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Combination of proteasome and HDAC inhibitors for therapy of uterine cervical cancer

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Abstract

PURPOSE—Cervical cancer cells are addicted to the expression of Human Papillomavirus (HPV) oncoproteins E6 and E7. The oncogenicity of E6 is mediated in part by targeting p53 and PDZ-family tumor suppressor proteins for rapid proteasomal degradation, whereas E7 oncoprotein acts in part by co-opting histone deacetylases (HDAC)1/2. Here, we examine the hypothesis that inhibition of proteasome function and HDAC activity would synergistically and specifically trigger cervical cancer cell death by the interruption of E6 and E7 signaling.

EXPERIMENTAL DESIGN—The sensitivity and molecular responses of keratinocytes and HPV-positive and negative cervical cancer cells and xenografts to combinations of proteasome and HDAC inhibitors were tested. The expression of HDAC1/2 in situ was examined in cervical cancer, its precursors and normal epithelium.

RESULTS—Cervical cancer cell lines exhibit greater sensitivity to proteasome inhibitors than HPV-negative cervical cancers or primary human keratinocytes. Treatment of cervical cancer cells with

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Conflict of Interest: A drug used in the study (Vorinostat) described in this article is manufactured by Merck & Co. RBSR is a paid consultant of Merck & Co. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

STATEMENT OF TRANSLATIONAL RELEVANCE

There are currently no virus-specific therapies for cervical cancer and the efficacy of standard surgical and chemo/radiotherapies is limited for advanced disease. Cervical cancer cells are addicted to the expression of Human Papillomavirus (HPV) oncoproteins E6 and E7. The oncogenicity of E6 is mediated in part by targeting p53 and PDZ-family tumor suppressor proteins for rapid proteasomal degradation, whereas E7 oncoprotein acts in part by co-opting histone deacetylases (HDAC)1/2.

A ‘first in class’ proteasome inhibitor, Bortezomib, was recently licensed for the treatment of multiple myeloma, and herein we examine its potential for therapeutic activity against cervical cancer, alone or in combination with hydroxamate-based pan-HDAC inhibitors, Trichostatin A and Vorinostat. Notably, Vorinostat is the ‘first in class’ HDAC inhibitor licensed in 2006 for the treatment of cutaneous T cell lymphoma. The synergistic killing observed suggests the potential of combinations these licensed proteasome and HDAC inhibitors for the treatment of cervical cancer.

Bortezomib elevated the level of p53 but not hDlg, hScribble or hMAGI. Immunohistochemical analysis revealed elevated HDAC1/2 expression in CIN and cervical carcinoma versus normal cervical epithelium. The combination of Bortezomib and HDAC inhibitors Trichostatin A (TSA) or Vorinostat show synergistic killing of HPV-positive, but not HPV-negative, cervical cancer cell lines. Similarly, treatment of HeLa xenografts with the combination of Bortezomib and TSA retarded tumor growth significantly more effectively than either agent alone.

CONCLUSIONS—A combination of proteasome and HDAC inhibitors, including Bortezomib and Vorinostat respectively, warrants exploration for the treatment of cervical cancer.

Keywords

Cervical cancer; E6; p53; Bortezomib; Vorinostat

INTRODUCTION

Persistent infection with an oncogenic type HPV, most commonly HPV16 and HPV18, is a necessary but insufficient cause of cervical cancer (1). HPV DNA is detected in 99.7% of cervical cancers (2), as well as a large proportion of other anogenital cancers, and also in a subset of head and neck cancers. While cytologic screening and HPV vaccines are effective preventive measures, there are currently no virus-specific therapies for cervical cancer and the efficacy of standard surgical and chemo/radiotherapies is limited for advanced disease. Expression of two viral oncogenes, E6 and E7, is critical for induction and maintenance of the transformed phenotype and is lacking from normal cells (3-6). This suggests E6 and E7 as logical targets for rational therapeutic approaches and that inhibitors should target the functions of both oncoproteins (4). While neither has intrinsic enzymic activity, genetic and biochemical studies have defined key cellular partners through which these viral proteins transform cells (7,8).

