Cancer Therapy: Preclinical

Vorinostat-Induced Apoptosis in Mantle Cell Lymphoma Is Mediated by Acetylation of Proapoptotic BH3-Only Gene Promoters

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Abstract

**Purpose:** Mantle cell lymphoma (MCL) is an aggressive B-cell neoplasm with generally poor prognosis, for which current therapies have shown limited efficacy. Vorinostat is a histone deacetylase inhibitor (HDACi) that has been approved for the treatment of cutaneous T-cell lymphoma. Our purpose was to describe the molecular mechanism whereby vorinostat induces apoptosis in MCL with particular emphasis on the role of proapoptotic BH3-only proteins.

**Experimental Design:** The sensitivity to vorinostat was analyzed in eight MCL cell lines and primary cells from 10 MCL patients. Determination of vorinostat mechanism of action was done by flow cytometry, immunoblotting, HDAC activity assay kit, quantitative reverse transcription PCR, chromatin immunoprecipitation, and siRNA-mediated transfection.

**Results:** Vorinostat inhibited total histone deacetylase activity leading to selective toxicity toward tumor cells. Vorinostat-mediated cell death implied the activation of mitochondrial apoptosis, as attested by BAX and BAK conformational changes, mitochondrial depolarization, reactive oxygen species generation, and subsequent caspase-dependent cell death. This phenomenon was linked to H4 hyperacetylation on promoter regions and consequent transcriptional activation of the proapoptotic BH3-only genes BIM, BMF, and NOXA. Selective knockdown of the three corresponding proteins rescued cells from vorinostat-induced apoptosis. Moreover, vorinostat enhanced the activity of the BH3-mimetic ABT-263 in MCL cells, leading to synergistic apoptosis induction.

**Conclusion:** These results indicated that transcriptional upregulation of BH3-only proteins plays an important role in the antitumoral activity of vorinostat in MCL, and that HDACi alone or in combination with BH3-mimeticizing agents may represent a promising therapeutic approach for MCL patients.

**Clin Cancer Res; 17(12); 3956–68. ©2011 AACR.**

Introduction

Mantle cell lymphoma (MCL) is an aggressive B-cell neoplasm which corresponds to 5% to 10% of all non-Hodgkin lymphomas. It is characterized by the overexpression of cyclin D1 resulting from the chromosomal translocation t(11;14)(q13;q32). Furthermore, MCL cells harbor a high number of additional chromosomal and molecular alterations that may confer the aggressive behavior to the tumor. Conventional chemotherapy obtains frequent remissions (60%-90%) which are usually very short (1–2 years). High intensive regimens, including autologous and allogeneic stem cell transplantation, improve the outcome but only in a limited proportion of patients, due to the high median age of MCL diagnosis (>60 years; ref. 1). Recent studies have documented clinical responses in MCL following treatment with novel agents such as the mTOR kinase inhibitor temsirolimus, the proteasome inhibitor bortezomib, and the immunomodulatory agent lenalidomide. However, none of these agents provide long-term benefit and patients eventually relapse (2). Hence, novel targeted treatments are urgently needed.

Aberrant loss of histone acetylation is a common feature in cancer cells, particularly in leukemia and lymphoma (3). Acetylation of lysine residues within the H3 and H4 histone tails of the nucleosomes constitutes an important epigenetic mechanism whereby gene expression is controlled. Such status is regulated by histone deacetylases (HDAC) and histone acetyltransferases. HDACs mediate the removal of the acetyl groups from the lysine residues in histone tails, thus deacetylating chromatin. Hypoacetylation of histones on gene promoters is associated with a condensed and inactive chromatin conformation and repressed transcription, because it becomes inaccessible for transcription factors. The potential reversibility of the epigenetic abnormalities that occur in cancer cells...
Vorinostat Signaling in MCL

MCL is a B-lymphoid neoplasm with poor response to current therapies, thus novel therapeutic strategies are needed. Vorinostat is an HDACi that has been approved for the treatment of cutaneous T-cell lymphoma. Although it has shown clinically promising results, its mechanism of action has not been fully elucidated. In this article, we describe the mechanisms underlying vorinostat antitumoral activity in cell lines and primary MCL. Our results suggest that transcriptional upregulation of BH3-only proteins plays an important role in the antitumoral activity of vorinostat in MCL, thereby facilitating the antitumoral activity of the BH3-mimetic compound ABT-263. We thus provide a better comprehension of vorinostat mechanism of action and the basis for its rational use alone or in combination, a concern that may hopefully improve the outcome of MCL patients.

Translational Relevance

MCL is a B-lymphoid neoplasm with poor response to current therapies, thus novel therapeutic strategies are needed. Vorinostat is an HDACi that has been approved for the treatment of cutaneous T-cell lymphoma. Although it has shown clinically promising results, its mechanism of action has not been fully elucidated. In this article, we describe the mechanisms underlying vorinostat antitumoral activity in cell lines and primary MCL. Our results suggest that transcriptional upregulation of BH3-only proteins plays an important role in the antitumoral activity of vorinostat in MCL, thereby facilitating the antitumoral activity of the BH3-mimetic compound ABT-263. We thus provide a better comprehension of vorinostat mechanism of action and the basis for its rational use alone or in combination, a concern that may hopefully improve the outcome of MCL patients.

