Combined proteasome and histone deacetylase inhibition in non-small cell lung cancer

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Objective: Inhibitors of histone deacetylases are potent inducers of cell-cycle arrest and apoptosis in certain malignancies. We have previously demonstrated that chemotherapy activates the antiapoptotic transcription factor nuclear factor κ B in non-small cell lung cancer and fails to induce significant levels of apoptosis. We hypothesize that nuclear factor κ B inhibition with the proteasome inhibitor bortezomib (formerly known as PS-341) will sensitize non-small cell lung cancer cells to histone deacetylase inhibitor-mediated apoptosis.

Methods: Tumorigenic non-small cell lung cancer cells (A549, H358, and H460) were treated with bortezomib, followed by the histone deacetylase inhibitor sodium butyrate. After treatment, nuclear factor κ B transcriptional activity was measured by using a luciferase reporter assay and transcription of the nuclear factor κ B-dependent gene *IL8*. Apoptosis was determined on the basis of caspase-3 activation and DNA fragmentation. Western blot analyses for the cell-cycle regulatory proteins p21 and p53 were performed, and cell-cycle alterations were determined by means of FACS analysis. Experiments were performed in triplicate, and statistical significance was determined by using unpaired *t* tests.

Results: Butyrate increased nuclear factor κ B transcriptional activity 4-fold relative to that seen in control cells ($P = .05$) in all non-small cell lung cancer cell lines. Treatment with bortezomib reduced butyrate-induced activation of nuclear factor κ B to baseline levels. The proteins p21 and p53 were stabilized after treatment with bortezomib, correlating with a G₂/M cell-cycle arrest. Treatment with butyrate alone resulted in minimal apoptosis, but combined histone deacetylase and proteasome inhibition increased apoptosis 3- to 4-fold ($P = .02$).

Conclusions: Combined molecular targeting of histone deacetylases and proteasomes synergistically induced apoptosis in non-small cell lung cancer. Pharmacologic nuclear factor κ B suppression through proteasome inhibition, followed by treatment with histone deacetylase inhibitors, might represent a novel treatment strategy for patients with non-small cell lung cancer.

There has been a significant lack of improvement in the long-term survival of patients given a diagnosis of non-small cell lung cancer (NSCLC) over the past 30 years. Initial enthusiasm for combination third-generation chemotherapeutic agents has been tempered by the work of the recent Eastern Cooperative Oncology Group trial¹ and others,² which has shown that the benefits of chemotherapy for locoregionally advanced or metastatic NSCLC are marginal (an increase in survival

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of 2-3 months) in good-performance-status patients. This suggests that chemotherapy for advanced or recurrent NSCLC has likely reached a plateau, with little differences noted among various combinations of drugs.³ The lack of improvement in overall survival and the failure of current systemic therapies have mandated that new approaches to this disease be explored.

Advances in the understanding of acetylation and deacetylation of nucleosome core histones have prompted investigators to evaluate the role of histone deacetylase (HDAC) inhibitors as novel anticancer agents.⁴⁻⁷ HDACs have been associated with several well-characterized tumor suppressor genes (p53 and Rb) and the oncogene Myc.⁴ In addition, gene silencing by HDAC complexes has been shown to be an important mechanism in the development of acute promyelocytic leukemia, acute myeloid leukemia, and certain lymphomas.^{8,9} The inhibition of gene silencing by HDACs has resulted in the evaluation of several structurally dissimilar inhibitors of HDAC activity. HDAC inhibitors have been shown to induce tumor cells to undergo cell-cycle arrest,^{10,11} to upregulate p21 and p53 expression,^{10,12} to inhibit angiogenesis,¹³ and to promote apoptosis.^{6,14} The HDAC inhibitors phenylbutyrate, depsipeptide, and suberoylanilide hydroxamic acid (SAHA) are all currently in phase I or II clinical trials.⁵

In addition to exploring novel anticancer agents, such as HDAC inhibitors, another approach to improving the treatment of NSCLC is to identify and subsequently exploit the tumor biology of NSCLC by evaluating cell-signaling pathways that modulate resistance to current therapies. More than 80% of NSCLC tumor specimens overexpress subunits of the nuclear factor (NF) κ B family relative to adjacent normal lung tissue, suggesting that this antiapoptotic transcription factor is dysregulated in NSCLC.¹⁵ Our laboratory has previously reported that the transcription factor NF- κ B is upregulated in NSCLC after treatment with chemotherapy and that inhibition of NF- κ B with an adenovirally delivered dominant-negative inhibitor of NF- κ B markedly sensitized NSCLC cells to caspase-mediated apoptosis.^{16,17} Moreover, we have also recently found that various HDAC inhibitors activate NF- κ B–dependent transcription through Akt-mediated phosphorylation of the coactivator protein p300.¹⁸ Thus inhibition of the antiapoptotic NF- κ B pathway combined with a genotoxic stressor appears to be a plausible treatment strategy for patients with NSCLC.