E6 exerts one important aspect of its oncogenic activity by binding to the HECT domain E3 ubiquitin ligase E6-AP (and possibly other ubiquitin ligases) and redirects its activity towards p53 and PDZ family proteins, including hDlg, hScribble, and hMAGI, for rapid proteasomal degradation (9,10). This reduces the level of these key cellular cell cycle regulators without their mutation (11). Therefore, treatment with proteasome inhibitors might potentially recover near normal levels of wild type p53 and PDZ tumor suppressor proteins, and thereby trigger cell death. Absent of E6, p53 protein stability is regulated by competition between E3 ligase-mediated ubiquitination and histone acetyl transferase (HAT)-mediated acetylation of key lysine residues. Acetylation of lysine by HATs prevents ubiquitination of p53 by MDM2, thus stabilizing p53, whereas their de-acetylation by a histone deacetylase 1 (HDAC1)-containing complex has the opposite effect. Indeed, de-acetylation of p53 represses p53-dependent transcriptional activation, apoptosis and growth arrest. This suggests that inhibition of p53 de-acetylation may further enhance both the level of p53 and in the response cell cycle arrest and apoptosis.

The E7 oncoprotein also binds to multiple functional partners, notably pRB and the class I histone deacetylases HDAC1 and HDAC2 (12-14). E7 destabilizes pRB levels through a cullin 2-mediated proteasomal degradation (15). E7 binds indirectly to both HDAC1 and HDAC2 via sequences in the zinc-finger domain of E7 (16,17). Mutations within the zinc-finger domain of E7 do not affect binding to and degradation of pRb, but do abrogate the ability of E7 to immortalize cells, suggesting that both activities of E7 are required for immortalization (18) (17). The binding of HDAC1 and HDAC2 to E7 is mediated by association with the Mi2 β protein, a component of the mammalian NuRD chromatin remodeling complex. Class I HDACs, including HDAC1, HDAC2, HDAC3, and HDAC8, are found in complexes with

other transcriptional corepressors including mSin3 and SMRT/N-corepressor (19,20). HDACs regulate the activity of numerous promoters including those that are E2F-dependent. E7 directs HDAC activity to the E2F2 promoter increasing its expression (21). In addition, HDACs can directly deacetylate E2F proteins, although the function of this modification is not well understood (21). Finzer et al demonstrated that HDAC inhibitor treatment induces an intrinsic type of apoptosis in HPV-positive cells by disrupting the mitochondrial transmembrane potential (22,23). This was only detected in E7, but not in E6 oncogene-expressing cells. HDAC inhibition led to a time-dependent degradation of the pocket proteins pRb, p107 and p130, releasing 'free' E2F1 following initial G1 arrest. Inhibition of proteasomal proteolysis, but not of caspase activity rescued E7-directed pRb from degradation and functionally restored its inhibitory effect on the cyclin E gene, known to be suppressed by pRb-E2F1 in conjunction with HDAC1.

We hypothesized that inhibition of key E6 and E7 functions by treatment with proteasome and HDAC inhibitors might provide synergistic killing of cervical cancer cells while sparing normal cells that lack these viral oncogenes. A 'first in class' proteasome inhibitor, Bortezomib (also known as PS-341 and marketed as Velcade), was recently licensed for the treatment of multiple myeloma, and herein we examine its potential for therapeutic activity against cervical cancer, alone or in combination with hydroxamate-based pan-HDAC inhibitors, Trichostatin A and Vorinostat. Notably, Vorinostat (also known as suberoylanilide hydroxamic acid (SAHA) and sold as Zolinza (Merck & Co.)), is the 'first in class' HDAC inhibitor licensed in 2006 for the treatment of cutaneous T cell lymphoma.

MATERIALS AND METHODS

Animals

Six-week old female immunodeficient BNX (beige nude xid) mice were obtained from National Cancer Institute-Frederick (Frederick, MD) and maintained in a pathogen-free animal facility at least 1 week before use. All animal studies were done in accordance with institutional guidelines.

Human specimens

Studies using human tissue were performed with the approval of the Johns Hopkins Institutional Review board. Fresh and archival tissues were obtained from the Department of Pathology of the Johns Hopkins Hospital and the latter assembled in tissue microarrays by a core facility.

