

Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF- κ B–dependent DNA methyltransferase activity in acute myeloid leukemia

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Bortezomib reversibly inhibits 26S proteasomal degradation, interferes with NF- κ B, and exhibits antitumor activity in human malignancies. Zinc finger protein Sp1 transactivates *DNMT1* gene in mice and is functionally regulated through protein abundance, posttranslational modifications (ie, ubiquitination), or interaction with other transcription factors (ie, NF- κ B). We hypothesize that inhibition of proteasomal degradation and Sp1/NF- κ B-mediated transactivation may impair aberrant DNA methyltransferase activity. We show here that, in addition to inducing

accumulation of polyubiquitinated proteins and abolishment of NF- κ B activities, bortezomib decreases Sp1 protein levels, disrupts the physical interaction of Sp1/NF- κ B, and prevents binding of the Sp1/NF- κ B complex to the *DNMT1* gene promoter. Abrogation of Sp1/NF- κ B complex by bortezomib causes transcriptional repression of *DNMT1* gene and downregulation of DNMT1 protein, which in turn induces global DNA hypomethylation in vitro and in vivo and re-expression of epigenetically silenced genes in human cancer cells. The involvement of

Sp1/NF- κ B in *DNMT1* regulation is further demonstrated by the observation that Sp1 knockdown using mithramycin A or shRNA decreases DNMT1 protein levels, which instead are increased by Sp1 or NF- κ B overexpression. Our results unveil the Sp1/NF- κ B pathway as a modulator of DNA methyltransferase activity in human cancer and identify bortezomib as a novel epigenetic-targeting drug. (Blood. 2008; 111:2364-2373)

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Introduction

Methylation of CpG islands in promoter region of genes is due to enzymatic addition of a methyl (CH₃) group at the carbon 5 position of cytosine and has been shown to inhibit gene transcription.¹ This enzymatic reaction is mediated by DNA methyltransferases (DNMTs: DNMT1, 3a, and 3b) that use *s*-adenosylmethionine (SAM) as a methyl donor. While DNMT3a and 3b are important to establish novel methylation sites on nascent DNA, DNMT1 plays a critical housekeeping role in maintaining established patterns of DNA methylation in dividing cells.² *DNMTs* have recently been found to be overexpressed in human acute myeloid leukemia (AML) and solid tumors, thereby supporting a role of these enzymes in the development and maintenance of the neoplastic phenotype.^{3,4} Inhibition of DNMT1 by antisense or shRNA oligonucleotides or nucleoside analogs (eg, 5-aza-2'-deoxycytidine [decitabine]) induces DNA hypomethylation and reactivation of hypermethylated tumor suppressor genes in leukemia cells.⁵⁻¹¹ This ultimately restores normal patterns of cell proliferation, differentiation, and apoptosis, which in turn leads to a significant antitumor activity.

To date, 2 hypomethylating nucleoside analogs (decitabine and 5-azacitidine, referred to hereafter as azanucleosides) have been approved by the FDA for the treatment of myelodysplastic

syndromes (MDSs) and are currently in clinical trials for other types of cancers.^{12,13} An accepted mechanism for the antitumor activity of these agents is their incorporation into newly synthesized DNA strands followed by covalent binding, sequestration, and depletion of the DNMT enzymes.^{12,13} Clinical responses to azanucleosides, however, appear to be restricted to a minority of hematopoietic malignancies, which are characterized by a relatively high proliferative cell fraction.¹⁴ Thus, development of novel hypomethylating compounds with mechanisms of action distinct from azanucleosides may broaden the therapeutic toolbox targeting epigenetic aberrations in human cancer.

Recent studies suggest that *DNMT1* expression is tightly regulated during normal cell growth, and its transcription is modulated by the Sp1 protein in mice.¹⁵ Sp1 is a ubiquitous zinc finger transcription factor that binds GC-rich *cis*-acting elements ((G/A)(G/A)GGCC(G/T)(G/A)(G/A)) in the promoter region of inducible genes.¹⁶ The complexity of the regulatory functions mediated by Sp1 can be explained by a variety of posttranslational modifications (ie, ubiquitination, glycosylation, phosphorylation) of this protein^{16,17} and/or its physical interaction with other transcription factors, such as those of the NF- κ B family^{18,19} that are constitutively activated in AML and controlled by the proteasomal degradation.^{20,21}

Submitted August 31, 2007; accepted December 9, 2007. Prepublished online as *Blood* First Edition paper, December 14, 2007; DOI 10.1182/blood-2007-08-110171.