Patients, Materials, and Methods

Cell lines

Eight human MCL cell lines (GRANTA-519, Z-138, HBL-2, JVM-2, JEKO-1, UPN-1, MAVER-1, and REC-1) were used (Table 1). Their genetic characteristics were previously described (13). Cell lines were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% to 20% heat-inactivated FBS, 2 mmol/L glucose, 50 μg/mL penicillin–streptomycin (Invitrogen), and maintained in a humidified incubator at 37°C with 5% carbon dioxide. All cells were routinely tested for Mycoplasma infection by PCR and the identity of all cell lines was verified by using AmpFISTR identifier kit (Applied Biosystems).

Isolation and culture of primary cells

Tumor cells from 10 patients diagnosed of MCL, according to the World Health Organization classification criteria (14), who were either untreated or had not received treatment for the previous 3 months, were used. The study was done in accordance with protocols approved by the Ethics Committee of the Hospital Clinic (Barcelona, Spain), and all patients signed an informed consent according to the Declaration of Helsinki. The characteristics of these cases are listed in Table 1. For all samples, cyclin D1 overexpression was determined by immunohistochemistry or realtime PCR. Tumor cells from patients and peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll sedimentation (GE Healthcare), and conserved within the Hematopathology Biobank of our institution (CDB Biobank/IDIBAPS-Hospital Clinic Biobank). Cells were either used directly or cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (Sigma), 60% FBS and 30% RPMI 1640. Freezing/thawing manipulations did not influence cell response (15) and cells were cultured in a supplemented RPMI medium likewise cell lines.

Cytotoxic studies and analysis of apoptotic features by flow cytometry

MCL samples were treated as indicated with vorinostat (kindly provided by Merck & Co.) and the BH3-mimetic ABT-263 (Selleck Chemicals). When specified, cells were preincubated for 1 hour with 50 μmol/L of the pan-caspase inhibitor benzoylcyarboxyl-Val-Ala-Asp-fluoro-methylketone (z-VAD.fmk; Bachem) previous to drug addition. Cell viability was quantified after dual staining of external exposure of phosphatidylserine (PS) residues with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI; Bender Medsystems). For the analysis of apoptosis in CD3 + and CD19 + subpopulations, PBMCs were labeled simultaneously with anti-CD3–FITC and anti-CD19–phycoerythrin (PE; Becton Dickinson) antibodies.
and Annexin V–allophycocyanin (APC; Bender Medsystems). Loss of mitochondrial membrane potential (Δy_m) and reactive oxygen species (ROS) production were evaluated after 30 minutes of staining cells at 37°C with 20 nmol/L 3,3’-diexyloxacarbocyanine iodide [DiOC6(3)] and 2 μmol/L dihydroethidine (DHE; Invitrogen), respectively. For the detection of active caspase 3 and conformational changes of BAX and BAK, cells were fixed with 4% paraformaldehyde (USB Corporation), permeabilized with saponin 0.1% and albumin 0.5% (Sigma) in PBS, and labeled for 45 minutes with 1 mg/mL of antibodies against the active forms of caspase 3 (clone C92–605), BAX (clone 6A7; BD Pharmingen) and BAK (clone Ab-1; Oncogene Research), respectively. FITC-conjugated anti-rabbit and anti-mouse secondary antibodies (Sigma) were added afterwards. A total of 10,000 to 25,000 stained cells per sample were acquired and analyzed in a FACScan or FACSCalibur flow cytometer by using CellQuest and Paint-A-Gate softwares (Becton Dickinson). Lethal dose 50 (LD50) was defined as the concentration of drug required to reduce cell viability by 50%. For drug combinations, combination indexes (CI) were calculated with the CalcuSyn software version 2.0 (Biosoft) by using the Chou and Talalay algorithm. The interaction between 2 drugs was considered synergistic when CI < 1.

**HDAC activity assay**

Cells were lysed in Radio-ImmunoPrecipitation Assay buffer (Sigma) supplemented with protease and phosphatase inhibitors as follows: 1 mmol/L phenylmethanesulfonyl fluoride, 2 mmol/L sodium pyrophosphate decahydrate, 2 mmol/L sodium beta-glycerophosphate, 1 mmol/L sodium fluoride (NaF), 1 mmol/L sodium orthovanadate (Na3VO4), 10 μg/mL aprotinin and 10 μg/mL leupeptin (Sigma). Whole protein lysates were quantified by using standard Bradford protein assay (Bio-Rad Laboratories), and deacetylase activity was determined as previously described.

