased viral replication following bortezomib treatment. U251T3 cells were pretreated with/without 0.1 μmol/L of 17-AAG or geldanamycin (1 μmol/L) for 1 hour and then treated with 12 nmol/L bortezomib for 16 hours before 34.5ENVE infection (MOI = 0.01). Forty-eight hours later cells and media were harvested and viral titers were measured by standard plaque assay. Data points represent the mean, and error bars indicate ± SD for each group. Asterisks indicate statistically significant differences between indicated pairs. D, Western blot analysis for HSV polymerase (UL30). U251T3 cells were pretreated with 2 or 12 nmol/L of bortezomib for 16 hours, followed by drug washout and treatment with 34.5ENVE of 0.1 MOI. Four hours later, cells were harvested and proteins were fractionated for nuclear (N.F) and cytoplasmic extracts (C.F). Lamin B and GAPDH antibody was used as loading control for N.F. and C.F., respectively.
bortezomib and oHSV both promising and intriguing. Here, our results demonstrate increased virus replication and cancer cell killing when cells are pretreated with bortezomib before oHSV infection. In infected macrophages and dendritic cells in vivo, proteasome-mediated degradation of viral capsid proteins has also been shown to release viral genomic DNA into the cytoplasm activating antiviral IFN responses in macrophages post HSV-1 and CMV infections and can aid in viral clearance (7). Immune suppression with bortezomib may account for the increased virus reactivation observed in patients and in vivo tumor-bearing mice in our experiments. In the context of our study, we have not investigated the effect of bortezomib on antiviral and antitumor immune responses that are generated with oHSV treatment. Future studies to investigate this interaction would be of interest.

Resistance to bortezomib remains a clinical challenge and the induction of the UPR has been correlated with the development of resistance to apoptosis initiated by proteasome inhibition (34). The UPR leads to the induction of HSPs: a group of ubiquitously expressed chaperon proteins that ensure correct folding and prevent aggregation of specific target proteins. These chaperone proteins coordinate and rescue ER stress and are thought to contribute toward bortezomib resistance (34). Interestingly, HSP90 is also utilized by HSV-1–encoded DNA polymerase for its proper localization to the nucleus (35, 36). Our results show that the induction of HSP90 as part of the UPR...
Figure 6. Histologic characterization of tumor tissues. Athymic nude mice were subcutaneously implanted with CAL27 and treated as in Fig. 5. Mice were injected intratumorally twice with $1 \times 10^7$ pfu of oHSV every other day. Three days later from second virus injection, tumors were harvested and sectioned. Data shown are representative images of H&E- and HSV-stained tumor sections. Original magnification $= \times 10$ and $\times 20$, as indicated. Scale bar $= 100$ μm.

Activated by bortezomib is responsible for increased virus replication, and HSP90 inhibitors ablated the synergistic cell killing and increased viral replication induced by bortezomib. Together these results indicate that the combination of oHSV therapy with bortezomib is an attractive strategy to enhance therapy or deal with the development of resistance to bortezomib treatment. Here, we show that the UPR induced by bortezomib increases viral replication in vitro. It is interesting to speculate whether different proteasome inhibitors with different affinities for distinct proteolytic activities of the proteasome and/or their different half-life of each inhibitor may account for the increased oHSV replication observed in bortezomib-treated cells but inhibition with wild-type virus observed with other proteasome inhibitors. In this study, the lack of bortezomib alone to show therapeutic efficacy in mice bearing intracranial glioma is consistent with the lack of response observed in patients with glioma after bortezomib treatment (37).

However, the enhanced survival with combination treatment is consistent with our results which show that even sublethal doses of bortezomib can also increase viral replication and improve cell killing in vitro. Although it is interesting to speculate that tumors which can induce UPR in response to bortezomib would be most sensitive to combination of bortezomib and oHSV, future studies will identify biomarkers to predict tumor types that will be most sensitive to this combination treatment.

Recently, bortezomib was found to synergize with oncolytic reovirus therapy in the treatment of multiple myeloma by induction of ER stress and NOXA-dependent cellular apoptosis (15). Although reovirus induces ER stress in infected cells, HSV-1 is known to hijack cellular pathways to override ER stress signaling and maintain ER homeostasis (38). Here, we show that combination of bortezomib and oHSV did not exacerbate ER stress in cells compared with cells treated with only bortezomib. To our knowledge, this is the first report showing synergy between oHSV and bortezomib. This study offers a novel therapeutic treatment strategy for cancer therapy that can be rapidly translated in patients with multiple solid tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
Any opinions, findings, and conclusions expressed in this material are those of the authors and do not necessarily reflect those of the Pelotonia Fellowship Program.

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