Vorinostat and bortezomib exert synergistic antiproliferative and proapoptotic effects in colon cancer cell models

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Abstract
Despite the availability of several Food and Drug Administration-approved drugs, advanced inoperable colorectal cancer remains incurable. In this study, we focused on the development of combined molecular targeted therapies against colon cancer by testing the efficacy of the combination of the histone deacetylase inhibitor vorinostat with the proteasome inhibitor bortezomib to determine if this resulted in synergistic antitumor effects against colorectal cancer. The effects of the histone deacetylase inhibitor vorinostat in combination with the proteasome inhibitor bortezomib on the growth of two colorectal cancer cell lines were assessed with regard to proliferation, cell cycle arrest, and apoptosis. Treatment with the combination of vorinostat and bortezomib resulted in a synergistic decrease in proliferation of both colorectal cancer cell lines compared with treatment with single agents alone. This inhibition was associated with a synergistic increase in apoptosis as measured by caspase-3/7 activity and cleaved poly(ADP-ribose) polymerase. In addition, we observed an increase in the proapoptotic protein BIM and an increase in apoptosis as measured by caspase-3/7 activity and cleaved poly(ADP-ribose) polymerase. These data indicate that combination treatment with vorinostat and bortezomib result in synergistic antiproliferative and proapoptotic effects against colon cancer cell lines, providing a rational basis for the clinical use of this combination for the treatment of colorectal cancer. [Mol Cancer Ther 2009;8(2):342–9]

Introduction
Colorectal cancer is the third most prevalent cancer type in both men and women in the United States, accounting for 10% of estimated new cases, and is the third leading cause of cancer deaths among men and women (1). Colorectal cancer is currently managed with chemotherapy combinations using both “cytotoxics” (5-fluouracil, oxaliplatin, and irinotecan) and “biologics” targeting either the epidermal growth factor receptor (cetuximab and panitumumab) or the vascular endothelial growth factor (bevacizumab; ref. 2). Although multiple large clinical trials are under way evaluating the best way to combine and sequence these agents, unfortunately advanced unresectable colorectal cancer remains incurable with a yearly U.S. mortality of >50,000. Moreover, cytotoxic agents in particular have potentially serious side effects such as neutropenia and diarrhea with irinotecan, where the 60-day all-cause mortality approaches 6% in clinical trials (3). Clearly, new targets and new approaches to treatment are needed. One approach to overcome these limitations is the use of biologics in combination, with the goal of achieving synergistic anticancer activity that is greater than the single agents administered alone.

Histone acetylation is a post-translational modification of lysine residues in nucleosomal histone proteins that affects chromatin structure and thereby gene regulation. The acetylation status of histones is modulated by histone acetyl transferases and histone deacetylases (HDAC). Histone acetyl transferases are generally thought to be transcriptional activators because acetylated chromatin is transcriptionally active, whereas HDACs are considered transcriptional repressors as deacetylation of histones is associated with transcriptional repression. Recently, HDAC activity has been shown to be up-regulated in cancer cells and it has been theorized that this results in repression of tumor suppressor gene products such as p53, making HDACs an attractive drug target. In cell culture models, HDAC inhibitors (HDACi) have been shown to decrease proliferation rates, induce apoptosis, and induce autophagy-related cell death of several cancer cell lines. Due to their relative specificity toward cancer cells, HDACi represent a new class of cancer treatment agents that are generally well tolerated. One such compound, vorinostat, (suberoylanilide hydroxamic acid; Merck & Co.), has shown encouraging activity in early studies against several cancers including B-cell lymphoma, metastatic mesothelioma, papillary thyroid carcinoma, non-small cell lung carcinoma, and head and neck cancer and is currently approved for the treatment of cutaneous T-cell lymphoma (4, 5).