<table>
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<th>MCL cell line</th>
<th>LD50 24 h (μmol/L)</th>
<th>% response (5 μmol/L) 24 h</th>
<th>LD50 48 h (μmol/L)</th>
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<th>Disease status</th>
<th>Morphologic variant</th>
<th>% of tumor cells</th>
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*Percentage of cytotoxicity induced by vorinostat at the indicated doses and times.
bp53 mutational status detected by FISH and direct sequencing.
*Levels of mRNA (arbitrary units) assessed by quantitative PCR using as a calibrator the mean value of all MCL cell lines.
*Mutations not analyzed.
*CD19+ tumor cells quantified by flow cytometry.

Abbreviations: NR, not reached; Del indicates deletion; Mut, mutation; Wt, wild type; Upd, uniparental disomy; Ampl, amplification.
was measured employing a colorimetric Histone Deacetylase Assay Kit (Millipore), following manufacturer’s instructions. The assay is based on the detection of p-nitroanilide which is released on incubation of the deacetylated substrate with the activator solution turning out color. Colorimetric detection was read at 415 nm by using a spectrophotometer. Basal HDAC activities were referred to a HeLa nuclear extract and percent inhibition of treated cells was compared with untreated controls.

**Western blot analysis**

Total protein extracts were obtained after cell lysis in triton buffer [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mmol/L EDTA and 1% Triton X-100] supplemented with protease and phosphatase inhibitors as previously. Whole protein lysates were quantified by using standard Bradford protein assay, subsequently denatured with protease and phosphatase inhibitors as previously. Whole protein lysates were separated on a 12% to 15% SDS-PAGE and transferred to an Immobilon P membrane (Millipore). Membranes were blocked in TBS-Tween 20 containing 5% nonfat dry milk. For protein immunodetection, membranes were incubated with the specific primary antibodies and horseradish peroxidase-labeled secondary antibodies. Chemiluminescence detection was done by using ECL detection system (Pierce) in a mini-LAS4000 Fujiﬁlm device. Membranes were probed with primary antibodies against: HDAC1, HDAC2, HDAC3, HDAC4, HDAC5 (Cell Signaling Technology), HDAC6 (clone A01; Abnova), acetyl lysine 4 from histone H3 (acetyl-H3), acetyl lysine 12 from histone H4 (acetyl-H4; Upstate), histone H3, histone H4, MCL-1 (clone S-19), BCL-XL (clone S-18), BCL-2 (clone 100; Santa Cruz Biotechnology Inc.), BIM (Calbiochem), BMF (clone 9G10) and NOXA (clone 114C307; Enzo Life Sciences). Anti-mouse IgG (Sigma), anti-rabbit IgG (Sigma and Cell Signaling Technologies) and anti-rat IgG (Santa Cruz Biotechnology Inc.) were used as secondary antibodies. Equal protein loading was conﬁrmed by probing membranes with anti-β-actin or anti-α-tubulin antibodies (Sigma). For densitometric quantification, relative protein levels of HDACs were referred to β-actin by using Image Gauge software (Fujifilm).

**mRNA quantification by real-time PCR**

Total RNA was extracted using TRIZOL method (Invitrogen) according to manufacturer’s instructions. One microgram of RNA was retrotranscribed to cDNA with M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Roche). BMF, BIM, NOXA, p27, and Ki67 mRNA expression was analyzed in duplicate by quantitative real-time PCR (qRT-PCR) on the ABI Prism 7900HT sequence detection system (Applied Biosystems) by using predesigned Assay-On-Demand probes (Applied Biosystems). The relative expression of each gene was quantified by the comparative cycle threshold (Ct) method (ΔΔCt) by using β-actin as endogenous control. Expression levels are given in arbitrary units, taking as a reference the control sample (untreated cells) or the mean of values of all MCL cell lines for p27 and Ki67.

