Romidepsin and Belinostat Synergize the Antineoplastic Effect of Bortezomib in Mantle Cell Lymphoma

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Abstract

Purpose: Romidepsin and belinostat are inhibitors of histone deacetylases (HDACI). HDACIs are known to induce cell death in malignant cells through multiple mechanisms, including upregulation of death receptors and induction of cell cycle arrest. They are also known to be prodifferentiating. Mantle cell lymphoma (MCL) is an aggressive subtype of non-Hodgkin lymphoma characterized by the (11;14) (q13;q32) translocation leading to the overexpression of cyclin D1.

Experimental Design: Assays for cytotoxicity including mathematical analysis for synergism, flow cytometry, immunoblottings, and a xenograft severe combined immunodeficient beige mouse model were used to explore the in vitro and in vivo activity of romidepsin and/or belinostat alone or in combination with the proteasome inhibitor bortezomib in MCL.

Results: In vitro, romidepsin and belinostat exhibited concentration-dependent cytotoxicity against a panel of MCL cell lines. Both HDACI showed strong synergism when combined with the proteasome inhibitor bortezomib in MCL. An HDACI plus bortezomib also induced potent mitochondrial membrane depolarization and apoptosis, whereas no significant apoptosis was observed in peripheral blood mononuclear cells from healthy donors with the combination. These events were associated with a decrease in cyclin D1 and Bcl-XL, and an increase in accumulation of acetylated histone H3, acetylated α-tubulin, and Noxa in cell lines. In a severe combined immunodeficient beige mouse model of MCL, the addition of belinostat to bortezomib enhanced efficacy compared with either drug alone.

Conclusions: Collectively, these data strongly suggest that HDACI such as romidepsin or belinostat in combination with a proteasome inhibitor could represent a novel and rationale platform for the treatment of MCL. Clin Cancer Res; 16(2); 554–65. ©2010 AACR.

Mantle cell lymphoma (MCL) represents a distinct subtype of aggressive lymphoma, characterized by gross cell cycle dysregulation. The pathognomonic lesion in the disease is the reciprocal translocation t(11;14)(q13;q32) leading to the juxtaposition of the cyclin D1 gene downstream of the immunoglobulin heavy chain gene promoter (1). This genetic event leads to constitutive overexpression of cyclin D1. In addition, many forms of MCL exhibit loss of the cyclin-dependent kinase (cdk) inhibitors p27 and p21, leading to further dysregulation of cell cycle control (2). Although the precise mechanism explaining the loss of p27 remains to be clarified, several lines of evidence suggest that overexpression of Skp2, a component of the ubiquitin E3-ligase that targets p27 for proteolytic degradation, may contribute to the loss of cell cycle inhibition (3). The collective importance of these genetic lesions has been supported by additional lines of data showing the importance of proliferation in MCL. Data by Rosenwald and colleagues (4) identified a "proliferation signature" in MCL, in which the patients with the most highly proliferative disease experienced the poorest prognosis, whereas patients with the least proliferative disease exhibited a superior survival. This biology has also been validated by Ki-67 immunohistochemical staining, which also correlated overall survival with the proliferative index in MCL (5). These data suggest that by merely "lowering" the proliferative signature of the disease, it might be possible to alter the natural history and overall prognosis of the disease.

In addition to the lesions involved in cell cycle control, it is also clear that aberrant expression of various Bcl-2 family members, including Mcl-1, and deficiencies in BH3-only proteins such as Bim and Noxa may render these cells relatively resistant to apoptosis (6). Pharmacologic strategies that directly address these specific lesions represent an innovative strategy for targeting "targeted therapy" to a specific disease context.

The proteasome inhibitor bortezomib was approved for the treatment of relapsed or refractory MCL based on the
HDAC Inhibitors in Mantle Cell Lymphoma

Translational Relevance

Mantle cell lymphoma (MCL) is a relatively rare, although challenging, form of non–Hodgkin’s lymphoma. Recently, several promising new drugs with important single-agent activity have begun to teach us about the unique biology of this disease. The combination of a proteasome inhibitor and HDAC inhibitor represents a novel and theoretically highly targeted drug combination suited to address the unique pathogenetic lesions indicative of MCL. In this article, we show the remarkable synergy between these two classes of drugs in MCL, establishing the fact they are able to “turn off” cyclin D1 expression and “turn on” NOXA in a manner that is synergistic in vitro and in vivo. These data establish the class effects of these agents providing a clear-cut rational for moving these combinations into clinical trials for patients with MCL. In addition, they provide insights into possible biomarkers of activity that could be explored in future clinical studies.