NF- κ B is normally sequestered in the cytoplasm by the inhibitory protein I κ B. NF- κ B activation occurs by either cytokines or intracellular stress signals that result in Akt, IKK, or both kinase-mediated phosphorylation of I κ B. Specifically, phosphorylation of I κ B on serines 32 and 36 marks I κ B for ubiquitination and degradation by the 26S proteasome. I κ B degradation facilitates nuclear translocation of the transcriptionally active NF- κ B isoform p50/p65, leading

to transcription of NF- κ B–dependent antiapoptotic genes. Thus inhibition of proteasome-mediated degradation of I κ B would prevent subsequent translocation of NF- κ B to the nucleus and would be one approach to inhibit stimulus-induced NF- κ B activation. There are several small-molecule proteasome inhibitors currently undergoing evaluation, including bortezomib (Velcade, formerly known as PS-341)¹⁹ and MG-132.²⁰ Although no phase I studies have been performed with MG-132, phase I studies have been completed with bortezomib, and they suggest that it is well tolerated. In addition, it appears to have moderate efficacy in treating multiple myeloma and chronic lymphocytic leukemia.^{21,22}

Therefore the purpose of this study was to determine whether the small-molecule proteasome inhibitor bortezomib would abrogate HDAC inhibitor–induced NF- κ B activation in NSCLC and whether combined proteasome and HDAC inhibition would sensitize NSCLC cells to undergo apoptosis.

Methods

Cell Culture, Reagents, and Plasmid Constructs

Human NSCLC lines (NCI-H358, NCI-H460, and NCI-A549) were grown in RPMI-1640 (Life Technologies, Inc, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah) and penicillin-streptomycin. These cell lines were chosen because they comprise a representative panel of clinical NSCLC on the basis of their varying expression levels of p53, p16, and Rb proteins. The 3 $\times$ - κ B luciferase reporter construct contains NF- κ B DNA-binding consensus sites originally identified in the MHC class I promoter fused upstream to firefly luciferase. The Gal-4 luciferase construct contains 4 Gal-4 DNA consensus binding sites derived from the yeast GAL-4 gene promoter cloned upstream of luciferase cDNA. The Gal4-p65 fusion protein has the yeast Gal-4 DNA-binding domain fused to full-length p65 (1-551) and was previously described. Antibodies against p21 and p53 were obtained from Santa Cruz biotechnologies (Santa Cruz, Calif). Sodium butyrate and MG-132 were purchased from Sigma-Aldrich (St Louis, Mo), and bortezomib was kindly provided through a materials transfer agreement with Millennium Pharmaceuticals (Cambridge, Mass).

Cell Viability Assay

NSCLC cells were treated with sodium butyrate at the appropriate dose for 24 or 36 hours. Cell viability was determined by sequentially incubating the cells in 1% glutaraldehyde, 0.5% crystal violet, and distilled water at room temperature for 15 minutes each. Cells were then thoroughly dried, and crystal violet dye was eluted by incubating the cells in Sorenson's solution (30 mmol/L sodium citrate, 0.02 mol/L HCl, and 50% ethanol) at room temperature for 15 minutes. Optical density of the eluted dye was measured at a wavelength of 570 nm.

HDAC Assays

NSCLC cells, plated in 100-mm dishes for 18 hours, were left untreated or were cultured in the presence of sodium butyrate (500 μ mol/L). Eighteen hours after the addition of the HDAC inhibitor,

cells were harvested, and nuclear extracts were isolated by using previously described protocols.¹⁶ Nuclear protein samples were quantified by using the Pierce BCA protein assay dye reagent (Pierce, Rockford, Ill). Extracts (25 μ g) were analyzed for HDAC activity by using the HDAC kit from Biomol (Plymouth Meeting, Pa), as directed by the manufacturer.