Bortezomib is a potent inhibitor of the 26S proteasome, the primary subcellular component of the protein degradation pathway that regulates the turnover of proteins
involved in cell cycle progression and apoptosis, such as the p21 cyclin-dependent kinase inhibitor, cyclins, and IkB, a regulator of nuclear factor-κB transcriptional activity (6). Bortezomib is currently approved for the treatment of multiple myeloma and has shown evidence of activity in other malignancies such as renal cell carcinoma, advanced non-small cell lung cancer, and mantle cell lymphoma (7, 8). Moreover, recent *in vitro* studies have shown that simultaneous targeting of HDACs and the 26S proteasome produce a synergistic inhibition of cell proliferation and induction of apoptotic cell death in pancreatic cancer cell lines (9), multiple myeloma (10), lung cancer, and hepatoma cell lines (11, 12) when compared with either single agent. In the present study, we examined the effectiveness of combining HDAC inhibition via vorinostat, with proteasome inhibition via bortezomib, on colorectal cancer cell lines *in vitro* with respect to their potential synergistic effects on cell proliferation. Our hypothesis was that these two agents would act synergistically to inhibit growth and induce apoptosis in colorectal cancer cell lines. We also sought to investigate the molecular mechanisms of synergy by assessing effects on cell cycle and downstream effector pathways. The intent of these studies was to provide supporting data for a mechanism-based regimen in the treatment of colorectal cancer.

**Materials and Methods**

**Cell Lines and Culture**

The human colon cancer cell lines HCT116 (p53 wild-type; ras mutated) and HT29 (p53 mutated; ras wild-type) were obtained from the American Type Culture Collection. Cells were grown in RPMI supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin and were maintained at 37°C in an incubator under an atmosphere containing 5% CO₂. The cells were routinely screened for the presence of *Mycoplasma* (MycopAlert; Cambrex BioScience) and were exposed to drugs when they reached ~70% confluence. Vorinostat was provided by Merck & Co. and prepared as a 10 mmol/L stock solution in DMSO. Bortezomib was purchased from Millennium Pharmaceuticals and prepared as 2.6 mmol/L stock solution in sterile saline.

**Evaluation of Cytotoxicity and Combination Effects**

Cytotoxic effects were determined using the sulforhodamine B method (13). Briefly, cells in logarithmic growth phase were transferred to 96-well flat-bottomed plates with lids. Cell suspensions (100 μL/mL) containing 5,000 viable HT29 or HCT116 cells were plated into each well and incubated overnight before exposure with different concentrations of drugs. Initially, HT29 and HCT116 cells were exposed for 24, 48, and 72 h to increasing concentrations of vorinostat (0-10 μmol/L) and bortezomib (0-0.5 μmol/L). After drug treatment, medium was removed and cells were fixed with cold 10% TCA for 30 min at 4°C. Cells were then washed with water and stained with 0.4% sulforhodamine B (Fisher Scientific) for 30 min at room temperature and washed again with 1% acetic acid followed by stain solubilization with 10 mmol/L Tris at room temperature. The plate was then read on a plate reader (Biotek Synergy 2) set at an absorbance wavelength of 565 nm. Cell proliferation curves were derived from the raw absorbance data. For determining synergy, vorinostat and bortezomib were tested at three different concentrations, 0.5, 1, and 3 and 0.05, 0.1, and 1 μmol/L, respectively, in all possible combinations. The results of the combined treatment were analyzed according to the isobolographic method of Chou and Talalay (14) using the Calcsyn software program (Biosoft). The resulting combination index (CI) was used as a quantitative measure of the degree of interaction between the two drugs. CI = 1 indicates additivity, CI > 1 indicates antagonism, and CI < 1 indicates synergism.

**Induction of Apoptosis**

Cells (5,000 per well) were seeded in 96-well, white-walled plates and allowed to attach for 24 h. The cells were then treated with vorinostat (0.5 and 3 μmol/L) or bortezomib (0.05 and 0.1 μmol/L) and in all possible combinations for 24 h, after which apoptosis was determined by the measurement of caspase-3 and -7 activity using a lumino metric Caspase-Glo-3/7 assay (Promega) according to the manufacturer’s protocol and read using a 96-well plate reader. Cellular apoptosis was expressed as the fold increase over untreated control cells.