consistent observations from independent phase 2 studies (7–10) including one registration-directed phase 2 study (11). In addition, bortezomib has been shown to complement a host of other targeted drugs including Bcl-2–targeted agents such as obatoclax mesylate; ABT-737, and oblimersen sodium (7, 12–14). Although there are a multitude of biological effects induced by inhibition of cellular proteasome, accumulation of the cell cycle–dependent kinase inhibitors such as p27, p21, p53; inhibition of NF-κB; and accumulation of proapoptotic proteins such as Noxa have been consistently shown in many cell line models (15).

The acetylation and deacetylation of histones regulate transcriptional activation by mediating chromatin condensation and decondensation (16). The acetylation status of histones and nonhistone proteins is determined by the balance between histone acetyl transferase and HDACs (17–20). For example, nonhistone protein targets of HDACs include transcription factors (such as p53, c-Myc, Bcl-6, NF-κB), transcriptional regulators (such as Rb), signal transduction mediators (STAT3), DNA repair enzymes, nuclear import regulators, chaperone proteins (HSP90), structural proteins (α-tubulin), and mediators of inflammation (21). Maintaining acetylation of histone as a therapeutically rational condition in cancer was considered after the discovery of the HDAC inhibitor (HDACi) trichostatin A as early as 1990 (22). Since then, many different HDACis have been identified and developed, including noncancer-related drugs not previously recognized as having HDACi activity (23). Although many of these agents are progressing through, vorinostat, a hydroxamic acid analogue, was the first and, to date, only HDACi approved for the treatment of cancer (cutaneous T-cell lymphoma; refs. 24–26). Although it has been difficult to ascribe a singular mechanism of action to the HDACi in any specific biological context, the biological effect of these drugs has been shown to include cell cycle arrest, terminal differentiation, and induction of apoptosis, depending on the concentration of drug and the cell model studied.

The immediate rationale for combining these two classes of drugs in MCL revolves around recent observations that HDACis, such as vorinostat and LBH-589, may be able to “turn off” cyclin D1. Ellis et al. (27) showed in a phase 1 experience that LBH-589 consistently produced downregulation of cyclin D1 in punch biopsies of patients with cutaneous T-cell lymphoma treated with LBH-589 by gene expression array. Similarly, vorinostat, trichostatin A, and even the aliphatic acids have been shown to reduce intracellular cyclin D1 protein in MCL cell lines, but not in cell lines of acute myeloid leukemia. These observations were shown to be dependent on concentrations of drugs that also induced accumulation of acetylated histone H3 (28, 29). Although the precise mechanism of this downregulation remains to be established, the observation creates a unique opportunity to both turn off cyclin D1 and “turn on” cdk inhibitors such as p21 and p27 in a disease characterized by these very lesions. Belinostat and romidepsin are both pan-HDACis that now in advanced stage clinical trials. Given the activity of proteasome inhibitors in MCL, and its ability to enforce accumulation of cdk inhibitors, we sought to explore the merits of this combination in models of MCL as well as in normal peripheral blood mononuclear cells (PBMC) from healthy donors.

Materials and Methods

Cells and cell lines. HBL-2, Jeko-1, and Granta-519 are well-characterized MCL lines (30, 31). Mononuclear cells from PBMC samples of healthy donors were purchased from Allcells. All cell lines were grown as described previously (12, 13).

Materials. All reagents for Western blotting were obtained from Bio-Rad Laboratories and Pierce Biotechnology, Inc.; DMSO was obtained from Sigma. Drugs were obtained as follows: romidepsin was from Gloucester Pharmaceuticals, Inc.; belinostat was from TopoTarget; and bortezomib was from the institutional research pharmacy.