Transfection and Luciferase Reporter Gene Assays

NSCLC cells at 60% to 80% confluency were transiently transfected by using Polyfect reagent (Qiagen, Valencia, Calif) according to the manufacturer's instructions. Briefly, plasmid constructs (0.75–1 μ g of DNA per well of a 12-well plate) were diluted in serum-free media and mixed with the Polyfect reagent (4 μ L per well). Complexes were allowed to form for 10 minutes before the addition of complete media containing 10% fetal bovine serum. The cells were washed once with 1 $\times$ phosphate-buffered saline (PBS), and Polyfect-DNA complexes were added to the cells and placed in a humidified incubator at 37°C with 5% CO₂. Six hours after the start of transfection, additional complete media containing the appropriate pharmacologic agent or agents for the experimental conditions were added to the cells. Twenty-four hours after transfection, cells were washed once with 1 $\times$ PBS and lysed in luciferase reporter buffer (Promega, Madison, Wis). Cells were then snap-frozen and thawed in liquid nitrogen and a 37°C water bath, respectively. Extracts were collected and cleared by means of centrifugation at 14,000 rpm. Protein concentrations were determined with a Pierce BCA protein assay kit reagent (Pierce). Luciferase assays were performed by using the substrate D-Luciferin (Promega), relative light units were measured with an AutoLumat LB953 luminometer, and luminescence was normalized to protein concentrations.

Western Blot Analysis

Logarithmically growing NSCLC cells were either left alone or were treated with butyrate, bortezomib, or both for 12 hours. Cells were harvested, and whole-cell lysates were prepared with RIPA buffer (150 mmol/L NaCl, 25 mmol/L Tris, 5 nmol/L ethylenediamine tetraacetic acid, 1% NP-40, and 0.5% deoxycholate). Cellular proteins were quantified by using the Pierce BCA protein assay kit. Proteins (50 μ g per well) were resolved on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Primary antibodies against p21 and p53 were used for immunoblotting.

Northern Blot Analysis

Logarithmically growing NSCLC cells were either left alone or were treated with sodium butyrate, bortezomib, or both for 12 hours. Total RNAs were isolated with Trizol reagent (Life Technologies Inc). RNAs (10 μ g per lane) were resolved on a denaturing 1.8% agarose-formaldehyde gel, transferred to Hybond membrane (NEN/Perkin Elmer Life Sciences, Boston, Mass), and cross-linked. Gene expression was determined by analyzing Northern blots with phosphorous 32 random-labeled probes generated from *IL8* or *GAPDH* cDNAs. After an 8-hour hybridization period, the blots were washed twice in 2 $\times$ standard saline citrate/0.1% sodium dodecylsulfate for 15 minutes at room temperature and twice in 0.1 $\times$ standard saline citrate/0.1% sodium dodecylsulfate for 15 minutes at 65°C. Northern blots were then analyzed by means of autoradiography.

FACS Analysis

NSCLC cells were treated with the appropriate pharmacologic agents for either 12 or 36 hours. Cells were then washed once with 1 $\times$ PBS, followed by trypsinization and resuspension in ice-cold 70% ethanol for 24 hours or longer. Fixed cells were then incubated for 12 hours in a propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/mL RNase, and 0.02 mg/mL propidium iodide), and DNA content was determined the following day by means of FACS analysis.

Apoptosis Assays

NSCLC cells were plated at 5×10^5 cells per well in a 12-well plate. Twelve hours after plating, cells were either left alone or treated with sodium butyrate, bortezomib, or both. After 30 hours of treatment, cells were harvested, and apoptosis was quantified by means of detection of caspase-3 activation and DNA fragmentation. Caspase-3 activity was determined by means of the addition of an APC-DEVD protein conjugate (Calbiochem, San Diego, Calif) to cellular lysates containing 25 μ g of protein. Fluorescence of caspase-3–cleaved protein conjugates was determined fluorometrically. DNA fragmentation was determined by means of evaluation of cellular nucleosome formation with the Cell Death Detection ELISA Plus kit (Roche, Indianapolis, Ind), according to the manufacturer's instructions.

Statistical Analysis

Statistical differences between treatment groups were determined by using a 2-tailed unpaired Student *t* test when appropriate.