**Chromatin immunoprecipitation**

The assay was conducted using an acetyl-histone H4 immunoprecipitation assay kit (Millipore) following manufacturer’s instructions. Cells (2 × 10^6–6 × 10^6) were fixed in 0.5% formaldehyde for 10 minutes and lysed. Chromatin was sonicated in a Branson sonifier to an average length of 0.2 to 2 kb. Chromatin was then immunoprecipitated with an anti-acetylated histone H4 antibody, which recognizes transcriptionally active chromatin regions, or beads-only for the negative control. After washing, crosslinking was reversed and DNA was purified with phenol/chloroform and ethanol precipitation. Isolated DNA was analyzed by RT-PCR on the ABI Prism 7900HT sequence detection system (Applied Biosystems) according to manufacturer’s instructions. One microgram of RNA was retro transcribed to cDNA with M-MLV reverse transcriptase (Invitrogen) and random hexamer primers (Roche). BMF, BIM, NOXA, p27, and Ki67 mRNA expression was analyzed in duplicate by quantitative real-time PCR (qRT-PCR) on the ABI Prism 7900HT sequence detection system (Applied Biosystems) by using predesigned Assay-On-Demand probes (Applied Biosystems). The relative expression of each gene was quantified by the comparative cycle threshold (Ct) method (ΔΔCt) by using β-actin as endogenous control. Expression levels are given in arbitrary units, taking as a reference the control sample (untreated cells) or the mean of values of all MCL cell lines for p27 and Ki67.
Figure 2. Decrease in HDAC activity by vorinostat and HDAC characterization in MCL. A, JEKO-1 (left) and REC-1 (right) were treated with vorinostat at the specified doses and times. Acetylated levels of histones H3 and H4 (acetyl-H3 and acetyl-H4) were analyzed by Western blotting. Histones H3 and H4 were probed as a loading control. B, JEKO-1, REC-1, and primary MCL#6 cells were incubated with 1 μmol/L vorinostat for the indicated times. HDAC activity was determined as described in “Patients, Materials, and Methods” and expressed respect to untreated cells at 0 hours (100%). Results represent the mean ± SEM of 3 independent experiments. C, correlation of basal HDAC activity, referred to HeLa nuclear extract (100%), and % of response to vorinostat 5 μmol/L after 24 hours of incubation in primary and MCL cell lines. D, Western blot analysis of basal HDAC1, 2, 3, 4, 5, and 6 levels in primary and MCL cell lines (top). Relative protein quantification of HDACs in primary and MCL cell lines in correlation with % of response to 24-hour exposure of vorinostat 5 μmol/L (bottom).
GGCTGCAAGAATCAAGT-3' and 5'-TGGCGGGAGGGAAGGGTTT-3'; BIM: 5'-CTGTGTCTGCAGTTTGCAGAATCAAGT-3' and 5'-GCTGCAGAATCAAGT-3'). The relative amount of immunoprecipitated DNA was quantified by the ΔΔCt method by using RNase P (Applied Biosystems) from input samples as a calibrator. Acetylation levels are given in arbitrary units using as a reference the control condition (untreated cells).

**siRNA assays**

Five million cells were cultured without antibiotics and washed with FBS-free medium. Then, cells were resuspended in 100 μL of Ingenol Electroporation Solution (Mirus) containing a mix of 3 different oligonucleotides (2.5–3 μmol/L) for each gene. Commercially available Silencer Select Pre-designed siRNAs for BIM, BMF, and NOXA and a nonsilencing negative control (Ambion) were used. A custom siRNA for BMF (target sequence: 5'-AAAGGTGTCATGCTGCCTTGT-3'; Qiagen) was also added to the mix. Cells were transfected in a Nucleofector II device (Lonza) by using M-013 and A-032 programs for 2 cell lines used above, as well as of a representative MCL donor. Sensitivity to vorinostat was measured by 100 μL of Ingenol Electroporation Solution containing a mix of 3 different oligonucleotides (2.5–3 μmol/L) for each gene. Commercially available Silencer Select Pre-designed siRNAs for BIM, BMF, and NOXA and a nonsilencing negative control (Ambion) were used. A custom siRNA for BMF (target sequence: 5'-AAAGGTGTCATGCTGCCTTGT-3'; Qiagen) was also added to the mix. Cells were transfected in a Nucleofector II device (Lonza) by using M-013 and A-032 programs for 2 cell lines used above, as well as of a representative MCL donor.

**Statistical analysis**

The data are depicted as the mean ± SD or SEM of 3 independent experiments. All statistical analyses were done by using GraphPad Prism 4.0 software (GraphPad Software). Comparison between 2 groups of samples was evaluated by nonparametric Mann–Whitney test and 2-way ANOVA to determine how response is affected by 2 factors. Nonparametric Spearman test was used to assess statistical significance of correlation. Results were considered statistically significant when P < 0.05 (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

**Results**

**Vorinostat induces selective MCL tumor cell death**

Eight MCL cell lines were exposed to vorinostat for 24, 48, or 72 hours, at doses ranging from 0.1 to 25 μmol/L. The LD50 for these MCL cell lines at 24 and 48 hours were listed in Table 1. No association was observed between sensitivity to vorinostat and known genetic alterations or mRNA levels of Ki67 and p27 (Table 1). REC-1, UPN-1, MAVER-1, and JEKO-1 were the most sensitive, showing a LD50 ≤ 2.5 μmol/L after 24 hours of vorinostat treatment. JVM-2 and HBL-2 exhibited low sensitivity to vorinostat with a LD50 > 10 μmol/L, whereas the LD50 could not be reached in GRANTA-519 and Z-138 cell lines. Importantly, the cytotoxic effect of vorinostat increased greatly after 48 and 72 hours of incubation, reaching physiologically achievable concentrations (16) in all cell lines except GRANTA-519, which was the least-sensitive with a LD50 around 5.30 μmol/L at 48 hours.

In cells from 10 MCL patients (Table 1), the sensitivity to vorinostat (range 0.1–25 μmol/L) after 24 hours of incubation was also heterogeneous. Seven cases (no. 1, 2, 4, 5, 6, 8, and 10) were sensitive to vorinostat (mean LD50 2.24 ± 1.45 μmol/L), whereas 3 cases (no. 3, 7, and 9) showed low cytotoxicity (LD50 > 10 μmol/L). Again, no association was observed between the response to vorinostat and the genetic alterations identified in these MCL cases or Ki67 and p27 levels (Table 1).