Cytotoxicity assays. For all in vitro assays, cells were counted, incubated, and processed as described previously (12, 13). Romidepsin and belinostat were diluted in DMSO that was maintained at a final concentration of <0.5%. Romidepsin and belinostat were added at concentrations from 1 nmol/L to 10 μmol/L. For combination experiments with bortezomib, the final concentrations of each drug were selected to approximate the IC_{10–60} for each drug. For all cytotoxicity experiments, Cell-Titer-Glo Reagent (Promega Corp.) and a Synergy
HT Multi-Detection Microplate Reader (Biotek Instruments, Inc.) were used as described previously (12, 13). Each experiment was done at least in duplicate and repeated at least twice. Data are presented as averages ± SD.

Flow cytometry. Cells were seeded at a density of $6 \times 10^5$/mL and incubated with concentrations of romidepsin or belinostat with or without bortezomib at concentrations approximating the IC$_{10-30}$ for 24 h. To determine changes in the transmembrane mitochondrial membrane potential ($\Delta \psi_m$), cells were stained with 1.25 μg/mL of JC-1 dye (Invitrogen) for 30 min at 37°C in normal growth medium, then washed once in warm PBS, resuspended in 200 μL of medium, and analyzed using a FACSCalibur Flow Cytometer (BD). Carbonyl cyanide m-chlorophenylhydrazone (Sigma Immunochemicals) was used as a positive control for loss of membrane potential. A minimum of 3 × 10$^5$ events were acquired from each sample. Median values obtained from the FL1 and FL2 channels after standard gating of forward and side scatter were used to calculate the normalized Δψ$_m$. For detection of apoptosis, Yo-Pro-1 and propidium iodide (PI) were used (Invitrogen). After incubation with romidepsin or belinostat plus or minus bortezomib, cells were washed and resuspended in cold PBS. One microliter of Yo-Pro-1 and 1 μL of PI were added to each 1 mL of cell suspension. The fluorescence signals were acquired by a FACSCalibur System. All data from flow cytometry were analyzed with the Flowjo software. Each experiment was done at least in duplicate and repeated at least twice. Data are presented as averages ± SD.

Western blot analysis. Cells were incubated with the approximate IC$_{10-30}$ of each drug alone (bortezomib, romidepsin, and belinostat) and in combination (HDACI ± bortezomib) under normal growth conditions for 24 h. Proteins from total cell lysates were resolved on 12% to 20% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in phosphate-buffered saline and 0.05% Triton X-100 containing 5% skim milk powder, and were then probed overnight with specific primary antibodies. Antibodies were detected with the corresponding horseradish peroxidase–linked secondary antibodies. Blots were developed using SuperSignal West Pico chemiluminescent substrate detection reagents. The membranes were exposed to X-ray films for various time intervals. The images were captured with a GS-800 calibrated densitometer (Bio-Rad), and the ratios were quantified by densiometric analyses within the linear range of each captured signal. The monoclonal and polyclonal antibodies used were as follows: cyclin D1, acetylated histone H3, acetylated α-tubulin 1, Bcl-X$_L$ (all from Cell Signaling Technology), Noxa (Imgenex Corp.), and β-actin (clone AC-74, Sigma).

Mouse xenograft models. In vivo experiments were done as described previously (12, 13). In brief, 6- to 8-wk-old beige mice with severe combined immunodeficiency (SCID; Charles River Laboratories) were injected with 1 × 10$^7$ HBL-2 cells in their flanks through a s.c. route. When tumor volumes approached 50 mm$^3$, mice were separated into treatment groups of 9 to 10 mice each. Tumors were assessed using the two largest perpendicular axes ($L$ length; $W$ width) as measured with standard calipers. Tumor volume was calculated using the formula 4/3$\pi l w$, where $l = (L + W)/4$. Tumor-bearing mice were assessed for weight loss and tumor volume at least thrice weekly. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm, or when the tumor volume exceeded 2,000 mm$^3$ in accordance with institutional guidelines. Complete response was defined as nonpalpable tumor. In a first series of experiments, mice not bearing tumors were treated with belinostat plus bortezomib to explore the toxicity of the combination. Belinostat was administered by i.p. injection at a dose up to 40 mg/kg/d for 7 d [highest dose level explored in the study from Plumb et al. (ref. 32)]. Bortezomib was administered by i.p. injection at 0.5 mg/kg on days 1, 4, 8, and 11. In a subsequent xenograft experiment, belinostat was administered at 35 mg/kg/d for 7 d and bortezomib was given by i.p. injection at 0.5 mg/kg on days 1, 4, 8, and 11. Belinostat was added to a mixture of 10% DMSO and 90% sterile water. Control groups were treated with the vehicle solution.

