Chronic lymphocytic leukemia (CLL) is a generally incurable, accumulative disease of relatively mature B lymphocytes. Standard treatment of CLL includes the use of alkylating agents (e.g., chlorambucil and cytoxan), corticosteroids, nucleoside analogues (e.g., fludarabine), and antibodies directed against CLL surface proteins [i.e., CD20 (rituximab)], alone or in combination (1). However, it remains uncertain whether these therapies are able to extend survival of CLL patients. Thus, current attempts have focused on development of new therapies involving small-molecule inhibitors that specifically target survival signaling pathways (1). Although the pathogenesis of CLL is unclear, there is evidence that dysregulation of the nuclear factor-κB (NF-κB) pathways may be involved. NF-κB represents a family of heterodimeric transcription factors, of which RelA(p65)/p50 is the most abundant, which regulate diverse cellular processes, including proliferation, differentiation, inflammatory responses, and cell survival (2). In B-CLL cells, the NF-κB pathway has been implicated in mediating diverse survival signals involving pathogenesis of CLL (3, 4). Notably, CLL cells exhibit pronounced constitutive activation of NF-κB (5) and, in preclinical studies, display marked sensitivity to NF-κB inhibitors (4, 6). Thus, attention has focused on novel agents targeting such survival pathway.

Proteasome inhibitors, including bortezomib (Velcade; formerly known as PS-341), represent a class of agents that inhibit the 20S proteasome and thereby block the elimination of diverse cellular proteins targeted for degradation (7). In preclinical studies, bortezomib preferentially kills transformed cells compared with their normal counterparts (8). Proteasome inhibitors, such as bortezomib, have been developed as anticancer agents based on their ability to block proteasomal degradation of the NF-κB–inhibitory protein IκB, an action that traps NF-κB in the cytoplasm and prevents its nuclear translocation and activation (9). Bortezomib has shown remarkable activity in multiple myeloma (10), a B-cell
malignancy characterized by constitutive NF-κB activation. In view of a requirement for NF-κB in CLL cell survival (5), bortezomib represented a logical candidate agent for the treatment of this disease. Indeed, bortezomib and more recently another novel proteasome inhibitor, NPI-0052, are potent inducers of apoptosis in CLL cells in vitro (11, 12). However, clinical experience with bortezomib in CLL is limited, and initial evidence suggests that in this disease bortezomib has modest to minimal activity as a single agent (13, 14), thereby further exploration of this agent in combination with other agents is warranted.

Romidepsin (depsipeptide; FK228) and belinostat (PXD101) are inhibitors of histone deacetylases (HDAC), proteins that, in conjunction with histone acetyltransferases, regularly regulate acetylation of lysine residues residing on the positively charged histone tails of nucleosomes. Belinostat, like other hydroxamic acid analogues, is a pan-HDAC inhibitor (HDACi; ref. 15), which inhibits class I and II HDACs [e.g., reflected by increased acetylation of histone H3 and H4 (16) as well as α-tubulin (17), respectively]. In contrast, romidepsin is thought to inhibit primarily class I (e.g., HDAC1 and HDAC2) versus class II HDACs (e.g., HDAC4 and HDAC6; ref. 18). By inhibiting HDACs, HDACi promote histone acetylation, which leads to a more open chromatin configuration that generally favors gene transcription. However, HDACi induce cell death in neoplastic cells through multiple mechanisms, including up-regulation of death receptors, disruption of Hsp90 function, and generation of reactive oxygen species (15, 19). Notably, HDACi block RelA deacetylation mediated by class I HDACs, thereby increases NF-κB/DNA binding and its transcriptional activity (20); conversely, inhibiting this process lowers the threshold for HDACi lethality (21, 22). HDACi, including romidepsin, have shown single-agent efficacy in cutaneous T-cell lymphoma (23). Single-agent romidepsin has also shown modest activity in CLL (24), although this agent, like other HDACi (e.g., MS-275), markedly induces CLL cell apoptosis in preclinical settings (25–27). Such findings have stimulated efforts to improve the therapeutic efficacy of HDACi by combining them with other drugs, including other targeted agents (19).

Previous studies by several groups, including ours, have shown that in malignant hematopoietic cells (i.e., leukemia and myeloma) coadministration of proteasome inhibitors with various classes of HDACi, including hydroxamate (e.g., vorinostat, belinostat, LBH589, and tubacin), cyclic tetrapeptide (e.g., romidepsin), benzamidine (e.g., MS-275), and short-chain fatty acid (e.g., butyrate and valproic acid), results in a dramatic increase in cell death (28–32). One possible mechanism for this phenomenon is that HDACi, by acetylating Hsp90 and/or other proteins (e.g., tubulin), disrupt protein folding and the formation of aggresomes, subcellular structures responsible for clearance of misfolded proteins (30, 31). Furthermore, interference with proteasome function (i.e., by proteasome inhibitors) in the face of dysregulated protein folding and trafficking/removing (e.g., by HDACi) has been shown to efficiently trigger the apoptotic response (30–32). As NF-κB activation represents a determinant of HDACi effectiveness in induction of apoptosis (21, 33), an alternative mechanism is that NF-κB inactivation (i.e., by bortezomib) might interrupt cytoprotective NF-κB–dependent responses, thereby enhancing HDACi lethality (22, 28).