**Flow Cytometric Analysis of Cell Cycle Distribution**

Cells (2 × 10⁵ per well) were seeded in 6-well plates and allowed to attach for 24 h. The cells were then treated with vorinostat (0.5 and 3 μmol/L) or bortezomib (0.05 and 0.1 μmol/L) and in all possible combinations of the two agents for 24 h, washed in PBS, and resuspended in Krishanaposs stain and allowed to incubate at 4°C for 24 h before analysis by flow cytometry at the University of Colorado Cancer Center Flow Cytometry Core Facility.

**Immunoblotting**

Cells were seeded into 6-well plates 24 h before treatment and exposed to each drug alone or the combination for an additional 24 h. After treatment, cells were scraped into radioimmunoprecipitation assay buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. The total protein in samples was determined using the BioRad D₆₇ Protein Assay. Total protein (30 μg) was loaded onto a 4% to 20% gradient gel, electrophoresed, and then transferred to Immobilon-P (Millipore). The membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in TBS containing Tween 20 (0.1%) before overnight incubation at 4°C with one of the following antibodies (all from Cell Signaling): anti-p21 mouse monoclonal antibody (1:5,000), anti-cyclin D1 rabbit polyclonal antibody (1:5,000), anti-p27 rabbit polyclonal antibody (1:2,000), anti-Bim (1:5,000), anti-Bcl-xL (1:5,000), and anti-poly(ADP-ribose) polymerase (PARP; 1:5,000). After the primary antibody, blots were washed 3 × 20 min in TBS-Tween 20 (0.1%), incubated with the appropriate secondary anti-rabbit or anti-mouse IgG horseradish peroxidase-linked antibody at 1:20,000 (Jackson Immunoresearch), and then transferred to Immobilon-Western.
Results

**Vorinostat and Bortezomib Interact Synergistically to Inhibit Colon Cancer Cell Proliferation**

In several *in vitro* cancer model systems, dual inhibition of HDACs and the 26S proteasome has been shown to result in synergistic inhibition of proliferation and induction of apoptotic cell death when compared with inhibition of each of these alone (9–12). To evaluate the potential synergistic effects of vorinostat and bortezomib against colorectal cancer cell lines, HCT116 and HT29 cells were exposed for 24 and 72 h to increasing concentrations of vorinostat or bortezomib or the combination, and effects on proliferation were assessed by the sulforhodamine B assay (13). Due to a high degree cellular toxicity at the 72 h time point, we chose to perform subsequent experiments at the 24 h time point. We determined that both HCT116 and HT29 cells were moderately resistant to vorinostat with IC_{50} values of >5.0 μmol/L, whereas both cell lines were more sensitive to bortezomib with IC_{50} values of 0.1 and 0.5 μmol/L, respectively (Fig. 1A and B). Based on these results, three concentrations of each compound were then chosen (0.5, 1.0, and 3.0 μmol/L vorinostat and 0.05, 0.1, and 1.0 μmol/L bortezomib) and assessed for synergy. As depicted in Fig. 2C and D, a 24 h exposure to the combination of vorinostat and bortezomib resulted in clear synergy with CI < 1 in both HT29 and HCT116 cells. Interestingly, the HCT116 cells appeared to be more sensitive to the combination with CI values of 0.07 to 0.4 compared with the HT29 cells (CI = 0.2-0.9).