Statistical analysis. The IC$_{50s}$ were calculated by fitting dose-response curves (33). Confidence intervals (CI) are shown in between parenthesis. Drug-drug interactions were computed using the relative risk ratio (RRR) analysis (GraphPad), with a RRR of <1 defining synergism, a RRR of 1 defining additivity, and a RRR of >1 defining antagonism. Data from the in vitro assays were analyzed using a t test with a robust variance estimate. For the mouse experiments, the tumor volumes and area under the curves were log transformed and evaluated using ANOVA for four-way comparison and Wilcoxon test for pairwise comparisons. Median absolute deviation was used as a measurement of variability. All significance testing was done at the $P < 0.05$ level.

Results

Romidepsin and belinostat interact synergistically with bortezomib in MCL cell lines. The IC$_{50}$ of romidepsin and belinostat after a 24 h of exposure across a panel of MCL cell lines was in the 10 to 100 nmol/L range for romidepsin, and 1 to 100 μmol/L range for belinostat (Fig. 1A-B). For HBL-2, Jeko-1, and Granta-519, the IC$_{50}$ values for romidepsin were 4.3 nmol/L (CI, 3.6-5.2), 11 nmol/L (CI, 6.7-18.2), and 58.5 nmol/L (CI, 26-131), respectively. For belinostat, the corresponding IC$_{50}$ values for the same lines were 0.4 μmol/L (CI, 0.3-0.7), 0.2 μmol/L (CI, 0.1-0.7), and 56.3 μmol/L (CI, 30.7-103.5), respectively.

Formal synergy analyses were done using HBL-2, Jeko-1, and Granta-519 cells treated with different concentrations of romidepsin or belinostat (approximating the IC$_{10-60}$) in combination with bortezomib at 3, 3.5, or 4 nmol/L for

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Paoluzi et al.
24 hours. In all cell lines, a synergistic cytotoxic effect was observed when combining either HDACI with bortezomib through a range of concentrations. The RRR analysis showed very strong synergism (RRR < 0.3) in virtually all combinations of HDACI and bortezomib (Table 1A-B). The observed RRR were as follows: belinostat + bortezomib: RRR ≤ 0.7 in HBL-2, RRR ≤ 0.5 in Jeko-1, and RRR ≤ 0.4 in Granta-519; romidepsin + bortezomib: RRR ≤ 0.8 in HBL-2, RRR ≤ 0.8 in Jeko-1, and RRR ≤ 0.1 in Granta-519 (Fig. 1B). The combination of an HDACI plus bortezomib at concentrations approximating the IC10-60 for each drug resulted in a cell viability ranging between 9% and 30% in Jeko-1, 2% and 40% in HBL-2, and 3.5% and 18% for Granta-519. Figure 2 presents the cell viability data after treatment with the single agents or the combination with a fixed concentration of bortezomib (3.5 or 4 nmol/L depending on the cell line). All the explored combinations showed impressive cytotoxicity with no more than 20% to 25% cell viability at 24 hours in all three cell lines. The corresponding RRRs revealed very strong synergism in all cases (RRR ≤ 0.3).

**Romidepsin or belinostat plus bortezomib enhance apoptosis in MCL cell lines.** The combination of romidepsin or belinostat (IC10-40) and bortezomib (IC10-20) for 24 hours showed potent induction of apoptosis in HBL-2 and Jeko-1 cell lines. When HBL-2 or Jeko-1 cells were treated with romidepsin (2.5-6 nmol/L) or belinostat (100-600 nmol/L) plus bortezomib (3-3.5 nmol/L), statistically significant apoptosis was observed in all the combination groups compared with the individual drugs and the control in both cell lines (P ≤ 0.007 for HBL-2, P ≤ 0.001 for Jeko-1; Fig. 3). The combination of romidepsin plus bortezomib increased the percentage of apoptotic plus dead cells up to about 70% in HBL-2 (P = 0.001-0.007) and 80% (P = 0.001) in Jeko-1 (Fig. 3A). The combination of belinostat plus bortezomib increased the same percentage up to 80% in both cell lines (P = 0.002-0.003 for HBL-2, P = 0.001 for Jeko-1; Fig. 3B).