Importantly, when compared with normal T (CD3+) and B (CD19+) lymphocytes from 3 different healthy donors, sensitive MCL primary samples were significantly more responsive to vorinostat (***, P < 0.001) at doses up to 25 μmol/L (Fig. 1), thus showing the specificity of the drug toward malignant B cells.

These results showed that vorinostat exerted a selective, heterogeneous, and time- and dose-dependent cytotoxic effect in MCL cells.

**Sensitivity to vorinostat is independent of basal HDAC activity and HDAC protein levels in MCL**

To establish the relationship between vorinostat toxicity and histone acetylation, we analyzed the expression of acetylated histones H3 (acetyl-H3) and H4 (acetyl-H4), after vorinostat treatment in 2 representative cell lines, JEKO-1 and REC-1. Acetylation of both histones occurred in a dose-dependent manner, as soon as 6 hours of treatment for H4 and at 24 hours for H3 (Fig. 2A).

To further confirm that vorinostat was efficiently inhibiting HDAC activity in MCL, we monitored the evolution of total HDAC activity following vorinostat exposure of the 2 cell lines used above, as well as of a representative MCL primary culture (Fig. 2A). Vorinostat inhibited HDAC activity in MCL cells after only 1 hour of incubation and this effect was sustained within 6 hours of treatment with the drug (Fig. 2B).

In an attempt to determine whether the differences in sensitivity to vorinostat could be due to distinct basal HDAC activities among MCL samples, we then compared the inherent HDAC activity in a set of primary MCL cells (n = 9) and MCL cell lines (n = 8), using HeLa nuclear extracts as a reference control (100% activity). As shown in Figure 2C, no significant correlation was observed between cytopathic effect after 24-hour exposure to vorinostat 5 μmol/L and basal HDAC activities. Next, we monitored by Western blot the basal protein expression levels of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC6 in 8 MCL cell lines and 8 primary MCL tumors (#1–8; Fig. 2D). After densitometric quantification of HDAC protein levels, the values were normalized to β-actin. Of note, MCL cell lines harbored significantly higher levels of HDAC2 (***, P < 0.001) and HDAC4 (*, P < 0.05) than primary tumors, whereas HDAC5 and HDAC6 expression was undetectable in primary MCL tumors. However, no significant differences in HDAC protein levels were found regarding sensitivity to vorinostat.

These data confirmed that vorinostat was efficiently inhibiting HDACs in MCL, although neither basal HDAC activities nor basal HDAC protein expression could predict cell response to vorinostat.
Vorinostat induces activation of the mitochondrial apoptotic pathway in MCL cells

We observed that vorinostat treatment led to typical events occurring during mitochondria-dependent apoptosis in MCL cells, including BAX and BAK conformational changes, ΔΨm loss and caspase 3 activation along with PS residues exposure. In addition, vorinostat toxicity was accompanied by a remarkable production of ROS (Fig. 3A).

**Figure 3.** Mitochondrial apoptotic cell death induced by vorinostat. A, JEKO-1 cells were treated with vorinostat 2 μmol/L for 24 hours. When indicated, cells were previously incubated with the pan-caspase inhibitor z-VAD.fmk (50 μmol/L). BAX/BAK conformational changes, loss of ΔΨm, caspase 3 activation, PS residues exposure, and ROS production were determined by flow cytometry as described in “Patients, Materials, and Methods.” The percentages inside each chart refer to the population in black. These experiments have been carried out twice with similar results and a representative experiment is shown.

B, BMF, NOXA, and BIM mRNA levels were analyzed by qRT-PCR after treating samples (REC-1, JEKO-1, GRANTA-519, and MCL#6) with vorinostat for 6 hours. Untreated cells were used as a reference control. C, Western blot analysis of BCL-2 family proteins following 6 hours of vorinostat exposure in REC-1, JEKO-1, GRANTA-519, and MCL#6. α-Tubulin was probed as an equal loading control.
Mitochondrial alterations preceded the activation of caspases, as BAX/BAK conformational changes and mitochondrial depolarization were not inhibited by preincubating MCL cells with the pan-caspase inhibitor z-VAD.fmk (Fig. 3A). Of note, the generation of ROS was completely prevented in the presence of z-VAD.fmk (Fig. 3A), indicating that this phenomenon was a mere consequence of caspase activation instead of a primary event.

To investigate the upstream events involved in vorinostat-induced apoptosis, we carried out the expression analysis of 3 BH3-only proteins (BIM, BMF, and NOXA) by qRT-PCR in MCL cells (REC-1, JEKO-1, GRANTA-519, and primary MCL#6). We observed a constant upregulation of BIM, BMF, and NOXA after treatment of MCL cells with vorinostat (Fig. 3B). After 6 hours of incubation, vorinostat was able to induce an 11-fold and 7-fold increase in BMF mRNA levels in JEKO-1 and REC-1 cells, respectively, whereas this effect was slightly lower in primary MCL#6 (3-fold). NOXA mRNA was also enhanced about 4 times in JEKO-1 and a 2-fold increase was observed in REC-1 and primary cells. Finally, about 2-fold increase in BIM mRNA levels was also detected in REC-1 and primary cells (Fig. 3B), whereas this transcript was undetectable in the JEKO-1 cell line as a consequence of a reported homozygous deletion at 2q13-q21 where BIM is located (17). In contrast, the low sensitive cell line GRANTA-519 failed to induce any of the BH3-only proteins (Fig. 3B).