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The 26S ubiquitin-proteasome, formed by the 20S core complex and the 19S regulatory particle, is present in both the cytoplasm and the nucleus of all eukaryotic cells²²⁻²⁷ and modulates both the levels and functions of many proteins involved in cell cycle progression, differentiation, apoptosis, and adhesion to the microenvironment.^{22,27-31} The proteolytic activity of 26S proteasome also plays a key role in regulating gene transcription by modulating coactivator recruitment, RNA transcriptional elongation, and protein posttranslational modifications.^{32,33} Disorders in ubiquitin-dependent proteolysis appear to be integral to the neoplastic phenotype, and newly developed inhibitors of the proteasome system have been demonstrated to effectively induce apoptosis in malignant cells.³⁴⁻³⁸

Bortezomib, the first proteasome inhibitor approved by the FDA for clinical use in multiple myeloma, is a dipeptidyl boronic acid that reversibly inhibits proteasome activity via formation of a pseudotetrahedral complex between the threonine amino-terminal group and the boronic acid pharmacophore.³⁸ It has been shown to inhibit NF- κ B activation by blocking the ubiquitination and proteasomal degradation of the endogenous NF- κ B inhibitor I- κ B.^{35,39} We postulated that exposure to bortezomib may cause disruption of the NF- κ B and Sp1 interplay, leading in turn to *DNMT1* down-regulation and DNA hypomethylation. Consistent with our hypothesis, we demonstrated here, for the first time, that bortezomib is a potent inhibitor of DNA methylation in malignant cells by interfering with Sp1/NF- κ B DNA-binding activity, which in turn results in decreased *DNMT1* expression, DNA hypomethylation, and transcription of methylation-silenced genes. These findings support bortezomib as a novel, nonazanucleoside therapeutic agent to target aberrant DNA hypermethylation in cancer.

Methods

Plasmid and cell lines

Construction of the human *Sp1* in Epstein-Barr virus (EBV)/retroviral hybrid vector (Pinco-Sp1) was established as previously described.⁴⁰ Retroviral infection to obtain Pinco-Sp1 or Pinco alone stably expressed in 293T cells was performed as previously reported.^{41,42} Two *Sp1* shRNA constructs were obtained by cloning the 2 correspondent annealed oligos into a pSuper.retro.neo + GFP vector (OligoEngine, Seattle, WA).^{43,44} The sequences of the 2 oligos for *Sp1* shRNA-1 were 5'-GATCCCCAAGTGTTCG-TGAGGAGTGTCAAGAGACTCCTCATGAAGCGCTTTTTTTA-3' and 5'-AGCTTAAAAAAGCGCTTCATGAGGAGTGTCTCTGAACACTCCT-CACGAAACACTTGGG-3'. The sequences of the 2 oligos for *Sp1* shRNA-2 were 5'-GATCCCCTCATCTATGGGTGAAATGTTCAAGAGACATTTCA-TCCATGGAGTGATTTTTTA-3' and 5'-AGCTTAAAAATCACTCCATGGAT-GAAATGTCTCTTGAACATTCACCCATAGAATGAGGG-3'. Cell lines were grown in DMEM supplemented with 10% (293T, HCT116) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) or in RPMI 1640 supplemented with 15% (Kasumi-1) or 10% (MV4-11, K562, ML-1) FBS (Invitrogen). Human bone marrow cells, which were obtained through the Ohio State Leukemia Tissue Bank from patients who gave informed consent in accordance with the Declaration of Helsinki on an IRB-approved protocol, were grown in RPMI 1640 supplemented with 15% human serum and granulocyte-macrophage colony-stimulating factor (GM-CSF) plus Cytokine Cocktail (R&D Systems, Minneapolis, MN).

Chemicals and antibodies

Bortezomib is commercially available from Millennium Pharmaceuticals (Cambridge, MA) and decitabine was purchased from Sigma-Aldrich (St Louis, MO). These compounds were dissolved in PBS sterilized by filtration through a 0.22- μ m syringe filter and stored at -80°C . The antibodies used were as follows: anti-HDAC1 (Upstate Biotechnology, Billerica, MA); anti-Sp1, β -actin, and rabbit IgG (Santa Cruz Biotechnol-

ogy, Santa Cruz, CA); anti-DNMT1 (New England Biolabs, Beverly, MA); and NF- κ B (p65) (Cell Signaling Technology, Danvers, MA).