**Induction of Apoptosis Is Potentiated with the Combination of Vorinostat and Bortezomib**

Next, we assessed the ability of these compounds, individually and in combination, to induce apoptosis in HT29 and HCT116 cells by measuring the activity of caspase-3 and -7, enzymes that play a central role in the execution phase of cell apoptosis. As shown in Fig. 3A and B, single-agent exposure to either dose of vorinostat or bortezomib led to a modest but not significant increase in caspase-3/7 activity in either HT29 or HCT116 cells. However, when either cell line was treated with vorinostat and bortezomib in combination, a dramatic increase in caspase-3/7 activity was observed in all combinations, indicating a synergistic increase in cells undergoing apoptosis (Fig. 3A and B). To confirm these biochemical effects, we evaluated the expression of PARP, an enzyme whose cleavage is reported to be required for the late stages of apoptosis (15). Whole-cell extracts of HT29 and HCT116 cells treated as described previously were subjected to immunoblotting with an antibody that recognizes both full-length and cleaved PARP (Fig. 3C and D). Consistent with the caspase-3/7 data, there was a marked increase in cleaved PARP in the cells exposed to the combination compared with the single agents in both cell lines that reached statistical significance (P < 0.001). Additionally, to investigate the mechanisms by which the combination of vorinostat and bortezomib induced apoptosis in HT29 and HCT116 cells, we assessed potential changes in known proapoptotic and antiapoptotic molecules, BIM and Bcl-xL, respectively. Surprisingly, treatment with vorinostat and bortezomib in combination had a minimal effect on the...
intracellular levels of the antiapoptotic protein, Bcl-xL, and the levels of BIM, a proapoptotic BH3-only protein, with single-agent or combination exposure in both cell lines when compared with the control (Fig. 3C and D).

**Cell Cycle Effects of Vorinostat and Bortezomib**

Previous studies have shown that HDACi and proteasome inhibitors administered in combination exhibit cell cycle regulatory effects that are reflected by inducing cell cycle arrest in the G2-M phase of the cell cycle (11). To determine if there were similar effects of these compounds in HT29 and HCT116 cells, we performed the single-agent and combination exposures and subjected the cells to cell cycle analysis by flow cytometry. As depicted in Fig. 4C and D, both cell lines treated with vorinostat or bortezomib for 24 h at the highest concentrations underwent a G2-M cell cycle arrest that was statistically significant, HT29 cells (P < 0.05 for vorinostat and P < 0.05 for bortezomib) as well as in the HCT116 cells (P < 0.05 for vorinostat and P < 0.01 for bortezomib). For example, HCT116 cells exposed to 0.01 μmol/L bortezomib for 24 h displayed 90% cells in G2-M compared to 25% in the control cells (P < 0.01). By contrast, although all combinations in both cell lines exhibited greater proportions of cells in G2-M compared with untreated controls, there was no evidence of an additive or synergistic effect of the combination compared with the single agents (P > 0.05 for all combinations compared with single agents).

**Analysis of Vorinostat and Bortezomib Effects on Prosurvival Signaling and Cell Cycle Regulatory Pathways in HT29 and HCT116 Cells**

Next, we analyzed the influence of these drugs, as single agents or in combination, on prosurvival signaling pathways in HT29 and HCT116 cells. We hypothesized that prosurvival proteins such as pAKT or pERK would be modulated up (reflecting a compensatory response) or down in cells undergoing apoptosis. Interestingly, no substantial changes in activation of these proteins were observed, except for a mild induction of pERK in bortezomib-exposed HCT116 cells (Fig. 5A and B). In some systems, it has been suggested that the proapoptotic effects of bortezomib are mediated through an increase in cyclin D1 and a subsequent decrease in the antiapoptotic factor Bcl-xL via a Stat3-dependent pathway (16). Although we did not observe any effects of bortezomib on cyclin D1 in either cell line, we did observe that both vorinostat and bortezomib treatment increased the levels of the cell cycle regulatory protein p21 alone and in combination (Fig. 5A and B). Notably, combination treatment also resulted in a marked increase in cleaved p21 (bottom band, ~14 kDa), which has been shown to be the result of caspase-3 activity on p21 and is suggestive of an acceleration of apoptotic cell death in cancer cells in response to anticancer agents (17). Consistent with its role as a HDACi, vorinostat treatment alone or in

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**Figure 2.** Effect of the combination of vorinostat and bortezomib on colon cancer cell lines. Approximately 5,000 HT29 or HCT116 cells were cultured in 96-well plates. Cells were treated with varying concentrations of vorinostat (0, 0.5, 1, and 3 μmol/L) and/or bortezomib (0, 0.05, 0.1, and 1 μmol/L) for 24 h. Raw proliferation data were expressed as percent viable cells for HT29 (A) and HCT116 (B). CI values for HT29 (C) and HCT116 (D) were calculated according to the Chou and Talalay mathematical model for drug interactions using CalcuSyn software.
combination increased the levels of acetylated histone H3 in both cell lines.