Cell culture

Cervical cancer cell lines HeLa, SiHa, CaSki, ME180, HT3 and C33A, were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 5% CO₂. Keratinocytes were obtained from Invitrogen and cultured in defined Keratinocyte-SFM

Drugs

The proteasome inhibitor Bortezomib (Millenium Pharmaceuticals, Inc. Cambridge, MA) was dissolved in 0.9% NaCl before each injection. The stock solutions of the HDACs inhibitors TSA and Vorinostat (Sigma, St. Louis MO) were dissolved in DMSO and then diluted into phosphate-buffered saline (PBS) before each injection

Xenograft murine model

Mice were inoculated s.c. in their right flank with 5×10^6 HeLa cells in 100 μ L DMEM. When tumor was measurable, mice were randomly assigned into four groups receiving Bortezomib only, TSA only, both Bortezomib and TSA, and PBS only. Treatment was given i.v. twice weekly via tail vein at 1 mg/kg Bortezomib, and s.c. twice weekly at left flank at 1 mg/kg TSA. The control group received the vehicle alone at the same schedule. Caliper measurements of the longest perpendicular tumor diameters were done every 2 days to estimate the tumor volume (mean $\pm$ SE; mm³), using the following formula: $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$, representing the three-dimensional volume of an ellipse. Animals were sacrificed when their tumors reached 2 cm.

Cell viability assay

Cell viability was determined by 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Roche Diagnostics GmbH, Mannheim, Germany). Cells seeded at the concentration of 1,000 per well in 100 μ L medium in 96-well plate were treated with proteasome inhibitors at specified concentrations. After the indicated periods, cells were incubated according to the manufacturer's protocol with the XTT labeling mixture for 4 hours. Formazan dye was quantified using a spectrophotometric plate reader to measure the absorbance at 450nm (ELISA reader 190; Molecular Devices, Sunnyvale, CA). All experiments were done in triplicate.

Antibodies and Western blot analysis

The following antibodies were used for detection with standard Western blot analysis techniques at the concentration recommended by the manufacturer: anti-p53, anti-hDlg-1 and anti-hScribble (Santa Cruz, Santa Cruz, CA), anti-hMAGI, monoclonal anti-Histone Deacetylase-1, 2, 6, anti- β -actin (Sigma, St. Louis, MO); and peroxidase-linked and peroxidase-linked anti-rabbit or anti-mouse IgG (Amersham, Piscataway, NJ).

Determination of apoptotic cells by flow cytometry

Induction of apoptosis was determined by Annexin-V/7-AAD staining and active caspase-3 staining. Annexin-V/7-AAD staining was done using Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) according to manufacturer's protocol. Briefly, 1×10^5 cells were re-suspended in Binding Buffer, 5 μ L of Annexin V-PE and 5 μ L of 7-AAD were then added into the cells which were then incubated at room temperature for 15 minutes, and analyzed by flow cytometry. Active caspase-3 staining was done using phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody (BD Pharmingen, San Diego, CA) according to the manufacturer's protocol. Briefly, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) for 20 minutes at 4°C. After washing, the cells were stained with phycoerythrin-conjugated rabbit anti-active caspase-3 monoclonal antibody using 20 μ L per 1×10^6 cells for 30 minutes at room temperature. Following incubation with the antibodies, the cells were washed, resuspended and analyzed by flow cytometry on a Becton Dickinson FACSCalibur. Data analysis was done with CellQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Paraffin-embedded tissue sections were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using an established method to assay for cell death-associated DNA double-strand breaks (24).

Immunohistochemistry of tissue microarrays

Immunohistochemical analysis of paraffin-embedded tissues was done as previously described (25). Briefly, 5µm tissue microarray sections were deparaffinized and rehydrated. Antigen retrieval was performed and slides were incubated for 5 min with 3% hydrogen peroxide, then washed and incubated in antibody dilution 1:250 for 60 min at room temperature. The avidin-biotin-peroxidase complex method of DAKO (Glostrup, Denmark) was used to visualize antibody binding, and slides were subsequently counterstained with hematoxylin. The staining was scored by three observers blind to specimen identity to obtain a consensus. Staining intensity was scored as negative (0), weak (1), intermediate (2) or strong (3).

Statistical analysis

Results are reported as mean $\pm$ SD. Unless otherwise indicated, statistical significance of difference was assessed by two-tailed Student's *t* test using Prism (V.4 Graphpad, San Diego, CA). The level of significance was set at $p < 0.05$. The combination index (CI) of Bortezomib and TSA or Vorinostat was calculated by the method of Chou and Talalay (26). The minimum CI was determined by fitting of a response surface to the data (27), and then calculating the CI using the fitted values. Synergy was also depicted by isobologram, in which the drug combinations leading to 50% loss in cell viability are plotted, or a bar chart if one (or both) of the agents fails to achieve this level.