Results

HDAC Inhibitors Fail to Induce Apoptosis in NSCLC Cells

HDAC enzymes reside within the nucleus, where they affect numerous pathways involved with transcription, cell-cycle regulation, differentiation, and apoptosis.^{4,6} Although it is currently not understood how the disruption of HDAC activity initiates apoptosis, HDAC inhibitors have been shown to induce eukaryotic cell death in vitro and in vivo.^{4,6} H358, H460, and A549 cells were treated with sodium butyrate at doses ranging from 0 to 5000 μ mol/L to determine whether the HDAC inhibitor sodium butyrate would induce apoptosis in NSCLC cell lines. Cell survival was determined by using a crystal violet assay. All NSCLC cell lines treated were resistant to sodium butyrate–induced death, even at the suprapharmacologic dose of 5000 μ mol/L (Figure 1, A). Cells were treated with butyrate, and nuclear extracts were prepared as previously described to determine whether sodium butyrate effectively inhibited HDAC activity in the NSCLC cell lines tested. Butyrate effectively inhibited 95% or more of the HDAC activity in each NSCLC cell line tested (Figure 1, B). These results suggest that the HDAC inhibitor sodium butyrate is limited in its ability to induce cell death in those NSCLC cell lines evaluated, despite effectively inhibiting HDAC activity.

Figure 1. A, NSCLC cells were treated with butyrate (0-5000 μmol/L) for 24 hours. Cell viability was determined on the basis of crystal violet uptake. B, NSCLC cells were incubated in the presence or absence of butyrate (500 μmol/L), and nuclear HDAC activity was determined.

Proteasome Inhibition Abrogates Sodium Butyrate-Induced Activation of NF-κB

The proteasome inhibitor bortezomib has been shown to inhibit cytosolic degradation of phosphorylated IκB, thereby preserving its inhibitory effects on NF-κB.²³ NSCLC cell lines were transiently transfected with either 3x-κB luciferase reporter constructs or cotransfected with a Gal4 luciferase reporter construct and a full-length p65 Gal4 expression construct to determine whether proteasome inhibition would reduce either basal or sodium butyrate-induced activation of NF-κB. Sodium butyrate significantly enhanced activation of NF-κB, as determined by the 3x-κB reporter gene, and this activation was completely abrogated by treatment with bortezomib (Figure 2, A). Similarly, bortezomib repressed the transactivation potential of p65 induced by sodium butyrate (Figure 2, B). Endogenous NF-κB activity was also determined by means of Northern blot analysis with radiolabeled probes specific for the tightly NF-κB-regulated endogenous gene *IL8*. Butyrate enhanced *IL8* transcription, but this increased transcription of *IL8* was reduced to basal levels after treatment with butyrate combined with bortezomib (Figure 2, C). Thus proteasome inhibition with the small-molecule inhibitor bortezomib sig-

Figure 2. A and B, NSCLC cells were transfected with either the 3x-κB reporter gene or cotransfected with Gal4-p65 and the Gal4-luciferase reporter gene and treated with nothing, butyrate, bortezomib (*bortz*), or butyrate and bortezomib (*P = .04). C, A549 cells were similarly treated, and Northern blot analysis for *IL8* was performed.

nificantly reduced activation of the transcription factor NF-κB.

Proteasome Inhibition Preserves Cell-Cycle Protein Levels and Induces a G₂/M Cell-Cycle Arrest

Previous reports have indicated that proteins involved in cell-cycle regulation are degraded by cytosolic proteasomes.¹⁹ Western blot analyses were performed on cells treated for 12 hours with sodium butyrate, bortezomib, or both to address the question of whether bortezomib would affect cell-cycle regulatory proteins and induce subsequent cell-cycle dysregulation in NSCLC. Sodium butyrate induced no appreciable increases in p21 and p53 protein levels. In contrast, bortezomib, alone or with butyrate, stabilized levels of p53 and p21 (Figure 3, A). FACS analysis was performed on each of the 3 cell lines with either sodium butyrate, bortezomib, or both for 12 hours to determine whether dysregulation of p21 or p53 levels correlated with alterations in the cell cycle. The 12-hour treatment period

Figure 3. A, H358, H460, and A549 cells were treated with nothing, bortezomib (*bortz*), bortezomib and butyrate, or butyrate alone. Western blots were performed with antibodies for p21, p53, and β -tubulin. B, NSCLC cells were treated as noted above and analyzed by means of FACS analysis.

was selected to allow time for cells to accumulate at a given arrest point but to prevent DNA fragmentation caused by apoptosis. Sodium butyrate alone did not appear to have a significant effect on cell-cycle regulation, but treatment with bortezomib was associated with a marked G_2/M arrest (Figure 3, B) in all cell lines.