Proteasome inhibition assay

MV4-11 cells were incubated with 60 nM bortezomib for the indicated time and stored at -80°C . A spectrofluorometric assay was used to assess the proteasome activity based on a previously described method.⁴⁵

In vivo xenograft models

Athymic nu/nu mice were purchased from Charles River (Wilmington, MA). Four- to 6-week-old animals ($n = 3$ to 5) were injected with 10^7 MV4-11 or K562 cells subcutaneously. When tumor size approached approximately 50 mm³, the animals received a single dose (2 mg/kg) of bortezomib or vehicle alone (intravenous bolus). After 48 hours, the tumors were excised for protein, RNA expression, and DNA methylation analysis.

Cell transfection, immunoprecipitation, and Western blot

Sp1 shRNA constructs were introduced into leukemia cell line by Nucleofector kits (Amaxa, Gaithersburg, MD) according to the manufacturer's instruction. Whole cellular lysates were prepared by harvesting the cells in $1 \times$ cell lysis buffer (20 mM HEPES [pH 7.6], 150 mM NaCl, and 0.1% NP40 supplemented with 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM NaF, 1 mM benzimidazole, and protease inhibitors [protease inhibitor cocktail set III; Calbiochem-Novabiochem, San Diego, CA]). Approximately 200 μ g nuclear extract or 1 mg total protein lysates was precleared with 70 μ L of 50% slurry of protein A or G agarose beads (Upstate Biotechnology) for 2 hours at 4°C . Agarose beads (70 μ L) were coated with 2 to 5 μ g antibodies at 4°C overnight. Precleared protein extracts were incubated with antibody-coated agarose beads for an additional 2 hours at 4°C . Bound proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Amersham, Piscataway, NJ). The protein blots were incubated with indicated antibodies, and signals were developed using a Chemiluminescent Detection kit (Amersham).

Quantification of global DNA methylation

DNA was prepared using a DNeasy tissue kit (QIAGEN, Valencia, CA) and hydrolyzed as previously described.⁴⁶ The hydrolyzed DNA was analyzed for DNA methylation by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) as previously described.⁴⁷

Electrophoretic mobility-shift assays and antibody-supershift assays

Three pairs of oligonucleotides derived from the human *DNMT1* promoter regions that contain putative Sp1-binding sites were chemically synthesized, and complementary oligos were annealed and labeled with ³²P-dCTP and Klenow. All reactions were processed on ice except for those indicated. The oligo sequences used were as follows: *DNMT1*/Sp1-1F: 5'-GGGCTCCGCGTGGGGGGGTGTGTGCCCGCCTTGCGC-3'; *DNMT1*/Sp1-1R: 5'-GCGCAAGGCGGGCACACACCCCCCCCAC-GCGGAG-3'; *DNMT1*/Sp1-2F: 5'-GGGCATGGCCGGCTCCGTTCCATCTTC-3'; *DNMT1*/Sp1-2R: 5'-GAAGGATG GAACGGAGCCG-GCCATG-5'; *DNMT1*/Sp1-3F: 5'-CCCGGACTGGGGTGGTAGA CGCCG-3'; *DNMT1*/Sp1-3R: 5'-GGGCGGCGTCTACCACCCAGTC-CGGG-3'. Total protein extracts were isolated from MV4-11 cells using M-PER (Mammalian Protein Extraction Reagent; Pierce, Rockford, IL), and nuclear extracts were prepared using NE-PER (Nuclear and Cytoplasmic Extraction Reagent; Pierce). Electrophoretic mobility-shift assays (EMSA) with total extracts or nuclear extracts and ³²P-labeled *DNMT1* promoter oligos were performed as previously described.⁴⁸ For antibody-supershift assays, 2 μ g antibodies (Sp1, NF- κ B [p65]) or control rabbit IgG was added after the binding reactions had proceeded for 10 minutes, and further incubated for another 20 minutes before gel loading. Alternatively, nuclear extracts were preincubated with individual antibody at 4°C for 16 hours before adding to the binding reactions.