**Romidepsin or belinostat plus bortezomib enhance disruption of Δψm in MCL cell lines.** Changes in Δψm represent an early event in the induction of apoptosis, and likely capture the effects of agents on various aspects of Bcl-2 family members. Treatment of Jeko-1 and HBL-2 cells with romidepsin (5-6 nmol/L) or belinostat (200-600 nmol/L) plus bortezomib (3.5 nmol/L) decreased the normalized Δψm in a time-dependent manner (Fig. 4A). After incubation with either romidepsin or belinostat plus bortezomib for 16 hours, both cell lines did not show any significant change in Δψm compared with the single agents and control. After 20 and 24 hours of incubation, the combination groups in both cell lines showed more than 60% and 80% decrease in Δψm, respectively, compared with the untreated controls. This level of effect was comparable with that observed after treatment with the positive control carbonyl cyanide m-chlorophenylhydrazine. In particular, for romidepsin + bortezomib, the P values in HBL-2 were ≤0.003 (20 hours) and ≤0.002 (24 hours), and ≤0.002 (20 hours) and ≤0.001 (24 hours) in Jeko-1. For belinostat + bortezomib, the P values in HBL-2 were ≤0.003 (20 hours) and ≤0.004 (24 hours), and ≤0.002 (20 hours) and ≤0.001 (24 hours) in Jeko-1 (Fig. 4A). These data are consistent with the cytotoxicity data that support the contention that a strong synergistic effect of the HDACI plus bortezomib requires relatively longer durations of exposure (up to 24 hours).

**Romidepsin or belinostat plus bortezomib do not enhance apoptosis to PBMC from healthy donors.** Concentrations of romidepsin and belinostat that produced synergy in
combination with bortezomib in cell lines of MCL were also tested in PBMC from healthy donors to explore potential toxicity against healthy cells. The observed synergistic effect in cell lines was specific to malignant cells, since the combination of romidepsin (2.5-5 nmol/L) or belinostat (400-600 nmol/L) plus bortezomib (3-5 nmol/L) did not show any significant apoptosis compared with the single agents alone and the control ($P \geq 0.82$). Apoptotic plus dead cells were $\sim 40\%$ in all groups (Fig. 4B).

**Influence of HDACIs plus bortezomib on proteins involved in cell cycle regulation and apoptosis.** Supplementary Fig. S1 and Fig. 5 show the results related to the immunoblottings for cyclin D1, acetylated histone H3, acetylated α-tubulin, Bcl-XL, Mcl-1, and Noxa before and after treatment with romidepsin or belinostat plus or minus bortezomib in two MCL cell lines (24-hour exposure). Following treatment with romidepsin at 4 or 6 nmol/L plus bortezomib, the expression of cyclin D1 was significantly decreased in HBL-2, whereas a similar effect was observed in Jeko-1 after treatment with romidepsin at 6 nmol/L with or without bortezomib. Belinostat decreased the expression of cyclin D1 in HBL-2, producing a near complete absence of this protein in the combination at 600 nmol/L. In Jeko-1, the combination of belinostat at 400 or 600 nmol/L plus bortezomib and the group treated with belinostat alone at 600 nmol/L produced a significant decrease in cyclin D1 compared with all other groups. An increase in histone H3 acetylation was observed after treatment with romidepsin or belinostat alone or in combination with bortezomib. In Jeko-1, the combination

### Table 1. RRR analysis in three cell lines of MCL after treatment with bortezomib plus belinostat (A) or romidepsin (B) at different concentrations for 24 h

**A. RRR analysis in three cell lines of MCL after treatment with bortezomib plus belinostat at different concentrations for 24 h**

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<th>Belinostat (μmol/L)</th>
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**B. RRR analysis in three cell lines of MCL after treatment with bortezomib plus romidepsin at different concentrations for 24 h**