RESULTS

Sensitivity of cervical cancer cell lines to proteasomal inhibition with Bortezomib is accompanied by increases in p53 but not PDZ protein levels

We examined the impact of Bortezomib treatment upon the viability of cultured cervical cancer cell lines. Bortezomib produced a dramatic drop in viability of CaSki, SiHa, ME180 or HeLa cells after 48h of treatment at nanomolar concentrations (Fig. 1A). In contrast to the HPV16-transformed CaSki and HPV18-transformed HeLa cells, the effect of Bortezomib upon the viability of primary human keratinocytes, or the HPV-negative cervical cancer line C33A was limited (Fig 1A). Likewise, ME180 cells, that contain HPV68, exhibit far greater susceptibility to Bortezomib treatment than the HPV-negative cervical cancer line HT-3. These findings imply that increased susceptibility to Bortezomib is associated with transformation by HPV, regardless of the oncogenic type.

Prior studies with other proteasome inhibitors have suggested that the HPV oncogene E6 exerts its effect by triggering the ubiquitination and subsequent proteasomal degradation of cellular tumor suppressor genes, notably p53 and PDZ family members. To identify potential mechanisms by which Bortezomib triggers the death of cervical cancer cells, we examined the levels of p53 in treated and untreated cells (Fig. 1B). Bortezomib treatment triggered a dramatic increase in wild type p53 levels in HeLa cells, and to a lesser extent in CaSki cells. A similar phenomenon was observed in primary keratinocytes, presumably by blockade of mdm2-dependent p53 degradation, whereas the levels of mutant p53 present in C33A cells remained similar despite Bortezomib treatment. Surprisingly, Bortezomib treatment exhibited little impact upon the levels of PDZ family members hMAGI, hScribble, or hDlg in any of the cell types tested regardless of HPV status (Fig. 1B). This may reflect inhibition of a different spectrum of proteasomal activities by Bortezomib, as compared with the proteasomal inhibitors used in previous studies of E6 regulation of PDZ proteins (28-30). Furthermore Bortezomib treatment of HeLa cells did not significantly alter pRB levels (not shown).

Overexpression of HDAC1, HDAC2 and HDAC6 in cervical cancer cells *in vivo* and *in vitro*

Since Bortezomib treatment was only partially effective in triggering the death of cervical cancer cells, we examined the possibility of inhibiting a transforming activity of HPV that was independent of proteasomal activity. E7 is known to interact with both HDAC1 and HDAC2, and mutational analysis of E7 suggests that this interaction may contribute to transformation. However it is unclear how frequently these HDACs are expressed in cervical cancer, and therefore we sought to address their expression pattern by immunohistochemistry. Commercial antisera to the nuclear class I histone deacetylases HDAC1 and HDAC2, as well as the cytoplasmic class II histone deacetylase HDAC6, are available and we confirmed their specificity by Western blot analysis (Fig. 2). Therefore to examine the potential of targeting these HDACs for treatment of cervical cancer we examined their expression level in squamous cell carcinoma (n=141 cases) and adenocarcinoma (n=24 cases) of the uterine cervix, as well as CIN (n=50) and normal cervical epithelium (n=8). Robust and consistent nuclear expression was observed for HDAC1 and HDAC2 in squamous cell carcinoma, adenocarcinoma and even CIN (Fig. 2). Interestingly, expression of HDAC1 and HDAC2 was apparent in the basal layer of normal cervical epithelium, but was rapidly lost at higher strata, consistent with an earlier study (31). The expression of HDAC6 was cytoplasmic, as expected for this class II HDAC (32), but was otherwise consistent with HDAC1 and HDAC2 (Fig. 2).