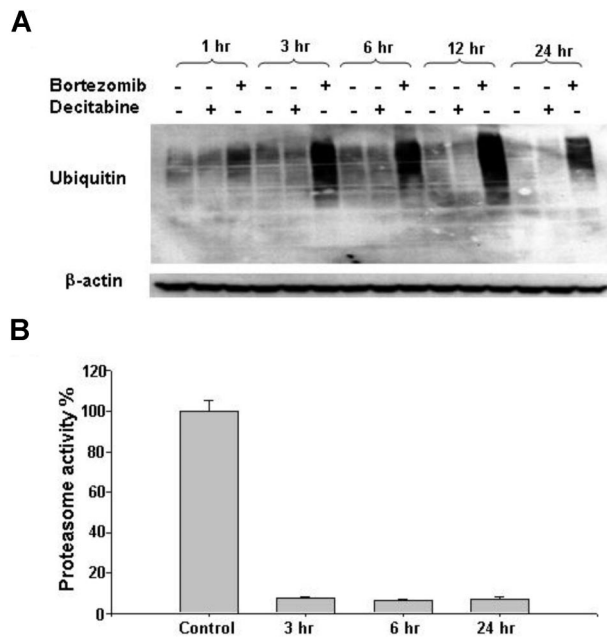


Figure 1. Inhibitory effect of bortezomib on 26S proteasome system in AML cells. (A) Accumulation of polyubiquitinated proteins after bortezomib treatment. MV4-11 cells were treated with 60 nM bortezomib or 2.5 μ M decitabine for the indicated time points. (B) Bortezomib (60 nM) exposure resulted in reduction of proteasome activity compared with untreated control. Error bars represent SD.

Quantitative real-time reverse-transcription–polymerase chain reaction

Quantitative real-time reverse-transcription–polymerase chain reaction (RT-PCR) for expression of *DNMT1*, *Sp1*, and *RIL* (primer and probes available upon request) was performed using 2 μ g total RNA prepared with Trizol reagent and reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen). The comparative cycle threshold (C_T) method was used to determine the expression levels of *DNMT1*, *Sp1*, and *RIL* relative to an internal control *18S*, as previously reported.⁴⁹

RIL gene-promoter methylation analysis

ML-1 cells were treated with bortezomib or decitabine for 24 or 48 hours and genomic DNA was extracted. DNA (1 μ g) from these cells was digested by *HpaII* or *BstI* for 2 hours. The digested DNA was cleaned by PCR purification kit (QIAGEN Science) and applied to PCR using primers specific for *RIL* gene promoter (primers available upon request). The cycle number and the amount of template were optimized to ensure assessment of amplicon levels within the linear range phase of the PCR. The PCR products were resolved on 1.0% agarose gel containing ethidium bromide and analyzed under UV light.

Results

Bortezomib inhibits the 26S ubiquitin-proteasome system and causes accumulation of polyubiquitinated proteins in AML cells

Bortezomib inhibits the 26S proteasome by binding to the chymotrypsin-like site in the 20S core complex. To test the proteasome inhibitory activity of this compound in human AML cells, we first assessed the levels of ubiquitinated proteins in MV4-11 cell line after incubation with bortezomib (60 nM) or decitabine (2.5 μ M), as a negative control. We observed that bortezomib, not decitabine, blocked ubiquitination-mediated protein degradation, leading in turn to the accumulation of polyubiquitinated proteins (Figure 1A).

Similar results were observed in other AML cell lines (ie, Kasumi-1 and K562) (data not shown). Consistent with increased levels of ubiquitinated proteins, exposure to 60 nM bortezomib for 3 or more hours resulted in approximately 90% reduction in 26S proteasome activity (Figure 1B). Of note, although cell viability decreased significantly at 72 hours, it was not affected before 12 hours and only partially decreased after 24-hour treatment with 20 nM or 60 nM bortezomib (data not shown), indicating that inhibition of proteasome activity precedes and, most likely, accounts for bortezomib-induced cell death.