Currently, no information is available about interactions between HDAC and proteasome inhibitors in CLL cells, particularly in primary CLL cells. To address this issue, we have examined responses to regimens combining bortezomib with romidepsin or belinostat, two clinically relevant novel HDACi, in primary samples obtained from CLL patients, as well as established CLL cell lines. Here, we report that low (i.e., nanomolar or submicromolar) concentrations of romidepsin and belinostat interact in a synergistic manner with very low concentrations of bortezomib to induce cell death in CLL cells. Furthermore, these effects are associated with NF-κB inactivation, down-regulation of antiapoptotic proteins (e.g., Mcl-1, Bcl-xL, and XIAP), and induction of the proapoptotic protein Bim.

Materials and Methods

Cells and reagents. Peripheral blood samples were obtained with informed consent from five CLL patients with approval from the institutional review board of Virginia Commonwealth University. Three patients were untreated (Rai stage 0–1), one patient (stage 1) had received prior chlorambucil, and one patient (stage 4) had received extensive prior therapy (fludarabine, rituximab, and CVP). Informed consent was provided according to the Declaration of Helsinki. Mononuclear cells were isolated by centrifugation at 400 × g for 38 min over Histopaque-1077 (Sigma Diagnostics). Viability of the cells was regularly >95% by trypan blue exclusion. Isolated cells are population of clonal B lymphocytes expressing both CD19 and CD5 (>95%), determined by flow cytometry using Simultest CDS/CD19 antibodies (Rector Dickinson). Primary CLL cells were incubated in RPMI 1640 containing 10% fetal bovine serum as described previously (34). The experiments generally involved a 24-h treatment interval to minimize spontaneous cell death (e.g., <10%), a phenomenon associated with longer exposure intervals (35). Human MEC-2 (chronic B-cell leukemia) and JVM-3 (B-prolymphocytic leukemia) cell lines were obtained from the German Collection of Microorganisms and Cell Cultures and maintained in Iscove’s MDM medium (containing 10% fetal bovine serum, 200 units/mL penicillin, and 200 µg/mL streptomycin) and RPMI 1640 as described above, respectively. Primary CLL cells were diluted to 1 × 10⁶/mL before drug treatment. All experiments for MEC-2 and JVM-3 cells were done using logarithmically growing cells (3 × 10⁵ to 5 × 10⁶/mL).

The proteasome inhibitor bortezomib was provided by Millennium. The HDACi romidepsin and belinostat were provided by Gloucester and CuraGen, respectively. These agents were dissolved in DMSO and stored at -20°C. Stock solutions of these agents were subsequently diluted with serum-free RPMI 1640 before use. In all experiments, the final concentration of DMSO did not exceed 0.1%.

Transient transfection. Constructs encoding green fluorescent protein (GFP)-tagged wild-type RelA or its acetylation site (inactive) mutants (i.e., K210R, K310R, and K218R/K221R/K310R triple mutation/KR) were kindly provided by Dr. Warner C. Greene (University of California, San Francisco, CA; ref. 36). Primary CLL cells (1 × 10⁶) were transfected with these constructs by Amaxa Nucleofector Device (program U-15) using Human B Cell Nucleofector kit (Amaxa GMBH). After incubation at 37°C for 6 h, transfected cells were treated with HDACi for another 24 h and then subjected to analysis of cell death by flow cytometry following 7-aminoactinomycin D (7AAD) staining.

Assessment of cell death. The extent of cell death was evaluated by 7AAD staining and flow cytometry. Briefly, cells were incubated with 0.5 µg/mL 7AAD (Sigma Diagnostics) at 37°C for 20 min, and then the percentage of cells exhibiting 7AAD positive was determined using Becton Dickinson FACScalibur.

Western blot analysis. Samples from whole-cell lysates were prepared, and 30 µg/condition of proteins were subjected to Western blot analysis as previously described in detail (34). The blots...
were probed with the appropriate dilution of primary antibody as follows. Where indicated, the blots were reprobed with anti-β-actin (Sigma Diagnostics) or anti-α-tubulin (Oncogene, Inc.) to ensure equal loading and transfer of proteins. The primary antibodies included the following: anti-acetylated (acetyl K310) p65 (Abcam); anti-caspase-9 and anti-caspase-3 (BD PharMingen); anti-cleaved caspase-9 (Asp175), anti-cleaved caspase-3 (Asp175), anti-phosphorylated β-Bo (Ser194), anti-p100/p52, and anti-Bcl-xl (Cell Signaling); anti-acetylated histone H3 and anti-β-Bo (Upstate Biotechnology); anti-p65, anti-Mcl-1, anti-A1, anti-c-FLIP, and anti-ICAM-1 (Santa Cruz Biotechnology); anti-cIAP1, anti-cIAP2, and anti-survivin (R&D Systems); anti-poly(ADP-ribose) polymerase (PARP; Biomol); anti-acetylated tubulin (Lys 40; Sigma Diagnostics); anti-human Bcl-2 oncoprotein (Dako); anti-XIAP (BD Transduction Laboratories); and anti-Bim (Calbiochem). In some cases, the density of blots was quantified using FluorChem 8800 Imaging System (Alpha Innotech) and VideoTesT-Master software (VideoTesT Ltd.).