Synergistic effect of proteasome and pan-HDAC inhibitors in inducing caspase-mediated apoptosis in cervical cancer cell lines

Trichostatin A (TSA), a potent and broad spectrum HDAC inhibitor, was previously shown to trigger cell cycle arrest and apoptosis in HeLa cells. Therefore we tested the combination of Bortezomib and TSA for possible synergistic killing of HeLa cells (Figure 3). Isobologram analysis indicates that rather than simple additive killing, the combination of Bortezomib and TSA is highly synergistic, consistent with inhibition of complementary transforming activities. Like TSA, Vorinostat is a hydroxamate-based pan-HDAC inhibitor. Potent synergistic killing of HeLa cells treated with Vorinostat and Bortezomib was also observed. This phenomenon was extended to HPV16-transformed SiHa and CaSki cells and HPV68-transformed ME180 cells. The optimal combination index (CI) for each cell line was achieved at the following concentrations: SiHa (CI=0.42) Bortezomib 6nM/ Vorinostat 50 μ M, HeLa (CI=0.52) Bortezomib 3.12nM/TSA 150 nM, HeLa (CI=0.42) Bortezomib 0.8 nM/ Vorinostat 0.8 μ M, ME180 (CI=0.6) Bortezomib 25 nM/ Vorinostat 12.5 μ M, CaSki (CI=0.1) Bortezomib 50nM/ Vorinostat 3.12 μ M. This synergistic killing was not observed in the HPV-negative (p53-mutant) cervical cancer lines C33A and HT-3.

While E7 interacts with the class I histone deacetylases HDAC1 and HDAC2, TSA and Vorinostat broadly inhibit class I and class II HDACs with nM Kis. Furthermore, we have shown that like HDAC1 and HDAC2, the class II histone deacetylase HDAC6 is also highly expressed in cervical cancer (Fig. 2). Therefore it is possible that the synergy with Bortezomib occurs through inhibition of HDAC6 activity (32-34), rather than HDAC1 and HDAC2. Indeed, there is strong precedent for this possibility from studies in multiple myeloma (33) and ovarian cancer (32). Combination therapy with Bortezomib and the HDAC6-specific inhibitor Tubacin exhibited synergistic killing of multiple myeloma by triggering massive accumulation of polyubiquitinated protein (33). However, we found that the Bortezomib and Tubacin combination did not provide synergistic killing of HeLa cells, suggesting that inhibition of HDAC6 does not contribute to the synergy of Bortezomib and TSA (Fig. 3B). In support of this, HDAC6 is not upregulated in CaSki cells (Figure 2B), although synergistic killing by Bortezomib and TSA is observed.

While combination treatment with Bortezomib and HDAC inhibitors kills HeLa cells, the pathway of cell death is not clear. We therefore examined the effect of Bortezomib and TSA

upon apoptosis in HeLa cells by Annexin V and 7-AAD double staining and flow cytometric analysis. Evidence of synergy was observed in the massive apoptosis triggered by treatment with Bortezomib and TSA in combination (Fig. 4A). Cell death by apoptosis and the synergy of these two agents was further confirmed by demonstrating that a high percentage of cells staining positive for active Caspase-3 (Fig. 4B).

***In vivo* activity of proteasome and pan-HDAC inhibitors against a cervical cancer xenograft**

We then examined whether Bortezomib and TSA could be used in combination to treat xenograft tumors in immunodeficient mice. Immunodeficient mice (15/group female BNX mice) were inoculated with HeLa cells, and when tumor became palpable, the mice were randomly assigned to four treatment arms: vehicle alone, 1mg/kg Bortezomib, TSA 1mg/kg or both 1mg/kg Bortezomib and TSA. Treatment with either the proteasome inhibitor alone, or the HDAC inhibitor alone slowed the rate of tumor growth and significantly prolonged survival of the mice. The effect of treatment with these agents together was to further slow tumor growth and prolong the survival of the mice. This was associated with marked apoptosis, as evidenced by TUNEL staining, in the tumors harvested upon reaching 2cm³ from the group treated with both agents as compared with the tumors treated with vehicle alone (Fig. 4C). Likewise, immunoblot analysis of tumor treated with either agent alone showed some increase in PARP cleavage as compared with vehicle-treated tumor, whereas PARP cleavage was more extensive in tumor harvested from animals treated with both agents (Fig. 4D).