Combined Proteasome and HDAC Inhibition Enhance Cell Death Through Apoptosis

On the basis of data presented in Figure 3, further experiments were conducted to determine whether inhibition of NF- κ B transcription with bortezomib would sensitize NSCLC cells to butyrate-induced cell death. NSCLC cells

Figure 4. NSCLC cells were treated with nothing, butyrate, bortezomib (*bortz*), or combined butyrate and bortezomib. Cell survival was determined by means of crystal violet assay (* $P = .01$, † $P = .06$).

were treated with nothing, butyrate, bortezomib, or both butyrate and bortezomib, and cell death was determined by means of the crystal violet assay. As shown in Figure 4, there is a significant decrease in NSCLC cell survival after combined proteasome and HDAC inhibition compared with after any of the other treatments ($P = .01$). DNA fragmentation and caspase-3 assays were performed to assess whether this decrease in cell survival was caused by apoptosis, as shown in Figure 5, A and B. Combined treatment with butyrate and bortezomib significantly increased DNA fragmentation relative to any of the other treatment groups ($P = .02$; Figure 5, A). Similar results were seen with caspase-3 activation after combined treatment with butyrate and bortezomib relative to the other treatment groups ($P = 0.03$; Figure 5, B). Thus combined HDAC and proteasome inhibition significantly increased DNA fragmentation and caspase-3 activation, which is consistent with cellular death occurring by means of apoptosis. FACS analysis was performed on the H358 NSCLC cells to assess the degree of apoptosis, and there was a 3- to 12-fold increase in apoptosis in the combined treatment group, as measured by the percentage of cells in the subG₀/G₁ phase.

Discussion

Current treatment options for patients with locoregionally advanced, metastatic, or recurrent NSCLC include chemotherapy, irradiation, palliative care, and, rarely, surgical intervention. Unfortunately, in the majority of cases these treatment strategies result in little improvement in survival, although symptom relief and quality of life might be im-

proved. Thus there is an increasing effort to better understand the biology of tumors, including their ability to grow and metastasize, as well as what genes or signal transduction pathways regulate their resistance to conventional therapies. Despite advances in understanding the biology of certain tumors (ie, chronic myeloid leukemia) and the successful clinical translation of those findings (ie, Gleevec, Novartis Pharmaceuticals), similar success stories have remained elusive for solid tumors, particularly lung cancer.

Histone acetylation-deacetylation controls gene transcription and is regulated by 2 families of enzymes, histone acetyltransferases and HDACs.^{4,5} Transcriptionally active genes are typically associated with increased histone acetylation, whereas low levels of acetylation correlate with transcriptional repression.⁴ Inhibition of HDAC activity by HDAC inhibitors has recently been shown to mediate tumor cell growth arrest, enhance apoptosis, and promote cell-cycle arrest.^{10-13,24} Encouraging in vitro and in vivo results have led to several Phase I trials with the HDAC inhibitors phenylbutyrate,²⁵ SAHA,²¹ and depsipeptide.²⁶ Although toxicity profiles with these agents have been favorable, it is unclear how effective these agents will be in affecting a tumor response. Using 3 different NSCLC cell lines and the HDAC inhibitor sodium butyrate, we were able to demonstrate that despite completely inhibiting all intranuclear deacetylase activity, all NSCLC cell lines evaluated were resistant to HDAC inhibitor-mediated cell death (Figure 1, B). These results have been confirmed with other HDAC inhibitors, including trichostatin A (TSA) and SAHA.¹⁸

Figure 5. A, NSCLC cells were treated with nothing, sodium butyrate, bortezomib (*bortz*), or butyrate and bortezomib. Apoptosis was assessed and quantified by means of DNA fragmentation (A, $*P = .02$), caspase-3 activation (B, $*P = .03$), and flow cytometry (C).