Sp1 protein binds to the *DNMT1* gene promoter in human cancer cells

It has been reported that *DNMT1* promoter activity is regulated by the Sp1 zinc finger protein in murine cells.¹⁵ Analysis of the human *DNMT1* promoter identified 4 putative binding sites for the transcription factor Sp1 (data not shown). Three double-stranded *DNMT1* promoter probes, which spanned different regions of the *DNMT1* promoter containing Sp1-binding sites, were generated for DNA-protein interaction studies. In electrophoretic mobility shift assays (EMSA) with nuclear (N) or total (T) lysates from MV4-11 cells, the use of all 3 probes yielded slower migrating DNA-protein complexes (Figure 2A lanes 3-4). The specificity of DNA binding was demonstrated by competition with 20-fold excess unlabeled *DNMT1* promoter probes (Figure 2A lane 2). In addition, unlabeled DNA oligos containing the consensus Sp1-binding site efficiently competed away protein binding to 2 of 3 probes (ie, *DNMT1*-Sp1-1 and -2 probes), supporting the specific interaction of *DNMT1* promoter with Sp1 protein (Figure 2A lane 1). These results indicated that Sp1 protein was enriched on *DNMT1* gene promoter.

To demonstrate that Sp1 protein is present in the DNA-protein complexes formed between the MV4-11 lysates and the *DNMT1* promoter probes, antibody-supershift assays were performed. Initial EMSA experiments with short (30 minutes) incubation of the binding reaction in the presence of anti-Sp1 antibody led to only a low amount of the complex being supershifted (not shown). Therefore, we next performed EMSA experiments with nuclear extracts preincubated with the anti-Sp1 antibody for 16 hours before adding the ³²P-labeled *DNMT1*/Sp1 probe. Pretreatment with anti-Sp1 antibody (Figure 2B lane 3) completely abolished the formation of only one of the 2 detectable DNA-protein complexes (ie, named C1), supporting that the antibody interfered with the ability of Sp1 to bind the *DNMT1* promoter. Our data, therefore, indicated that complex C1, but not complex C2, contained Sp1 protein. Furthermore, pretreatment with anti-NF- κ B (p65) antibody (Figure 2B lane 4) also completely abrogated the formation of complex C1, supporting that NF- κ B was part of the protein-binding complex in MV4-11 cell lysates and suggesting that NF- κ B interplays with Sp1 for *DNMT1* transactivation.

The physical interaction between Sp1 and NF- κ B was further supported by the observation that Sp1 was coimmunoprecipitated with NF- κ B (p65) in untreated MV4-11 cells (Figure 2C). In contrast, Sp1 and NF- κ B did not coimmunoprecipitate in bortezomib-treated cells (Figure 2C), indicating that bortezomib disrupted this protein-protein interaction. Furthermore, bortezomib exposure significantly reduced the DNA-binding activity of both Sp1 and NF- κ B, as shown in the EMSA experiments using consensus DNA-binding probes for these 2 proteins (Figure 2D lane B). In contrast, the DNA-protein complex remained intact in cells untreated (Figure 2D lane C) or treated (Figure 2D lane D) with decitabine.

Figure 2. Sp1 binds to the DNMT1 promoter. (A) Sp1 bound to putative Sp1-binding sites on DNMT1 promoter. EMSA assay of MV4-11 total extracts (T) or nuclear extracts (N) was performed using 3 different ³²P-labeled DNMT1 promoter probes: DNMT1-Sp1-1 (abbreviated as D1); DNMT1-Sp1-2 (D2); and DNMT1-Sp1-3 (D3). Unlabeled oligos containing the consensus binding sites for Sp1 (Sp1) or unlabeled DNMT1 promoter probes (D) were added for competition with the ³²P-labeled probes, thereby testing for DNA-binding specificity. (B) Antibody competition assays. Nuclear lysates were preincubated with Sp1 and NF-κB (p65) antibodies before formation of protein-DNA complex, thereby testing for protein-binding specificity. (C) Sp1 was physically associated with NF-κB and this association was disrupted by bortezomib. MV4-11 cells were treated with 60 nM bortezomib for 6 hours, and nuclear extracts were then applied to immunoprecipitation with NF-κB (p65) antibody. The immunocomplex was blotted with Sp1 antibody (top panel). Input controls (bottom panels). (D) Bortezomib inhibited NF-κB and Sp1 protein-binding activity. EMSA was performed with nuclear extracts prepared from MV4-11 cells untreated or treated with decitabine or bortezomib for 24 hours. DNA-consensus sequences containing Sp1- or NF-κB-binding sites were used as probes, shown on the top of each panel. C indicates untreated; D, decitabine (2.5 μM); and B, bortezomib (60 nM). Vertical lines have been inserted to indicate repositioned gel lanes.