Because p21 appeared to be markedly induced with the combination of vorinostat and bortezomib, we used a shRNA approach to knock down p21 expression in the two cell lines to further assess its role as a mediator of the combination effects of these two drugs. As depicted in Fig. 6, we were able to achieve a significant knockdown of p21 protein levels in both cell lines (Fig. 6A and B, immunoblots). Surprisingly, we did not observe proliferative differences between a scrambled shRNA and p21 shRNA stable transfectants, indicating that p21 induction may be a bystander effect rather than a mediator of the observed phenotypic changes (Fig. 6A and B).

Discussion

Despite the availability of several Food and Drug Administration-approved drugs, advanced inoperable colorectal cancer remains incurable. In this study, we focused on the development of combined molecular targeted therapies against colon cancer and other gastrointestinal malignancies. Based on previous studies in other tumor types, we hypothesized that the combination of HDACi vorinostat with the proteasome inhibitor bortezomib would result in synergistic antitumor effects against colorectal cancer. We show here that vorinostat can interact synergistically with bortezomib to inhibit proliferation and induce cell cycle arrest and apoptosis of human colon cancer cells in vitro.

Recently, there have been advances in the understanding of molecular mechanisms underlying certain cancers. One of these advances has been the understanding of the role of acetylation and deacetylation of nucleosome core histones and the subsequent alterations in expression of protooncogenes and tumor suppressor genes in neoplastic transformation. Stemming from this is a greater appreciation for the potential role of HDACi as novel anticancer agents. Suberoylanilide hydroxamic acid (vorinostat) is a well-known HDACi that induces differentiation and/or apoptosis in vitro and has shown activity in vivo in solid and hematologic tumors (4, 18, 19). Recently, new anticancer drugs targeting the proteasome have been developed. One of these, bortezomib (PS-341, Velcade), is a dipeptide inhibitor of the 26S proteasome, which as a single agent, has been approved for treatment of patients with refractory multiple myeloma, and has shown antitumor activity alone and in combination with other chemotherapy in patients with non-small cell lung carcinoma (7).

In this study, we showed that, as single agents, both vorinostat and bortezomib exerted potent antiproliferative effects on two well-established colorectal cancer cell lines. Vorinostat has been shown previously to have antiproliferative action against a wide variety of cancer cell lines including, bladder, pancreatic, thyroid, breast, and gliomas (19). Likewise, in vitro studies have shown that bortezomib has cytotoxic effects on other cancer cell lines derived from solid tumors of the prostate, lung, and breast (7). The mechanism(s) by which HDACi such as vorinostat exert their biological effects is not completely known and seems
to vary by tumor type. Vorinostat has been shown to increase expression of cyclin-dependent kinases such as p21CIP1/WAF1 in bladder (20) and p27KIP1 in gliomas (21) but not in pancreatic cancer cell lines (22). Bortezomib acts by inhibiting the formation of the proteasome, an enzyme complex that plays a major role in ubiquitin-mediated degradation of proteins that regulate cell cycle (p16, p21, and p27), tumor suppressors (p53), the inhibitor of nuclear factor-κB (IκB), c-myc, Bax, etc. Additionally, bortezomib can induce cell cycle arrest and induce apoptosis against a variety of cell lines in vitro and in vivo (6). Previous studies in other cancer model systems have suggested that these drugs are more effective when combined with other agents, providing a rational basis for the current study (9–12). Consistent with this observation, we found that concurrent administration of the combination of vorinostat and bortezomib resulted in a synergistic inhibition of proliferation in colon cancer cell lines compared with either agent alone.