NF-κB/luciferase reporter assay. 3×κB-Luc reporter and mutant (inactive) 3×κB-Luc were kindly provided by Dr. Albert S. Baldwin, Jr. (University of North Carolina, Chapel Hill, NC; ref. 37). Primary CLL cells (1 × 10^7) were transfected with 1 μg of 3×κB-Luc reporter or its mutant construct as described in “Transient transfection.” Cells were incubated for 6 h and subsequently treated with the indicated agents. After 18-h treatment, cells were harvested and subjected to a luciferase assay using a Luciferase Reporter Assay kit (BD Clontech) as per the manufacturer’s instructions. Relative luciferase activity was determined by normalizing to amount of total proteins. NF-κB activity was expressed as fold increase relative to untreated controls.

Statistical analysis. For analysis of cell death (7AAD/flow cytometry), values represent the mean ± SD for at least three separate experiments done in triplicate (for cultured cell lines) or at least one experiment, depending on cell availability, done in triplicate (for primary CLL cells). The significance of differences between experimental variables was determined using the Student’s t test. Analysis of synergism was done according to median dose-effect analysis (38) using the software program CalcuSyn (Biosoft).

**Results**

Coadministration of romidepsin and belinostat with bortezomib results in a dramatic increase in cell death in primary CLL cells. To examine interactions between HDACIs and bortezomib in primary CLL cells, cells from five CLL patients were isolated and simultaneously exposed to romidepsin (3-5 nmol/L) or belinostat (500 nmol/L) in the presence or absence of bortezomib (3-5 nmol/L) for 24 h, after which cell death was determined by 7AAD staining and flow cytometry. As shown in Fig. 1A, exposure to HDACIs or bortezomib individually resulted in minimal increases in cell death compared with controls (i.e., generally <20%), whereas combined exposure to either HDACI in conjunction with bortezomib resulted in a major potentiation of cell death (i.e., 45-80%). Figure 1B illustrates representative DNA histograms, revealing a major right shift in 7AAD uptake for cells coexposed to both agents. These results indicate that coadministration of romidepsin or belinostat with bortezomib results in a dramatic increase in cell death in primary human CLL cells.

Combination regimens of HDACIs and bortezomib induce a marked increase in caspase activation and PARP cleavage in primary CLL cells. After coexposure to bortezomib with romidepsin or belinostat for 24 h, primary CLL cells exhibited...
morphologically apoptotic changes (Supplementary Fig. S1). To gain insights into the apoptotic pathways involved in cell death induction by HDACIs and bortezomib in primary CLL cells, Western blot analysis was done to examine processing of caspases and degradation of PARP in two primary CLL samples for which sufficient numbers of cells were available. For these studies, cells were exposed for 24 h to 3 nmol/L bortezomib ± romidepsin or belinostat, after which cell lysates were subjected to Western blot analysis. As shown in Fig. 2, exposure to each agent alone resulted in marginal cleavage of procaspase-9 and procaspase-3 and minimal PARP degradation. In contrast, combined treatment resulted in clear increase in cleavage/activation of caspase-3 and caspase-9, and PARP degradation. These findings indicate that combined exposure of primary CLL cells to bortezomib with romidepsin or belinostat results in pronounced apoptosis, a phenomenon associated with activation of apoptotic pathway, reflected by caspase activation.

**HDACs and bortezomib synergistically induce apoptosis in prolymphocytic (JVM-3) and B-cell CLL (MEC-2) cell lines.** Parallel studies were done in two continuously cultured human cell lines (i.e., JVM-3 and MEC-2), which were derived from patients with a prolymphocytic form of CLL and B-cell CLL, respectively. As shown in Fig. 3A and B, exposure of cells to 3 to 5 nmol/L of romidepsin, 300 to 500 nmol/L of belinostat, or 3 to 5 nmol/L of bortezomib individually for 48 h (JVM-3) or 72 h (MEC-2) had modest effect on cell viability (i.e., generally <25%), whereas combined treatment resulted in a dramatic increase in cell death in both JVM-3 and MEC-2 cells (e.g., 50-65%). In addition, median dose-effect analysis of cell death induction following exposure of cells to HDACIs and bortezomib at a fixed ratio yielded combination index values significantly <1.0 in both lines, indicating synergistic interactions (Fig. 3C). Western blot analysis revealed that combined HDACI/bortezomib treatment resulted in a clear increase in cleavage/activation of caspase-9 and caspase-3, as well as PARP degradation, compared with effects of the agents administered individually (Fig. 3D). These findings suggest that combined treatment with HDACIs and bortezomib synergistically induces cell death in both primary CLL cells and cultured CLL cell lines through similar mechanisms involving mitochondria-related apoptotic pathways.