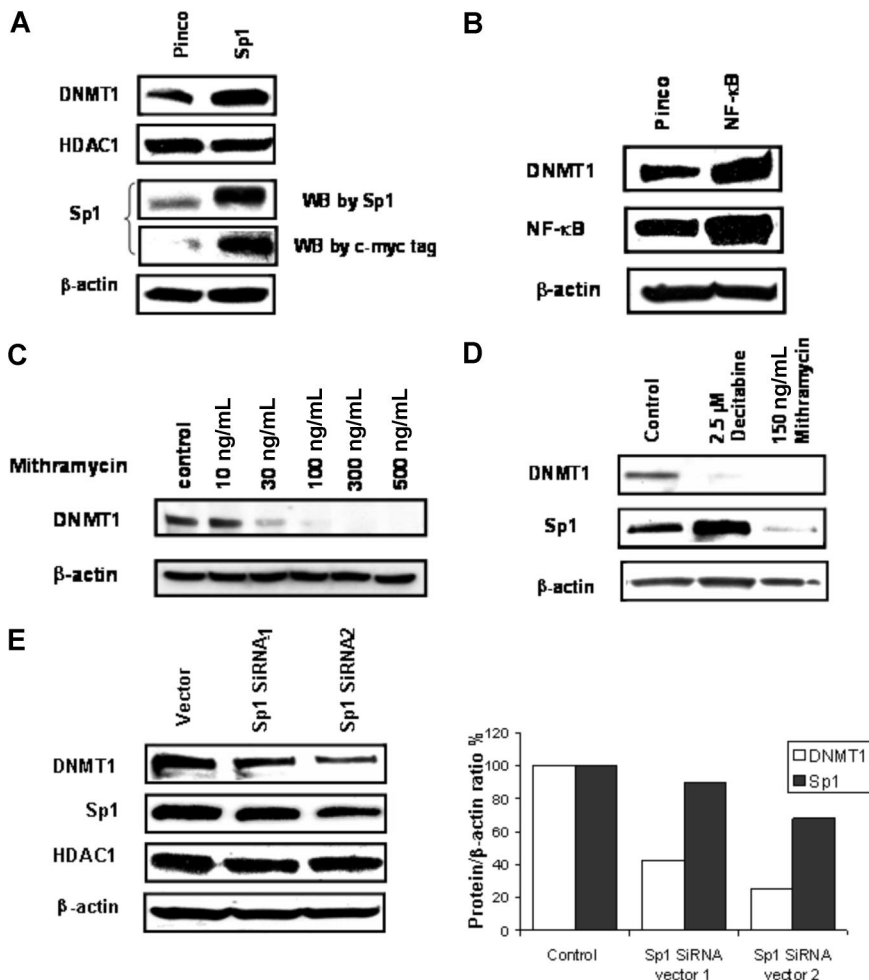
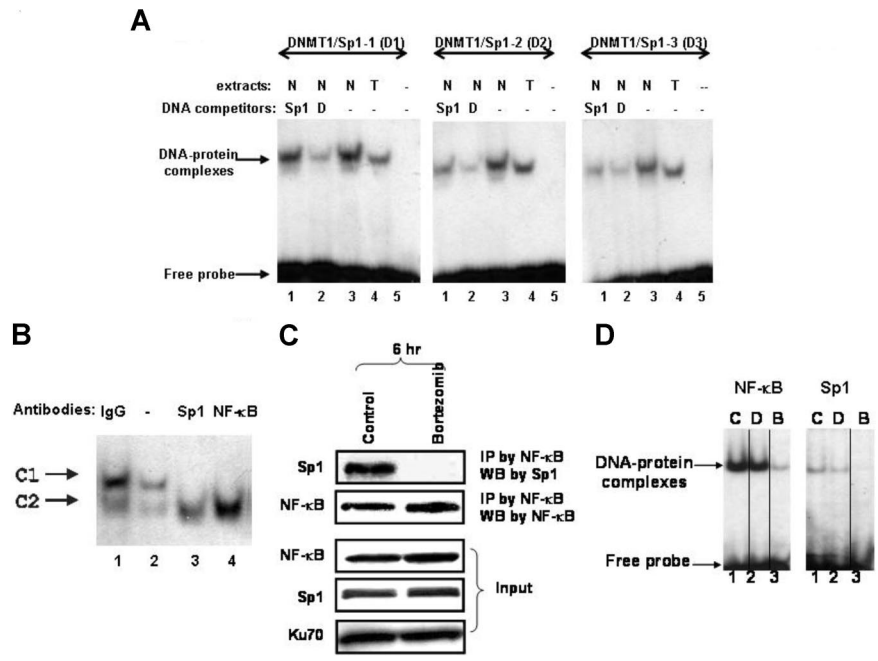