These findings were concordant with protein expression since after 6 hours of drug incubation induction of BIM, BMF, and NOXA proteins in REC-1 and primary cells (MCL#6) was found, as well as NOXA and BMF in JEKO-1. Similarly to mRNA, no upregulation of these proteins was found in GRANTA-519 (Fig. 3C). No changes in the antipapoptotic BCL-2 and BCL-X_L protein levels were detected, whereas MCL-1 accumulation was observed on vorinostat treatment.

Altogether, these results indicated that vorinostat exposure led to the upregulation of a set of proapoptotic BCL-2 family members, causing BAX/BAK activation, mitochondrial perturbation, and caspase-dependent death of MCL cells.

Vorinostat acetylates histones in the promoter regions of BIM, BMF, and NOXA

To explore the molecular mechanism through which vorinostat activated transcription of BMF, BIM, and NOXA genes, we considered the possibility that the compound was enhancing histone acetylation in their promoter regions. To investigate this hypothesis, we employed a chromatin immunoprecipitation (ChIP) assay. Quantitative PCR was used to measure the abundance of a DNA fragment from the BIM, BMF, and NOXA promoters that was associated with immunoprecipitated histones. As shown in Figure 4A, after 6 hours of incubation of REC-1 and JEKO-1 cells with vorinostat, there was a 2- to 6-fold increase in the acetylation levels of histone H4 at the promoter regions of these BH3-only genes. In contrast, a slight or almost undetectable increase in promoter acetylation of these genes was detected in the vorinostat-low sensitive cell line GRANTA-519, thus 3-fold less acetylated than sensitive cells (JEKO-1) exposed at the same dose (5 μmol/L). Notably, we found a significant correlation (*, P < 0.05) between induction of BMF and NOXA mRNA levels with H4 acetylation increase in their respective promoter regions after vorinostat treatment (Fig. 4B). A marginal significance (P = 0.08) was observed for BIM, probably due to the small number of samples analyzed, as JEKO-1 cells harbored a BIM deletion.

These findings suggested that transcriptional activation of the BH3-only genes by vorinostat resulted from histone acetylation in their promoter regions, and that this event was tightly linked to MCL susceptibility to vorinostat.

BIM, BMF, and NOXA cooperate in vorinostat-induced apoptosis in MCL cells

To ascertain if this increase in BH3-only proteins was functionally important for vorinostat-mediated apoptosis, we used a siRNA-mediated approach to knockdown BMF, BIM, and NOXA in REC-1 cells as well as BMF and NOXA in JEKO-1 cells. As shown in Figure 4C, transfection with siRNA oligonucleotides directed toward these genes significantly reduced mRNA (data not shown) and protein levels (Fig. 5A). Knockdown of BMF, BIM, or NOXA alone partially protected cells from vorinostat-induced apoptosis (Fig. 4D) being NOXA silencing more potent in interfering with vorinostat activity than BMF or BIM. Dual silencing (BMF+BIM, BMF+NOXA, and BIM+NOXA) provided an enhanced protection against HDACi-induced cell death when compared with individual gene knockdown. Importantly, we found that triple knockdown combination (BMF+BIM+NOXA) in REC-1 cells conferred the maximum protection to vorinostat-induced apoptosis in MCL cells (Fig. 4D).

These data strongly suggested that the activation of the 3 BH3-only proteins BMF, BIM, and NOXA was responsible for vorinostat antitumoral activity in MCL cells.

Vorinostat has a synergistic effect with the BH3-mimetic ABT-263 in MCL cells

Because vorinostat upregulated several BH3-only proteins, we then examined the effect of combining vorinostat with the BH3-mimetic ABT-263 in MCL cells. Simultaneous exposure of these cells to vorinostat (0.75–1 μmol/L) and ABT-263 (5–100 nmol/L) for 24 hours reduced cell viability more effectively than single drug treatment (Fig. 5A). Vorinostat/ABT-263 combination was found to evoke synergistic cell death in all the samples analyzed because the calculated CIs were lower than 1 in the great majority of the conditions. The highest synergistic effect was observed when combining vorinostat 1 μmol/L and ABT-263 50 nmol/L for JEKO-1 (CI = 0.328) and REC-1 (CI = 0.711) cells, and vorinostat 1 μmol/L and ABT-263 (5 or 10 nmol/L) for primary MCL samples (CI = 0.660 in MCL#6 and CI = 0.537 in MCL#8; Fig. 5A).