Belinostat but not romidepsin promotes α-tubulin acetylation in CLL cells, an effect that is not modified by bortezomib. The actions of HDACIs, particularly those that inhibit HDAC6, have been linked to acetylation of dynein motors and disruption of aggresome formation (39), events associated with synergistic interactions between HDAC and proteasome inhibitors (30–32). Consequently, the effects of romidepsin, an inhibitor of class I HDACs, and belinostat, a pan-HDACI, were examined in relation to acetylation of α-tubulin, which is mediated by the class II HDAC, HDAC6. Using antibodies specifically recognizing acetylated α-tubulin, Western blot analysis revealed that exposure to belinostat resulted in a clear increase in acetylated α-tubulin in CLL cells, the extent of which was not further modified by bortezomib (Fig. 4A). In contrast, treatment with romidepsin failed to do so (Fig. 4A). Exposure to either romidepsin or belinostat led to a roughly equivalent increase in histone H3 acetylation, which is mediated by class I HDACs (15), in all three CLL cell types (Fig. 4A). Taken together with the preceding findings, particularly the dramatic induction of cell death by the romidepsin/bortezomib regimen, these observations suggest that inhibition of HDAC6 by HDACIs is unlikely to be solely responsible for HDACI/bortezomib synergism in CLL cells.

**Bortezomib interrupts proteasome-dependent processing of NF-κB pathway proteins.** Among many other functions, proteasome-mediated protein processing plays an important role in regulation of NF-κB activation, a critical prosurvival event (2). In the canonical pathway, IκB kinase (IKK)-mediated phosphorylation of IκBα at Ser32 and Ser36 targets this protein for ubiquitination and proteasomal degradation, thereby releasing and activating NF-κB (RelA/p50 or NF-κB1; ref. 2). In addition, the proteasome also mediates processing of the precursor p100 protein into an active p52 form, thereby triggering the noncanonical pathway (RelB/p52 or NF-κB2; ref. 2), which is related to ligation of CD40 in B-cell CLL (3). To determine whether bortezomib enhances HDACI lethality in CLL cells by inhibiting proteasome-mediated processing of NF-κB pathway proteins, an important role in regulation of NF-κB activation, a critical prosurvival event (2). In the canonical pathway, IκB kinase (IKK)-mediated phosphorylation of IκBα at Ser32 and Ser36 targets this protein for ubiquitination and proteasomal degradation, thereby releasing and activating NF-κB (RelA/p50 or NF-κB1; ref. 2). In addition, the proteasome also mediates processing of the precursor p100 protein into an active p52 form, thereby triggering the noncanonical pathway (RelB/p52 or NF-κB2; ref. 2), which is related to ligation of CD40 in B-cell CLL (3). To determine whether bortezomib enhances HDACI lethality in CLL cells by inhibiting proteasome-mediated processing of NF-κB pathway proteins.
NF-κB–related proteins, Western blot analysis was done to monitor expression of phosphorylated IκBa and p100/p52. As shown in Fig. 4B, exposure to bortezomib resulted in a modest but discernible increase in the phosphorylated form of IκBa (e.g., 1.3- to 2.1-fold increase compared with untreated controls), manifested by the presence of a slowly migrating species in blots probed with antibodies recognizing both the phosphorylated and unphosphorylated forms of IκBa, presumably by inhibition of proteasomal degradation of phosphorylated IκBa (40). On the other hand, treatment with romidepsin or belinostat induced de novo synthesis of unphosphorylated IκBa (e.g., approximately a 4- or 2-fold increase compared with untreated controls in cell lines and primary CLL cells, respectively) by activating NF-κB, reflected by fast-migrating bands (arrows), a phenomenon blocked by bortezomib (see below). In addition, administration of bortezomib either alone or in combination with HDACIs also inhibited processing of p100 to its active form p52, manifested by decreased levels of p52 protein (e.g., 2- to 4-fold decrease compared with untreated controls; Fig. 4B), whereas coadministration of HDACIs had little effect on this event. Together, these results suggest that potentiation of HDACI lethality by bortezomib may involve interference with NF-κB pathways through inhibition of proteasome-mediated protein processing.

Bortezomib blocks HDACI-mediated RelA acetylation (Lys310) and NF-κB activation in CLL cells. In view of recent evidence that HDACIs activate NF-κB in association with RelA acetylation in human leukemia cells, and that blocking this process (i.e., by IKK inhibitors or an IκBa super-repressor) promotes apoptosis (21), attempts were made to determine whether this pathway might be involved in bortezomib/HDACI interactions in CLL cells. First, Western blot analysis was done to monitor cleavage of caspases and PARP degradation using the indicated primary antibodies. Each lane was loaded with 30 μg protein; blots were subsequently stripped and reprobed for expression of β-actin to ensure equivalent loading and transfer of protein. The results of a representative experiment are shown; an additional experiment yielded equivalent results.