<table>
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<th>Romidepsin (nmol/L)</th>
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**NOTE:** All ratios are <1 (synergy).
groups with romidepsin (4 or 6 nmol/L) or belinostat (400 or 600 nmol/L) showed some increased accumulation of acetylated H3 compared with romidepsin or belinostat alone, respectively. In HBL-2, the combination of belinostat at 400 nmol/L with bortezomib showed an increase in acetylated histone H3 compared with the control, belinostat, and bortezomib alone. The abundance of the antiapoptotic protein Bcl-XL showed some decrease after treatment with romidepsin at 6 nmol/L plus bortezomib compared with the other groups in Jeko-1, whereas in HBL-2, some decrease was observed in the groups treated with belinostat alone or in combination. The expression of the antiapoptotic protein Mcl-1 did not produce any significant change in either cell line. Noxa, a BH3-only proapoptotic protein that counteracts the antiapoptotic effect of Mcl-1, accumulated after treatment with romidepsin or belinostat plus bortezomib in both cell lines. In Jeko-1, significant accumulation of Noxa
was also observed in the groups treated with belinostat alone. The expression of acetylated α-tubulin showed an increase after treatment with belinostat plus or minus bortezomib in both cell lines. In HBL-2, there was a trend toward significant increase of Noxa in the combination groups with romidepsin compared with the drugs given alone or the control. In Jeko-1 the combination with romidepsin at 6 nmol/L seemed to slightly increase this protein compared with the other groups.

Belinostat enhances the activity of bortezomib in vivo. When belinostat was given at 40 mg/kg/d in combination with bortezomib at 0.5 mg/kg, 1 out 10 mice died on day 7 after receiving the last dose of treatment of drug, and 2 additional mice experienced significant weight loss (>10% of the initial weight). A dose reduction of ~10% (35 mg/kg/d) was subsequently explored and found to be better tolerated with only one mouse experiencing significant weight loss on day 8, which fully recovered by day 12.

The in vivo efficacy of belinostat at 35 mg/kg/d for 7 days in combination with bortezomib was investigated in a xenograft model of MCL (HBL-2; Fig. 6A). Statistical analysis (days 3, 8, and 12) from the beginning of the experiment revealed that the combination of belinostat and bortezomib was statistically superior to bortezomib alone ($P \leq 0.009$), belinostat alone ($P \leq 0.035$), and the control ($P \leq 0.003$; Table 2A-B). One mouse in the combination group experienced a complete response on day 8 that was lost by day 12, whereas an additional mouse experienced a complete response by day 12. Of note, no animals in any of the single agent or control groups experienced a complete remission. Thirty percent of the mice in the combination group experienced significant weight loss by day 12 (one mouse from day 3, one from day 8, and one from day 12), but no deaths were observed.

**Discussion**

The increasing clarification of the distinct molecular features of different NHL subtypes, coupled with an ever expanding understanding of the molecular pharmacology of many new drugs, has created a unique opportunity to formulate new concepts about the application of novel targeted drugs in cancer. The tailoring of these new agents based on their pharmacologic effects to specific disease contexts represents a rational strategy for developing novel...
platforms for treatment. MCL is a great example of a disease that has been largely redefined over the past few years. Understanding the molecular pathogenesis of the disease, coupled with an appreciation of the molecular pharmacology of the new agents, has created the opportunity to develop rational combinations of agents in this disease-specific context.

MCL is a disease characterized by gross cell cycle dysregulation. At the core of this dysregulation is the constitutive overexpression of cyclin D1, and the enhanced proteolytic degradation of cdk inhibitors such as p27 and p21. Lesions in apoptosis related to an imbalance in Mcl-1 and BH3-only mimetic proteins also contribute to an elevated apoptotic threshold. Although HDACIs and proteasome inhibitors may be associated with a plethora of biological effects, the observation that the former can turn off cyclin D1, and the latter force accumulation of cdk inhibitors, create a rational platform for the combination of these two drug classes in MCL.

Supporting the rationale further is the observation that both proteasome and HDACIs have been shown to have therapeutic value in the treatment of lymphoid malignancies (7, 24, 25–31). The biological effects of HDACIs seem to depend on a variety of factors, including the nature of the HDACI, its concentration and duration of exposure, and importantly, the cellular context. At least 12 different HDACIs are undergoing clinical trials in patients with hematologic and solid tumors. Vorinostat (suberoylanilide hydroxamic acid; 38) was the first-in-class agent approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (26). Belinostat and romidepsin are two new drugs now in registration-directed clinical trials, both of which are pan-Class I and II HDACIs (39).