DISCUSSION

Efforts at directly targeting E6 and E7 with small molecules have had limited success, probably because this approach is best suited to inhibition of enzymic activity rather than protein-protein interactions. Typically such protein-protein interactions are interrupted using peptides but their efficacy as *in vivo* reagents is often compromised by their inability to maintain secondary structure, susceptibility to proteolytic degradation and difficulties in penetrating cells. As an alternative, we have attempted to inhibit cellular enzymic activities, namely proteasomal proteolysis and histone deacetylation, that are both co-opted by and, as indicated by genetic analysis, essential to HPV oncoproteins during cellular transformation. While this approach loses some of the specificity of directly targeting the HPV oncoproteins, the possibility of a therapeutic index remains given the requirement for these cellular processes in E6/E7-mediated oncogenesis. This hypothesis is borne out in preferential ability of proteasome and HDAC inhibitors, alone but most particularly in combination, to trigger apoptosis in HPV-transformed, as compared to HPV negative cervical cancers cells or keratinocytes.

The ability of Bortezomib to trigger death of the cervical cancer cell lines is associated with a recovery in the levels of wild type p53 but no change in the levels of PDZ proteins known to be targeted by E6 for proteasomal degradation, or pRB. The failure of Bortezomib to detectably enhance the levels of PDZ protein or pRB in the cervical cancer lines tested implies that they are degraded via proteasomal activities distinct from those targeted by this inhibitor; notably Bortezomib effectively inhibits the chymotryptic activity of the proteasomal machinery, but does not impact their caspase activity, and actually enhances the tryptic activity (35). It is therefore possible that other proteasomal inhibitors targeting a broader spectrum of activities could be more effective against cervical cancer by recovering both p53 and PDZ proteins. The natural product NPI-0052 irreversibly inhibits all three proteasomal activities, and like Vorinostat, is orally bioavailable, suggesting the potential of this combination (36).

The relative insensitivity of HPV-negative cervical cancer lines HT-3 and C33A, which both carry p53 mutations, suggests that recovery of wild type p53 in HPV+ cervical cancers is relevant to the latter's sensitivity to Bortezomib. However, it is important to recognize that recovery of wild type p53 levels in cervical cancer by Bortezomib treatment is not likely to be

its only anti-tumor mechanism. Indeed, overexpression of p53 in cervical cancer cells only marginally slowed their proliferation (37), and Bortezomib is cytotoxic for a cancer cells of diverse origin that do not express p53 (25,38). Bortezomib will trigger the accumulation of potentially toxic levels of poly-ubiquitinated proteins. In addition, Bortezomib has been shown to inhibit neo-angiogenesis in multiple tumor models and to block hypoxic responses (39-41).

TSA and Vorinostat are chemically-related inhibitors of the HDAC family, including both the nuclear and cytoplasmic HDACs including HDAC6. However the EC50 of Vorinostat inhibition of HeLa cell growth is $3\pm 1\mu\text{M}$ as compared to $0.18\pm 0.1\mu\text{M}$ for TSA, reflecting its lower potency against purified HDAC protein (42). Accumulating evidence suggests that a lysosomal pathway can compensate for intracellular poly-ubiquitinated protein degradation when proteasome activity is insufficient (33,43-45). A critical component of the lysosomal protein degradation pathway is the microtubule-associated deacetylase HDAC6 that directly interacts with misfolded and/or poly-ubiquitinated proteins to target them for lysosome-mediated protein degradation via aggresome formation/autophagy (46-48). Because misfolded and ubiquitinated proteins are degraded via both proteasomes and HDAC6-dependent autophagy, simultaneous inhibition of proteasome and HDAC6 has been proposed as a new strategy to synergistically induce cell death in multiple myeloma and pancreatic cancer settings (33,34). However, here we show that inhibition of HDAC6 does not account for the synergy between Bortezomib and Vorinostat or TSA for killing cervical cancer cells. Rather, it suggests that inhibition of other HDACs is likely to be important, most likely HDAC1 and HDAC2 since they are known to interact indirectly with E7, and genetic evidence suggests that these interactions are critical for E7-mediated transformation. Furthermore, knockdown of HDAC1 or HDAC2 expression in HeLa cells suppresses proliferation and promotes autophagy and apoptosis of HeLa cells respectively, but knockdown of HDAC4 and HDAC7 had no discernable effect on proliferation (31,49,50). Nevertheless, further studies are needed to validate the central role of HDAC1 and HDAC2 inhibition in the synergistic killing of cervical cancer cells by TSA or Vorinostat and Bortezomib.