**Fig. 3.** HDACIs and bortezomib induce cell death in prolymphocytic (JVM-3) and B-cell CLL (MEC-2) cells in a highly synergistic manner. A, JVM-3 and MEC-2 cells were exposed for 48 h (JVM-3) or 72 h (MEC-2) to bortezomib (3 nmol/L for JVM-3 and 5 nmol/L for MEC-2) ± romidepsin (3 nmol/L for JVM-3 and 5 nmol/L for MEC-2). B, JVM-3 and MEC-2 cells were exposed for 48 h (JVM-3) or 72 h (MEC-2) to bortezomib (3 nmol/L for JVM-3 and 5 nmol/L for MEC-2) ± belinostat (300 nmol/L for JVM-3 and 500 nmol/L for MEC-2). A and B, at the end of the drug treatment period, cells were harvested and subjected to 7AAD staining and flow cytometric assay, and the percentage of dead cells (7AAD+) was determined. Columns, mean of three separate experiments done in triplicate; bars, SD. C, JVM-3 and MEC-2 cells were exposed to a range of concentrations of bortezomib (JVM-3, 3-5 nmol/L; MEC-2, 4-6 nmol/L) ± romidepsin (JVM-3, 3-5 nmol/L; MEC-2, 4-6 nmol/L) or bortezomib (4-5.5 nmol/L for both cell lines) ± belinostat (400-550 nmol/L for both cell lines) at fixed ratio, as indicated, for 48 h (JVM-3) or 72 h (MEC-2). At the end of the exposure intervals, the percentage of 7AAD+ cells was determined for each condition. Fractional effect values were determined by comparing results with those of untreated controls, and median dose-effect analysis was used to characterize the nature of the interaction between bortezomib and romidepsin or belinostat. Combination index (CI) values <1.0 denote a synergistic interaction. The results of representative experiments are shown; two additional studies yielded equivalent results. D, JVM-3 and MEC-2 cells were treated as described in A and B, after which Western blot analysis was done to monitor cleavage of caspases and PARP degradation using the indicated primary antibodies. Each lane was loaded with 30 μg protein; blots were subsequently stripped and reprobed for expression of β-actin to ensure equivalent loading and transfer of protein. The results of a representative experiment are shown; an additional experiment yielded equivalent results.
retention and transcriptional activation (36). As shown in Fig. 5A, exposure to either romidepsin or belinostat resulted in a clear increase in acetylated RelA, an event blocked by coadministration of bortezomib. In contrast, total levels of RelA remained unchanged in all cases. Furthermore, a previously described NF-κB luciferase reporter assay (21, 37) was used to monitor NF-κB activity in primary CLL cells following exposure (24 h) to romidepsin ± bortezomib. As shown in Fig. 5B, treatment with romidepsin led to a significant increase in NF-κB reporter activity, whereas bortezomib reduced basal NF-κB activity and completely blocked NF-κB activation by romidepsin in these cells. Furthermore, Western blot analysis was done to examine expression of NF-κB–dependent gene IκBα using antibodies recognizing the unphosphorylated form of this protein. As shown in Fig. 5C, exposure of CLL cells to either romidepsin or belinostat resulted in a marked increase in expression of unphosphorylated IκBα protein. In conjunction with the results shown in Fig. 4B, the increased IκBα protein presumably reflects de novo synthesis resulting from NF-κB activation by HDACIs (41). Notably, these events were largely abrogated by coadministration of bortezomib.

To test the functional significance of these events, primary CLL cells were transiently transfected with the constructs encoding GFP-tagged wild-type RelA or its acetylation mutants (K221R, K310R, or KR; ref. 36). After a 6-h recovery, cells were then exposed to 3 or 5 nmol/L of romidepsin for 24 h, after which the percentage of 7AAD+ cells was determined in the GFP+ cell population. The transfection efficiency, reflected by percentage of GFP+ cells, ranged from ~20% to 30% in these cells (Supplementary Fig. S2). As shown in Fig. 5D, CLL cells transfected with K221R, K310R, and particularly the triple mutant (KR) were significantly more sensitive to romidepsin than those transfected with wild-type RelA (P<0.01-0.001). Taken together, these findings are compatible with the notion that blockade of HDACI-mediated NF-κB activation by