On the basis of previous work by our group,^{16-18,23} we next sought to identify whether HDAC inhibition activated the antiapoptotic transcription factor NF- κ B. Recent studies have suggested that acetylation of specific lysine residues on the RelA/p65 subunit of NF- κ B controls DNA binding and facilitates I κ B-mediated nuclear exportation of NF- κ B, thus regulating NF- κ B-dependent transcription.²⁷ As shown in Figure 2, butyrate increased NF- κ B-dependent transcription, and this effect was ameliorated after treatment with the proteasome inhibitor. The exact mechanism by which HDAC inhibitors activate NF- κ B is unclear, although we have recently shown that it likely involves phosphory-

lation of the coactivator protein p300 by the serine-threonine kinase Akt.¹⁸

The 26S proteasome is the primary component of the protein degradation pathway of the cell, and its rapid and irreversible elimination of targeted proteins can activate or repress many cellular processes, including cell-cycle progression and apoptosis.¹⁹ We have previously shown that chemotherapy can activate NF- κ B by initiating phosphorylation of a cascade of cytosolic kinases culminating in the phosphorylation of I κ B, thus marking it for ubiquitination and subsequent degradation by the 26S proteasome.²³ Therefore proteasome inhibition permits cytosolic seques-

tration of NF- κ B by stabilizing I κ B and ameliorating all NF- κ B-dependent transcription. Several studies have shown that proteasome inhibition has antitumor activity both alone and combined with chemotherapy, radiation, or both.¹⁹ This encouraging antitumor activity has been shown to be the result of inhibition of NF- κ B, as well as cell-cycle arrest. The only proteasome inhibitor to enter clinical trial is bortezomib.^{19,21} Phase I trials with bortezomib have been completed and show that it is well tolerated.²¹ On the basis of these results and our desire to identify a small-molecule inhibitor of NF- κ B, experiments were performed to determine whether bortezomib would inhibit butyrate-induced NF- κ B activation. As shown in Figure 2, in all NSCLC cell lines evaluated, treatment with bortezomib significantly inhibited butyrate-induced NF- κ B-dependent transcription.

In addition to inhibiting NF- κ B activation, bortezomib also has been shown to stabilize the cell cycle proteins p53, p21, and p27.¹⁹ Similarly, HDAC inhibitors have been shown to stabilize p21, downregulate cyclins A and D, hypophosphorylate the tumor suppressor Rb, and induce both G₁ and S-phase cell-cycle arrest.^{3,4,12,24} Data shown in Figure 4 indicate that the HDAC inhibitor butyrate partially stabilized p21 in one cell line but not others and that bortezomib alone and bortezomib-butyrate stabilized both p21 and p53 protein levels. Other series have shown stabilization of p21 with HDAC inhibition alone,^{12,24} but we did not observe as robust of an increase as expected. This might relate the specific HDAC inhibitor or cell type used for experimentation. Importantly, combined HDAC and proteasome inhibition did demonstrate a significant G₂/M cell-cycle arrest by means of FACS analysis (Figure 4, B), which correlates with a previous report by Ling and colleagues.²⁸ Thus combined HDAC and proteasome inhibition stabilized p21 and p53 levels and induced a G₂/M phase arrest in our NSCLC cells.

In addition to demonstrating that combined HDAC and proteasome inhibition facilitates cell-cycle arrest, we also demonstrated that this combined treatment markedly enhanced NSCLC cell death. This cell death was caused, in part, by apoptosis, as measured by means of DNA fragmentation, caspase-3 activation, and FACS analysis. There was also a significant cell-cycle arrest after combined treatment, as noted in Figure 3. The observation of enhanced apoptosis in the setting of a cell-cycle arrest indicates that HDAC inhibitor-mediated apoptosis might not depend on cell-cycle progression, as is generally true for traditional chemotherapeutic agents. Although the exact mechanism of HDAC inhibitor-mediated apoptosis is not known, previous reports have found treatment with HDAC inhibitors to result in increased mitochondrial cytochrome c release, cleavage of the proapoptotic Bcl-2 family member BID, and generation of reactive oxygen species.¹⁴ We and others have also found that HDAC inhibitor-induced apoptosis can occur

independent of a death receptor pathway or the expression of functional p53.^{14,18} Finally, we have also shown that treatment with bortezomib and SAHA generates significant reactive oxygen species that are proapoptotic in NSCLC cells.