The combination of Bortezomib and TSA proved more effective in slowing the growth of HeLa xenografts and prolonging the survival of mice than either treatment alone. However, we did not observe synergy, but rather an additive effect. This may reflect use of maximal, rather than sub-maximal doses, and suggests that additional titrations may be required to demonstrate synergy. Furthermore, Vorinostat, or possibly newer HDAC1/2 specific inhibitors, may be a more effective in vivo than TSA, and clinical trials using Bortezomib and Vorinostat are ongoing in other cancer types. Finally, Bortezomib acts via several mechanisms to retard tumor growth in vivo, including blockade of neo-angiogenesis and hypoxic responses, that may potentially make synergy with TSA treatment in vivo less apparent than in vitro.

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Figure 1. Effect of Bortezomib upon viability and p53 and PDZ protein levels in cervical cancer cell lines

(A) Cultures of HPV-transformed cervical cancer cell lines (HeLa, CaSki, SiHa, ME180), or cervical cancer cell lines lacking HPV (C33A and HT-3), or primary human keratinocytes (from donor pools 1 or 2) were treated with the indicated concentration of Bortezomib for 24h. Cell viability was determined by XTT assay and plotted as a fraction of untreated control cultures. B. Western blot analysis of p53, hMAGI, hDlg, hScrib and β-actin (as a loading control) in primary human keratinocytes (Kerat.), HeLa, CaSki, and C33A cell cultures after 24h of treatment with 1. buffer alone, or 2. 5nM Bortezomib, 3. 50nM TSA, or 4. 5nM Bortezomib+50nM TSA.

Figure 2. Expression levels of HDAC1, HDAC2 and HDAC6 in normal cervical epithelium versus cervical cancer and its precursors

(A) Representative micrographs showing immunohistochemical staining of HDAC1, HDAC2 and HDAC6 in normal cervical epithelium, CIN, SCC and adenocarcinoma. HDAC1 and HDAC2 are nuclear class I HDACs, whereas HDAC6 is a cytoplasmic class II HDAC. (B) The mean and standard deviation of the staining intensity score (arbitrary units) for HDAC1, HDAC2 and HDAC6 was plotted for normal cervical epithelium (N=8), CIN (n=50), SCC (n=141), adenocarcinoma (n=24). (C) Western blot analysis of HDAC1, HDAC2 and HDAC6 protein levels in two independent pools of primary human keratinocyte cultures, and C33A, HeLa and CaSki cells. β-actin is provided as a loading control.

Figure 3. Bortezomib and hydroxamate-based pan-HDAC inhibitors (TSA and Vorinostat) synergistically kill HPV-transformed cervical cancer cells without effect on keratinocytes or HPV-negative cells

(A1-A5) Cultures of HeLa, CaSki, ME180 and SiHa, were treated with checker board dilution series of Bortezomib and HDAC inhibitors TSA, Vorinostat. Cell viability was measured by XTT assay and calculated as percent of control untreated cultures. Synergy was demonstrated by plotting the interaction between drugs in isobolograms. The dotted diagonal corresponds to an additive effect and points below the diagonal exhibit synergy. (B) Cultures of HeLa cells were treated with checker board dilution series of Bortezomib and HDAC6 specific inhibitor Tubacin. Cell viability was measured by XTT assay and calculated as percent of control untreated cultures. (C1-C3) Cultures of keratinocytes and HPV-negative cells C33A and HT3 were treated with checker board dilution series of Bortezomib and HDAC inhibitor Vorinostat. Cell viability was measured by XTT assay and calculated as percent of control untreated cultures.

Figure 4. Bortezomib and TSA synergistically trigger apoptosis in HeLa cells

HeLa cell cultures were treated with buffer alone, 5nM Bortezomib, 50nM TSA, or 5nM Bortezomib+50nM TSA for 24h. The cells were harvested and staining for (A) Annexin-V and permeability to 7-AAD, or (B) the active form of caspase-3. The percentage of cells within each gate is indicated.

Figure 5. Treatment of mice with Bortezomib and TSA retards growth of HeLa xenograft
Immunodeficient mice (15/group NIH III female mice) were inoculated with HeLa cells, and when tumor became palpable, the mice were randomly assigned to four treatment arms: vehicle alone, 1mg/kg Bortezomib, 1mg/kg TSA or both 1mg/kg Bortezomib and TSA twice weekly. (A) The tumor volume (A) and survival (B) was recorded. Tumors were harvested one day after the final treatment and subjected to TUNEL staining (C). Alternatively tumor lysates were probed for levels of PARP (D) by Western blot.