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**Fig. 4.** Treatment with belinostat but not romidepsin results in increased α-tubulin acetylation, whereas coadministration of bortezomib does not affect this event but blocks proteasomal process of NF-κB components. A, JVM-3, MEC-2, and primary CLL cells (patient #2) were treated as follows: JVM-3 (top), 3 nmol/L romidepsin ± 3 nmol/L romidepsin or 300 nmol/L belinostat for 48 h; MEC-2 (middle), 5 nmol/L bortezomib ± 5 nmol/L romidepsin or 500 nmol/L belinostat for 72 h; and CLL (bottom), 5 nmol/L bortezomib ± 5 nmol/L romidepsin or 500 nmol/L belinostat for 24 h. At the end of the incubation period, cells were lysed and subjected to Western blot analysis to monitor expression of acetylated α-tubulin (Ac-α-tub) and histone H3 (Ac-H3) as well as total levels of α-tubulin. B, JVM-3 and MEC-2 cells were treated as described in A, and primary CLL cells (patient #1) were exposed to 3 nmol/L romidepsin ± 3 nmol/L bortezomib or 3 nmol/L romidepsin for 24 h, after which Western blot analysis was done to examine expression of phosphorylated IκBα (Ser32) and p100/p52. Arrows, fast-migrating, newly synthesized unphosphorylated IκBα; *, IκBα (#); and p52 (‘) protein bands was quantified using an imaging system as described in Materials and Methods. Values reflect the ratio of integrated densitometric determinations between untreated and drug-treated cells. For A and B, each lane was loaded with 30 μg protein. The results of a representative experiment are shown; an additional study yielded equivalent results.
Bortezomib may contribute to the enhanced sensitivity of cells to HDACI-mediated lethality, actions analogous to those of IKK inhibitors or the IκBα super-repressor, as previously described (21).

Both romidepsin and belinostat induce expression of proapoptotic BH3-only protein Bim, whereas combined treatment with bortezomib results in downregulation of antiapoptotic proteins in CLL cells. Lastly, attempts were made to characterize the effects of combined treatment of CLL cells with HDACIs and bortezomib on the expression of various Bcl-2 family and IAP members. Individual or combined treatment of primary CLL cells resulted in multiple perturbations in expression of such antiapoptotic proteins. As shown in Fig. 6A, combined treatment with romidepsin and bortezomib resulted in marked down-regulation of Bcl-xL, A1, XIAP, cIAP1, c-FLIP, and ICAM-1. In contrast, no change in Bcl-2 expression was observed for any treatment. On the other hand, Mcl-1 levels were increased in cells exposed to bortezomib alone, presumably a result of inhibition of its proteasomal degradation (42, 43), whereas this increase was largely diminished by coadministration of romidepsin. In addition, cells exposed to both agents exhibited clearly discernible cleavage of survivin, manifested by fast-migrating species. Roughly equivalent effects on the expression of Bcl-xL, Mcl-1, and XIAP were observed in another primary CLL samples as well as in MEC-2 cells (Fig. 6B).

Notably, exposure to either romidepsin or belinostat, with or without bortezomib, markedly induced expression of the proapoptotic BH3-only Bcl-2 family member Bim (Fig. 6C) in

![Fig. 5. Coadministration of bortezomib diminishes HDACI-mediated RelA acetylation (Lys310) and NF-κB activation in CLL cells. A, JVM-3, MEC-2, and primary CLL cells (patients #1 and #2) were treated as follows: JVM-3 (top), 3 nmol/L bortezomib ± 3 nmol/L romidepsin or 500 nmol/L belinostat for 48 h; MEC-2 (top middle), 5 nmol/L bortezomib ± 5 nmol/L romidepsin or 500 nmol/L belinostat for 24 h; and CLL #1 (bottom), 3 nmol/L bortezomib ± 3 nmol/L romidepsin for 24 h. After treatment, cells were lysed and subjected to Western blot analysis to monitor expression of acetylated (Lys310) and total RelA. B, primary CLL cells (patient #1) were transiently transfected with either 3×kB luciferase reporter or its mutant (mt; inactive) counterpart as described in Materials and Methods. After incubation for 6 h, cells were exposed to 3 nmol/L romidepsin with or without 3 nmol/L bortezomib for 18 h. After treatment, cells were lysed and subjected to assay of luciferase activity. Relative luciferase activity was determined by normalizing to total protein. NF-κB activity was expressed as fold increase relative to the untreated controls. C, cells were treated as described in A, after which Western blot analysis was done to monitor expression of IκBα. D, primary CLL cells (patient #1) were transiently transfected with GFP-tagged wild-type (wt) RelA or its acetylation site mutants, including K221R, K310R, and K218R/K221R/K310R (KR) as described in Materials and Methods. After 6-h recovery, cells were exposed to 3 and 5 nmol/L of romidepsin for 24 h, respectively. At the end of treatment, cells were harvested and stained with 7AAD, after which the percentage of 7AAD+ cells in GFP+ population was determined by flow cytometry. For A and C, 30 μg protein was loaded in each lane. The results are representative of three separate experiments. B and D, columns, mean of experiments done in triplicate; bars, SD. Asterisk, significantly greater than the value for same treatment of cells transfected with wild-type RelA*.* P < 0.01; **, P < 0.001.
CLL cells. These results were consistent with previous reports showing induction of Bim by HDACIs (44). On the other hand, no change was noted in expression of other proapoptotic Bcl-2 family members (e.g., Bid, Bax, and Bak) in cells exposed to HDACIs and bortezomib, either alone or in combination (data not shown). Thus, apoptosis of CLL cells coexposed to bortezomib and HDACIs was associated with down-regulation of multiple antiapoptotic proteins, prevention of prosurvival Mcl-1 accumulation, and induction of proapoptotic Bim expression.