As shown in Figure 5B, combination of vorinostat (1 μmol/L) and ABT-263 (50 nmol/L) in JEKO-1 at 24 hours, induced a higher increase in cell death by enhancing
Figure 4. Vorinostat enhancement of histone acetylation within BH3-only gene promoters and functional assay. A, JEKO-1, REC-1, and GRANTA-519 cells were treated with vorinostat at the indicated doses for 6 hours. Acetylation of H4 histones on NOXA, BMF, and BIM promoters was evaluated by ChIP and quantified by RT-PCR (see “Patients, Materials, and Methods”). Untreated cells were used as a reference control. B, correlation between vorinostat-induced mRNA and vorinostat-induced promoter acetylation of BMF, NOXA, and BIM for REC-1, JEKO-1, and GRANTA-519 cell lines at 6 hours. Statistical significance was evaluated with nonparametric Spearman correlation test (*, $P < 0.05$, **, $P < 0.01$). C, JEKO-1 and REC-1 cells were transfected with BMF, BIM, and/or NOXA or nonsilencing (control) siRNA for 6 hours previously to vorinostat incubation (5 μmol/L for JEKO-1 and 2 μmol/L for REC-1) for 20 additional hours. Knockdown experiments were confirmed by Western blot analysis of BMF, BIM, and NOXA. α-Tubulin was probed as a loading control. D, cell viability was determined by Annexin V labeling. Bars represent the mean ± SD of apoptosis induced in vorinostat-treated cells minus control cells for each condition.
the typical mitochondrial hallmarks of apoptosis (BAX/BAK conformational changes, ΔΨm loss, caspase activation, and PS exposure) when compared with each drug alone.

Taken together, these results showed a synergistic interaction between vorinostat and ABT-263 that was a consequence of enhanced mitochondrial cell death.

**Discussion**

Overexpression of human HDACs is commonly found in several tumor models. This observation has led to the development of several HDACis with antitumoral activity in a wide range of neoplastic disorders including MCL.
(5;6), which poorly responds to common drugs (2). Multiple preclinical studies and clinical data support the use of HDACi in combination with other cancer therapies (18). Specifically, combinations with proteasome inhibitors (19–21), mTOR inhibitors (22, 23), and HSP90 inhibitors (24) have been proposed for MCL therapy.

Vorinostat is able to inhibit class I (HDAC1, 2, 3, and 8) and class II (HDAC6) HDACs (25). In MCL cells, vorinostat has been shown to cause a remarkable proliferation arrest, related to cyclin D1 downregulation through inhibition of PI3K/Akt/mTOR signaling (26), to upregulate the cell-cycle inhibitors p21 and p27 (27) and to ultimately lead to MCL cell death.

One of the most promising properties of HDACis is their ability to selectively induce apoptosis in malignant cells, while sparing the normal tissue (4). Importantly, herein we show that physiologically achievable low doses of vorinostat are effective in MCL cells, with LD50 similar to those reported previously (28), being this effect selective for tumor cells.

In this article, we have characterized the expression and activity of HDACs in MCL cells. Particularly, we have observed that HDAC2 and 4 levels are lower in primary MCL cells than in cell lines, whereas, as previously reported, we have found no HDAC6 expression in MCL primary cells (29), nor HDAC5. This last observation excludes a key role for these 2 proteins as relevant targets of vorinostat. In accordance, enzymatic assays have confirmed that this drug preferentially inhibits HDACs 1 and 3 (30). Nevertheless, our results indicate that sensitivity to vorinostat is not due to distinct basal HDAC protein/activity levels. We show that vorinostat can inhibit global HDAC activity by almost 50% in less than 6 hours in both MCL cell lines and primary cells, and consequently triggers the accumulation of acetylated histones H3 and H4.

Remarkably, our results show that vorinostat is able to induce accumulation of acetylated histone H4 on the proapoptotic BH3-only (BIM, BMF, and NOXA) gene promoters that, in turn, correlates with a transcriptional activation of these genes. Upregulation of BIM and BMF consequent to HDAC inhibition and enhanced promoter acetylation has been described in other models (31, 32). In this sense, it has been proposed a direct role for promoter deacetylation in the epigenetic silencing of these genes and malignant process, such as the repression of BIM in acute lymphoblastic leukemia and Burkitt lymphoma (32, 33). However, we cannot exclude a role for transcription factor activation on direct acetylation that could further enhance transcription. This has also been described for BIM, given that direct acetylation of E2F1 or Foxo3a transcription factors may enhance their activity consequently increasing target mRNA (34, 35). Importantly, in line with a direct consequence of promoter acetylation in vorinostat cytotoxicity, we have observed that histones of the BH3-only gene promoters are more resistant to acetylation in vorinostat-low sensitive samples, suggesting that this event, coupled to decreased transcriptional activation, could explain at least in part the diminished sensitivity to the drug.