Limitations of this experimental approach include the fact that only one HDAC and proteasome inhibitor were evaluated. However, we have observed nearly identical results with other HDAC inhibitors (TSA and SAHA) and the proteasome inhibitor MG-132. We chose to evaluate butyrate and bortezomib because both have been through Phase I clinical trials and because they have been the focus of other experiments in the laboratory. Another potential criticism of the study is that our findings are limited to our NSCLC cell-culture model system. In vivo experiments with NSCLC xenografts treated with combined HDAC and proteasome inhibitors are currently ongoing in the laboratory. Finally, we only used one dose of sodium butyrate, and there might have been further enhancement of apoptosis if a dose-response curve had been generated. We chose the dose of butyrate used in our experiments to be equivalent to the biologic active concentration of 0.5 mmol/L identified in the Phase I trial of butyrate in patients with refractory solid tumor malignancies.²⁵

To our knowledge, this is the first study to evaluate combined proteasome and HDAC inhibition in NSCLC, as well as in any other malignancy. In a disease in which meaningful treatment options have remained frustratingly few, pharmacomolecular treatment strategies are likely to hold promise for the future. The genesis of this line of investigation is based on our increasing understanding of the important role that NF- κ B cell-signaling cascades play in modulating resistance to both traditional and novel anti-cancer therapies. This preliminary report strongly suggests that combined proteasome and HDAC inhibition merits further investigation as a treatment strategy for patients with NSCLC and potentially other solid tumor malignancies.

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Discussion

Dr D. Nguyen (Bethesda, Md). Have you tested all the HDAC inhibitors like SAHA or TSA?

Dr Denlinger. Yes, we performed similar experiments with several HDAC inhibitors, including TSA and SAHA, and have observed very similar results with each of these compounds.

Dr Nguyen. Can you offer some thought about mechanisms why these drugs induce NF- κ B activation?

Dr Denlinger. Traditional chemotherapeutic agents activate NF- κ B by inducing nuclear translocation of the transcriptionally active p50/p65 heterodimer. In contrast, HDAC inhibitors appear to enhance NF- κ B-dependent gene transcription without inducing nuclear translocation. HDAC inhibitors activate NF- κ B by recruiting transcriptional coactivators to NF- κ B-dependent promoters. For example, we have recently demonstrated that the HDAC inhibitor butyrate facilitates recruitment of the transcriptional coactivator protein p300 to the IL-8 promoter. This correlates with enhanced transcription of the NF- κ B-dependent gene *IL8*. Therefore we believe that the HDAC inhibitors activate NF- κ B by enhancing the transcriptional activity of p50/p65 heterodimers present in the nucleus at baseline.

Dr Nguyen. You show in the Western blot about the p21 upregulation, and you show only one cell line. In the 460s, as I could see, there is not much increase in p21 at all. In my experience I have looked at different HDAC inhibitors, and it is uniformly increased in all lung cancer-associated mesothelioma, and usually upregulated p21 is a very good surrogate marker of HDAC activities in the cells. Have you tried other blots or an enzyme-linked immunosorbent assay to pick up p21 upregulation in the 460s?

Dr Denlinger. Could you rephrase that?

Dr Nguyen. Did you reblot the 460s with other HDAC inhibitors to see whether p21 also was upregulated?

Dr Denlinger. We also treated the H460 cell line with TSA and did not observe significant upregulation of p21, despite the fact that HDAC inhibitors have been shown to upregulate p21 in most NSCLC cell lines.

Dr Nguyen. The last thing I want to check with you is whether you have tried to block NF- κ B directly instead of using a proteasome inhibitor? As you know, a proteasome inhibitor would do a lot of other things in addition to blocking NF- κ B. Have you tried using other agents? There are pharmacologic agents that are very good at blocking binding of active NF- κ B through the DNA consensus sequence of the DNA-binding site of NF- κ B. Have you tried that yet?

Dr Denlinger. We have also combined HDAC inhibition with direct inhibition of NF- κ B by using a mutant I κ B that is incapable of phosphorylation and degradation. This I κ B mutant protein binds NF- κ B in the cytoplasm and likely depletes the nuclear NF- κ B population. We have shown that the mutant I κ B blocks HDAC inhibitor-induced activation of NF- κ B and sensitizes NSCLC to HDAC inhibitor-induced apoptosis. Importantly, this I κ B mutant functions at essentially the same level as the proteasome inhibitor bortezomib. Whereas the I κ B mutant cannot be phosphorylated at serines 32 and 36, which marks I κ B for degradation by the 26S proteasomes, bortezomib prevents degradation of endogenous I κ B despite its phosphorylation at serines 32 and 36. We prefer to experiment with the pharmacologic inhibitor of NF- κ B because it is likely to have greater potential for clinical application.