Discussion

The results of the present study indicate that two novel, clinically relevant HDACIs, romidepsin and belinostat, interact in a synergistic manner with bortezomib to induce cell death in both primary CLL cells as well as in CLL cell lines. Notably, such interactions occurred at very low drug concentrations (i.e., in the low nanomolar range for bortezomib and romidepsin and submicromolar concentrations for belinostat) and were accompanied by diverse changes in both survival and apoptotic signaling pathways. Induction of cell death by both HDACIs and proteasome inhibitors, such as bortezomib, proceeds through complex, multifactorial mechanisms (15, 19, 45). For example, previous studies have shown that CLL cells are quite sensitive to proteasome inhibitors in vitro (11), and diverse mechanisms have been invoked to account for this phenomenon, including interruption of NF-κB signaling (46) as well as down-regulation of c-FLIP accompanied by up-regulation of tumor necrosis factor–related apoptosis-inducing ligand and its death receptors (e.g., DR4 and DR5; ref. 47). Less is known about mechanisms responsible for HDACI-mediated lethality in CLL cells, although HDACIs (e.g., romidepsin and MS-275) have been shown to induce apoptosis in CLL cells in association with histone acetylation, caspase activation, and down-regulation of c-FLIP (25, 27). In view of the pleiotropic actions of both proteasome and HDACIs, it would hardly be surprising if interactions between these agents involved multiple mechanisms.

It is noteworthy that bortezomib blocked HDACI-mediated NF-κB activation, and this phenomenon is likely to contribute to potentiation of cell death. Although proteasome inhibitor lethality has been linked to numerous actions, our attention has focused on interruption of the NF-κB pathway, an event that dramatically enhances HDACI lethality as reported previously (28). Proteasome inhibitors block proteasomal degradation of the IκBα protein after its phosphorylation at Ser32/36 and ubiquitination, allowing IκBα to sequester NF-κB in the cytoplasm and prevent it from translocating to the nucleus, binding to DNA, and promoting gene transcription. Furthermore, CLL cells commonly exhibit high basal NF-κB activity and are dependent on an intact NF-κB pathway for survival (5). Recent findings have shown that HDACIs induce RelA acetylation and nuclear accumulation/activation and that interference with this process by either pharmacologic or genetic means dramatically increases HDACI lethality (21, 22). Acetylation of RelA exerts several critical actions that promote sustained NF-κB activation, including promoting of RelA–DNA binding and diminished association with IκBα (36). On the other hand, IKK inhibitors block RelA acetylation in cells exposed to HDACIs by inhibiting IKK-mediated IκBα acetylation.
phosphorylation and resulting proteasomal degradation and, in so doing, trap NF-κB in the cytoplasm (21). This action sequesters RelA from nuclear histone acetyltransferases (e.g., p300) and deacetylase (e.g., HDAC3), proteins that reciprocally mediate acetylation of RelA at Lys221, Lys310, and Lys218 (36). It is significant that, in CLL cells, both romidepsin and belinostat induced an increase in RelA acetylation at the Lys310 site, an event that was diminished by coadministration of bortezomib. In accord with these findings, bortezomib prevented HDACIs from induction of NF-κB activation and de novo expression of its target gene IκBα. In view of these events, it is likely that bortezomib mimics the actions of IKK inhibitors or expression of the IκBα super-repressor in potentiating HDACI lethality (21, 22), at least in CLL cells. The finding that CLL cells transfected with inactive, acetylation site mutants of RelA (i.e., K221R, K310R, or K218R/K221R/K310R) were significantly more sensitive to HDACI-mediated RelA acetylation and NF-κB activation contributes, at least in part, to the lethality of this regimen. However, given the pleiotropic actions of both HDAC and proteasome inhibitors, it is unlikely that interruption of NF-κB signaling represents the sole basis for synergistic interactions between these agents, and in all probability, additional mechanisms are involved.

IκBα is a downstream target of NF-κB whose expression directly reflects NF-κB activation (2). However, IκBα also acts functionally as an inhibitor of NF-κB and is subject to proteasomal degradation following phosphorylation at Ser32/36 (2). To examine IκBα expression, two distinct and specific antibodies were used to identify the phosphorylated (Ser32) and unphosphorylated forms of IκBα. As noted above, exposure to romidepsin and belinostat markedly increased expression of unphosphorylated IκBα but not the phosphorylated form, reflecting de novo synthesized protein stemming from HDACI-mediated NF-κB activation. However, de novo synthesized IκBα was not able to prevent NF-κB activation in HDACI-treated cells, most likely because it is unable to bind to acetylated RelA and export NF-κB from nucleus (36). On the other hand, treatment with bortezomib, either in the presence or absence of HDACIs, induced a modest but clear accumulation of phosphorylated IκBα that binds to and inhibits NF-κB (40), indicating inhibition of proteasomal degradation of this protein. Therefore, in CLL cells exposed to HDACIs, bortezomib is able to inhibit degradation of phosphorylated IκBα and, in so doing, prevents RelA nuclear translocation/acetylation and interferes with de novo expression of NF-κB–dependent genes, including IκBα.