A third rational that supports the potential combination of these drug classes revolves around the role of HDAC6. HDAC6 is a class II HDAC enzyme that deacetylates α-tubulin, leading to increased cell motility. Importantly, several investigators have shown that the induction of HDAC6 by proteasome inhibitors such as bortezomib may also account for some of the acquired drug resistance seen in cell lines treated with proteasome inhibitors, establishing the notion that inhibition of HDAC6 may circumnavigate the development of resistance to proteasome inhibitors such as bortezomib. HDAC6 plays an essential role in aggresomal protein degradation, a proteasome-independent pathway that eliminates misfolded polyubiquitinated proteins. HDAC6 can bind both polyubiquitinated proteins and dynein proteins, recruiting protein cargo to dynein motors that
transports misfolded proteins to aggresomes (41). Overexpression of HDAC6 leads to deacetylation of tubulin and increases cell motility (42, 43). Specific inhibition of HDAC6 activity or its downregulation has been shown to increase tubulin and HSP90 acetylation, which reduces cellular motility, and induces HSP90 client protein degradation, cell growth inhibition, and cell death (44). Inhibition of HDAC6 by either specific or pan-HDACIs can trigger different mechanisms of cell death. Hideshima et al. (40) showed that targeting both proteasome-dependent pathways with bortezomib and the aggresome pathway in tumor cells with a HDACI induces greater accumulation of polyubiquitinated proteins resulting in increased cellular stress and apoptosis. In addition to these effects of HDACI on the aggresome, some studies restricted to in vitro models of leukemia have suggested that HDACIs can induce apoptosis through inactivation of antiapoptotic Bcl-2 family members (45, 46).

The collective experimental data presented here support the potent activity of the new HDACIs romidepsin...
and belinostat in combination with the proteasome inhibitor bortezomib in MCL. The cytotoxicity assays established IC50 values in the nanomolar range for romidepsin and low micromolar range for belinostat across all MCL cell lines. To quantitate the synergistic interaction between the HDAC and proteasome inhibitor, intentionally subtherapeutic concentrations of each drug were studied. In the cytotoxicity assays, the combination of romidepsin or belinostat plus bortezomib showed potent synergism in all three cell lines of MCL. The RRR analysis showed very strong synergism (RRR < 0.1) in virtually all of the concentrations studied for the combination. RRR in this range are unusual even for the most established antineoplastic doublets. These observations were further supported by the changes in the \( \Delta \psi \text{m} \) as a function of the drug exposures. These experiments showed statistically significant changes in the \( \Delta \psi \text{m} \) with the combination of drugs that was time sensitive. The observed effect on the \( \Delta \psi \text{m} \) was most prominent after 20 hours of exposure with >60% of cells exhibiting \( \Delta \psi \text{m} \) in the combination groups in both cell lines. When used alone, neither HDAC-I nor bortezomib produced a significant change in \( \Delta \psi \text{m} \) or induction of apoptosis. The potent induction of \( \Delta \psi \text{m} \) and apoptosis in the MCL lines was restricted to the combination treatments. Importantly, when the same combinations of an HDAC-I plus bortezomib were evaluated on PBMC from healthy donors, none of the combinations were found to be more cytotoxic than any drug alone (\( P \geq 0.82 \)). These data suggest that this combination may also be associated with a good therapeutic index.

To explore the mechanistic basis for the synergy between the proteasome and HDACs, a series of immunoblottings for proteins involved in cell cycle regulation and apoptosis were done. Although more comprehensive studies on the effect of the drugs on the proteome need to be considered, these experiments suggest a prominent effect on cyclin D1, histone H3, HDAC6, Bcl-XL, and Noxa. Despite slight differences between the cell lines and the HDAC-I used, there was a consistent reduction in cyclin D1 and Bcl-XL with a consistent increase in acetylated histone H3, acetylated \( \alpha \)-tubulin, and Noxa in the cell lines treated with the HDAC-I, bortezomib, and the combination.