Mechanistically, the inhibition of proliferation could be due to a cytostatic effect or an increase in apoptosis. To further distinguish between these two, we measured markers of apoptosis as well as cell cycle regulators. The bulk of the inhibitory action of these drugs appears to be induction of apoptosis as we observed a synergistic increase in the activity of caspase-3 and -7, which are recognized markers of the late, execution phase of apoptosis. The intracellular pathways used by these drugs to induce apoptosis remain unclear and may be specific to any given cancer type. For example, treatment with vorinostat or bortezomib has been reported to cause alterations in the levels of apoptosis-regulating proteins including Bcl-xL (9), Bcl-2, and other Bcl-2-related proteins (10, 23). Our results indicate that an increase in the proapoptotic regulator protein BIM is involved in mediating the vorinostat/bortezomib apoptotic effect with no apparent role as an inhibitor of the Bcl-xL antiapoptotic regulator protein. BIM is a member of the BH3 domain death activator family that mediate cell death from physiologic stimuli such as cytokine deprivation, anoikis, a type of cell death characterized by detachment from the cell matrix, as well as by signals from activated oncogenes (24). Of all the BH3 family members, BIM and Puma are most critical for apoptosis induction because they have the ability to bind, and thereby antagonize, all the prosurvival proteins of the Bcl-2 family. Additionally, BH3 domain proteins have been shown to be important to cancer chemotherapy by mediating the cytotoxic responses elicited by these therapeutic agents. These findings have led to increased interest in developing “BH3 mimetics” as novel anticancer agents (25). Here, we have shown that
combination treatment with vorinostat and bortezomib produces a similar effect by raising intracellular levels of BIM endogenously. Interestingly, we observed a marked increase in acetylated H3 in cells exposed to the combination. These results suggest a role for bortezomib in the regulation of histone acetyl transferases and/or preferential degradation of acetylated H3 by the proteasome and could provide further insights into the mechanism of the observed synergistic effects. Although we observed very modest amounts of cleaved PARP when the colorectal cancer cell lines were treated with either agent alone, other groups have suggested that bortezomib may be acting to lower the apoptotic threshold, which permits synergistic action with addition of vorinostat (26).

In addressing the question of cytostatic effects of these drugs, we found that significant percentages of single agent-treated cells were arrested at G2-M cell cycle checkpoint; however, combination treatment did not significantly increase this effect. It is possible that the single-agent treatment in the HCT116 cell line has had a maximal effect on the percentage of cells at G2-M arrest (~75%) that cannot be further enhanced by combination treatment. Although this effect on cell cycle arrest has been observed previously in colorectal cancer cell lines with either agent alone (27, 28), to our knowledge, cell cycle analysis of these drugs combined has not been reported previously.

Activation of the growth factor/MEK/ERK pathway has been shown to be a critical mediator of antiapoptotic and prosurvival actions in numerous cancer model systems. Indeed, both HT29 and HCT116 colorectal cancer cell lines are characterized by constitutively active ERK phosphorylation and activity. Therefore, inhibition of ERK activity is a major goal in molecularly targeted therapies for colorectal cancer. Previous studies using HT29 cells have shown that coadministration of the MEK inhibitor PD184352 sensitizes cells to HDACi and subsequent cell death by apoptosis, suggesting that MEK inhibition is a requirement for optimal HDACi effects (29). In our study, treatment with vorinostat or bortezomib as single agents or in combination failed to result in lowered levels of pERK in either cell line tested. Despite this, we were able to achieve a synergistic effect on cell death via activation of caspase-3/7 with resulting apoptosis, indicating that inhibition of the MEK/ERK pathway is not an absolute requirement for the action of HDACi. Interestingly, although we observed a lack of inhibition of pERK with drug treatment, there was a modest increase in pERK levels in HCT116 cells treated with either dose of bortezomib alone. This effect of bortezomib on pERK levels has been reported previously and may be the reflective of sustained versus transient ERK activation, which has been associated with proapoptotic cell death (30).

In summary, our preclinical data suggest that combination therapy with vorinostat and bortezomib may be considered as an option for clinical treatment of colorectal cancer. Our studies indicate that the use of these drugs, administered simultaneously, results in significant synergy with respect to the inhibition of colorectal cancer cell proliferation and induction of BIM-mediated apoptosis in two well-characterized colorectal cancer cell lines in vitro. We are currently conducting in vivo colorectal cancer mouse...
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