CLL cells exposed to bortezomib and HDACIs displayed reduced expression of antiapoptotic proteins, including Bcl-xL, A1, XIAP, cIAP1, c-FLIP, as well as ICAM-1. Although the potential contribution of secondary, apoptosis-dependent changes in the expression of these proteins cannot be completely excluded, down-regulation of these antiapoptotic proteins may have lowered the threshold of CLL cells to prodeath signals, such as Bim induction. In addition to these proteins, McI-1 represents a target subject to ubiquitination and proteasomal degradation (42). As anticipated, levels of McI-1 protein were increased in CLL cells exposed to bortezomib alone, presumably due to inhibition of its proteasomal degradation (42). However, accumulation of McI-1 mediated by bortezomib was diminished by coadministration of HDACIs in CLL cells. It is possible that this phenomenon represents a secondary event related to activation of caspases as previously described (28). Mcl-1 has been shown to play a critical role in the survival of malignant hematopoietic cells, including CLL cells (48). Thus, up-regulation of Mcl-1 by proteasome inhibitors, such as bortezomib, may limit the proapoptotic actions of this agent. Consequently, it seems reasonable to speculate that the ability of HDACIs to diminish Mcl-1 accumulation in bortezomib-treated CLL cells, whether this represents a primary event, may facilitate cell death in cells cotreated with these agents.

Bim represents a critical proapoptotic BH3-only protein in mediating apoptosis induced by various stresses (44). It has recently been shown that HDACIs (e.g., vorinostat and trichostatin A) induce Bim expression at the transcriptional levels via an E2F1-dependent mechanism and that this event plays an important role in lethality of these agents (44). It is noteworthy that exposure of primary and cultured CLL cells to HDACIs, with or without bortezomib, resulted in a clear increase in expression of Bim. It remains to be determined whether romidepsin and belinostat induce Bim up-regulation through the same mechanism exhibited by vorinostat. Moreover, it is also uncertain whether Bim promotes apoptosis by directly activating multidomain proapoptotic molecules Bax/Bak, by antagonizing the antiapoptotic actions of antiapoptotic molecules such as Bcl-2 and Bcl-xL, or by a combination of these actions (49). Nevertheless, up-regulation of Bim by romidepsin or belinostat, in conjunction with down-regulation of antiapoptotic proteins in CLL cells coexposed to bortezomib, may serve to shift the balance from prosurvival to proapoptotic actions, leading to enhanced lethality for combination regimens.

Several recent reports have emphasized the potential role of simultaneous disruption of aggresome formation and proteasome function in synergistic interactions between proteasome inhibitors and HDACIs (30–32). Specifically, inhibition of HDAC6, a tubulin deacetylase of class II HDACs (15), disrupts the function of dynein motors and, by extension, interferes with aggresome formation, a critical alternative mechanism to dispose of misfolded protein in the setting of proteasome inhibition (39). Significantly, simultaneous inhibition of both processes leads to pronounced lethality (30–32). Consistent with these findings, treatment with belinostat, a pan-HDACI (15), resulted in a marked increase in tubulin acetylation, indicating inhibition of HDAC6. In contrast, it is noteworthy that romidepsin, which primarily inhibits class I HDACs (15), failed to do so. However, both HDACIs interacted in a synergistic manner with bortezomib to induce apoptosis, suggesting that interruption of aggresome formation may not be the sole mechanism responsible for interactions between HDACIs and proteasome inhibitors, at least in CLL cells.

In summary, the present findings indicate that clinically relevant HDACIs romidepsin and belinostat interact in a synergistic manner with bortezomib to induce cell death in primary and cultured CLL cells, most likely through multiple mechanisms, including inhibition of NF-κB, down-regulation of antiapoptotic proteins (Bcl-xL, A1, XIAP, cIAP1, c-FLIP, etc.), prevention of Mcl-1 accumulation, and induction of Bim. Although clinical studies of HDAC and proteasome inhibitors as single agents in CLL have yielded only modest results to date (14, 24), the finding that very low concentrations of these...
agents interact synergistically to kill primary CLL cells reveals the possibility that combination regimens may be significantly more effective in this disease. Whether this is the case will depend on multiple factors, including tolerability, selectivity, and in vivo activity. In this context, a recent pediatric leukemia phase I study of bortezomib revealed evidence of NF-κB inactivation in leukemic blasts (50). Accordingly, efforts to address these issues are currently under way.

References
Interactions between Bortezomib and Romidepsin and Belinostat in Chronic Lymphocytic Leukemia Cells


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