From a mechanistic perspective, high levels of expression of the antiapoptotic protein Mcl-1 has been shown to correlate with high-grade morphology and a high proliferative state in MCL (47). Noxa, a BH3-only proapoptotic protein that specifically interacts with and inactivates Mcl-1, seems to be emerging as a critical regulator of apoptosis in MCL (48). It has been reported that Noxa accumulation following bortezomib disrupts the Mcl-1–Bak complex by displacing free proapoptotic Bak. This event leads to conformational changes of Bak with activation of the intrinsic or mitochondrial pathway of apoptosis. Several studies have firmly shown strong induction of apoptosis after treatment with bortezomib irrespective of p53 status through a mechanism that involves increased accumulation of Noxa (49, 50). The immunoblottings

| A. Multiple comparison analysis of the median AUCs (MAD) at day 3, 8, and 12 |
|-------------------------------|-----------------|-----------------|-----------------|
| Treatment | Day 3 Median (mm³) ± MAD | Day 8 Median (mm³) ± MAD | Day 12 Median (mm³) ± MAD |
| Control | 81.6 ± 26.8 | 427.8 ± 265.8 | 1,352 ± 1,217.1 |
| B | 84.2 ± 66.7 | 276.9 ± 179.2 | 866.5 ± 864.3 |
| P | 83.3 ± 40.1 | 291.1 ± 121.7 | 752.9 ± 261.3 |
| B + P | 40.6 ± 27 | 53.8 ± 73.2 | 236.8 ± 317.8 |

| B. The \( P \) values for all comparison are shown. Statistically significant tumor shrinkage for the combination group compared with the single drugs and the control was observed at all time points (\( P < 0.03 \) in bold) |
|-------------------------------|-----------------|-----------------|-----------------|
| Treat 1 | Treat 2 | Day 3 \( P \) | Day 8 \( P \) | Day 12 \( P \) |
| Control | B | 0.740 | 0.529 | 0.394 |
| Control | P | 0.278 | 0.212 | 0.357 |
| B | P | 0.243 | 0.720 | 0.843 |
| Control | B+P | 0.001 | 0.003 | 0.003 |
| B | B+P | 0.001 | 0.004 | 0.009 |
| P | B+P | 0.035 | 0.003 | 0.023 |

NOTE: All significance testing is done at the \( P < 0.05 \) level.
Abbreviations: AUC, area under the curve; MAD, median absolute deviation; B, bortezomib; P, Belinostat.
performed here seem to suggest that a similar mechanism of apoptosis might be operative with the combination in MCL as well.

Before exploring the in vivo activity of belinostat and romidepsin in combination with bortezomib in a SCID beige xenograft model of MCL, we performed exploratory studies to optimize the toxicity profile of each HDACI given in combination. Plumb et al. (32) showed that a dose of belinostat up to 40 mg/kg/d administered i.p. for 1 week is safe in a nude mouse model. When giving belinostat at 40 mg/kg/d for 7 days in combination with bortezomib in a SCID beige model, we found that a 10% lower dose of belinostat was required to avoid excessive toxicity (no mouse deaths together with no more than 30% of the mice experiencing significant weight loss). In a subsequent xenograft model of MCL (HBL-2), the combination of belinostat at 35 mg/kg with bortezomib showed acceptable toxicity (transient weight loss in 3 of 10 mice but no deaths) compared with other doses and schedules. A statistically significant advantage for the combination group was shown (P ≤ 0.03), with two complete responses out of 10 mice.

To date, the in vivo experience with romidepsin is limited. Although exploratory dosing studies with romidepsin have been conducted, the optimal doses and schedules have yet to be identified. From a proof-of-principle perspective, the in vivo data of bortezomib plus belinostat support the in vitro data. Clearly, additional studies across the panoply of drugs now being developed as proteasome and HDACIs need to be conducted.

In conclusion, the combination of HDACIs such as romidepsin or belinostat and a proteasome inhibitor has shown to be markedly active across a panel of MCL cell lines without producing excessive toxicity in normal PBMCs from healthy donors. In addition, in vitro xenograft studies exploring the combination of the HDACI belinostat plus bortezomib confirmed the in vitro observations. Phase I to II studies have begun to explore this combination, with a focus on relevant pharmacokinetic and pharmacodynamic relationships. The consequence of many lines of data around the pivotal role of Noxa in MCL, especially as it relates to the use of proteasome and HDACIs, suggests that Noxa and the Δψm could be important biomarkers of response. Although no studies to date have addressed the potential value of identifying such biomarkers, it is clear that such surrogate markers of activity could, or perhaps should, be used to guide the pharmacologic dosing or schedule design of these agents to optimize the synergistic interaction of these novel drug classes.

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