Recent studies confirm that the upregulation of the proapoptotic BH3-only proteins represents a critical step during HDACi-mediated apoptosis. Accordingly, it has been reported that vorinostat treatment upregulated BIM (35, 36) and NOXA (36), and its suppression prevented the apoptotic effect of the drug. Concerning BMF, its role in HDACi-induced apoptosis has been controversial. Although studies done in leukemic cells have not found a relevant function of this protein (36), other reports have suggested that the induction of BMF was the crucial event in HDACi-mediated apoptosis in other tumor models (31). Also it has been reported that loss of BMF protects murine lymphocytes against HDACi-induced apoptosis (37). In this context, we show that, in all the MCL samples tested, both BMF and NOXA are induced following vorinostat exposure, and that BMF upregulation is conditioned to the presence or not of the homozygous deletion of BIM, which occurs in some MCL cases (17, 38). Our results show that specific siRNA knockdown of BMF, BIM, or NOXA results in a partial protection from vorinostat-induced apoptosis. Higher protective effect is achieved when preventing the upregulation of all 3 proapoptotic proteins. Thus, these results show that activation of these proteins is required for full vorinostat activity in MCL cells, being all of them functional, and that BMF, BIM, and NOXA may trigger the apoptotic process in a cooperated, rather than a redundant, manner.

As we show vorinostat to induce the typical hallmarks of mitochondrial cell death, including BCL-2 family modulation, it is reasonable to hypothesize that the induced BMF, BIM, and NOXA may directly counteract the antiapoptotic BCL-2-like proteins, leading to the conformational activation of the proapoptotic proteins BAX and BAK. In this regard, it has been postulated that HDACi exposure increases the amount of BIM bound to both BCL-2 and BCL-XL, but has little effect on BIM/MCL-1 binding (39). In parallel, upregulated BMF competitively displaces BIM from antiapoptotic proteins to activate BAX and BAK (40). Furthermore, NOXA induction after vorinostat treatment may be important to selectively bind to MCL-1 displacing BAK from MCL-1, as previously reported in MCL after bortezomib treatment (41, 42). This might counteract, in part, the accumulation of MCL-1 that we and others have found to be consequent to HDAC inhibition (43). We also show that antiapoptotic BCL-2 and BCL-XL are not downregulated after short incubation with vorinostat, although it has been described a decrease in BCL-XL levels after incubation with other HDACis (36).

Our data strongly support that vorinostat induces apoptosis in MCL cells primarily via the activation of the mitochondrial apoptotic pathway independently of p53, in agreement with a set of published studies (36, 44, 45). Accordingly, using a mouse model of B-cell lymphoma, it has been described that therapeutic response to HDACi does not require p53 activity or a functional death receptor pathway, but depends on the activation of the intrinsic apoptotic pathway (46). In addition, our results confirm...
that ROS production seems to be a mere consequence of caspase activation (36).

As vorinostat is capable to rapidly increase the expression of BIM, BMF, and NOXA, we tested the ability of BH3-only mimetic compounds to enhance the cytotoxic effect of vorinostat. ABT-263 is a small-molecule BH3-mimetic that recapitulates the capacity of BH3-only proteins to bind to the hydrophobic grooves of BCL-2, BCL-X\textsubscript{L}, and BCL-W, thereby disrupting their antiapoptotic functions (47). We have observed a synergistic effect of vorinostat with ABT-263 in MCL cells that may be explained by an increase in proapoptotic BH3-only activity that can collaborate to induce apoptosis. A synergistic effect of vorinostat has been reported previously with ABT-737 (39, 48) and GX15-070 (49, 50) in other models as well. It has been proposed a role for calpain activity and ER-located caspase signaling in the induction of both autophagy and apoptosis following this combination of drugs (49).

In summary, our data suggest that vorinostat may define an attractive therapeutic approach for the treatment of MCL. We identify BMF, BIM, and NOXA as transcriptionally targeted genes of vorinostat treatment in MCL cells, all them participating in committing the cell to die.

Disclosure of Potential Conflicts of Interest
The authors have no conflicts of interest to declare.

Acknowledgments
The authors thank Sandra Cabezas, Laura Jiménez, and Jocabed Boldán for expert technical assistance. Vorinostat was kindly provided by Merck & Co. This work was carried out (in part) at the Esther Koplowitz Center, Barcelona.

Grant Support
Ministerio de Ciencia e Innovación (SAF/09/9503), Generalitat Catalunya (2009-SGR-967), Redes Temáticas de Investigación Cooperativa de Cáncer from the Instituto de Salud Carlos III (RED 2006-20-014 to D. Colomer and 2006-20-039 to E. Campo), Fondo de Investigación Sanitaria (CP07/00072 and PI09/00060 to G. Ros). S. Xargay-Torrent and L. Rosich are recipients of predoctoral fellowships from Ministerio Ciencia e Innovación (FPI) and IDIAPRS, respectively.

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Received December 23, 2010; revised March 23, 2011; accepted April 20, 2011; published OnlineFirst June 7, 2011.

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Clinical Cancer Research.
Vorinostat-Induced Apoptosis in Mantle Cell Lymphoma Is Mediated by Acetylation of Proapoptotic BH3-Only Gene Promoters

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*Clin Cancer Res* 2011;17:3956-3968. Published OnlineFirst June 7, 2011.

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