Figure 3. Coregulation of DNMT1, Sp1, and NF-κB. (A) Western blot analysis of DNMT1 in 293T cells stably transfected with empty vector (Pinco) or Sp1-myc-tagged-Pinco. (B) Western blot analysis of DNMT1 expression in 293T cells transiently transfected with empty vector (Pinco) or NF-κB (p65)-Pinco. (C) Mithramycin A depleted Sp1 expression and down-regulated DNMT1 expression in MV4-11 cells. (D) Mithramycin A down-regulates both Sp1 and DNMT1 expression in AML primary cells. (E) Sp1 shRNA concurrently decreased Sp1 and DNMT1 proteins (left panel; immunoblot gel; right panel: quantification graph). MV4-11 cells were transfected with pSuper.retro.neo + GFP vector with DNA stuffer sequences (vector) or Sp1 shRNAs (1 or 2) and cultured for 2 weeks. Total cell lysates were subjected to Western blot for Sp1 and DNMT1.

Changes in Sp1 protein levels correlate with changes in DNMT1 transcription

To further support the hypothesis that the Sp1/NF- κ B complex coparticipates in *DNMT1* transcriptional activation, the *Sp1* gene was cloned into a viral vector (Pinco) and stable Sp1 expression in 293T cells was established. Increased levels of DNMT1 protein, but not histone deacetylase 1 (HDAC1), were observed in cells overexpressing Sp1 compared with cells transfected with the empty vector (Figure 3A). Notably, overexpression of NF- κ B (p65) in 293T cells also augmented the expression of DNMT1 protein (Figure 3B). Treatment of MV4-11 cells with mithramycin A, an inhibitor of Sp1-binding activity,⁵⁰ led to dose-dependent reduction of *DNMT1* levels (Figure 3C). Moreover, both DNMT1 and Sp1 protein levels were decreased in mithramycin A-treated AML primary cells (Figure 3D). Finally, when Sp1 expression was knocked down using Sp1 shRNA, a concurrent decrease in DNMT1, but not HDAC1 (Figure 3E), was observed, thereby supporting a role of Sp1 protein in regulating *DNMT1* expression.

Bortezomib-induced inhibition of the 26S proteasome represses Sp1 expression and abrogates its DNA-binding activity

We next studied whether bortezomib-induced proteasome inhibition alters Sp1 protein expression and activity. MV4-11 and Kasumi-1 cells were exposed to bortezomib (0, 1, 6, 20, 60, and 100 nM) for 24 hours and Sp1 expression was examined. Bortezomib treatment induced a significant decrease in Sp1 protein level in a dose-dependent manner (Figure 4A). Importantly, when the activity of bortezomib was tested *in vivo*, we observed more than 80% down-regulation of Sp1 levels in tumors from MV4-11 xenografts following 48-hour treatment with a single dose (2 mg/kg) of bortezomib, compared with tumors from untreated controls ($P < .001$, pairwise *t* test; Figure 4B). No toxicity was observed in the treated animals.

To determine the effect of bortezomib on the Sp1 binding to the *DNMT1* promoter, EMSAs were performed with nuclear extracts from MV4-11 cells treated with bortezomib (60 nM for 24 hours). As shown in Figure 4C, bortezomib decreased the Sp1 protein binding to the *DNMT1* promoter, while no obvious alteration of the Sp1 DNA binding was observed in decitabine-treated cells. In addition, Western blot analysis on the same extracts showed that bortezomib decreased Sp1 protein expression (data not shown).

Bortezomib causes down-regulation of DNMT1 gene expression

As Sp1 interacts with NF- κ B to transactivate *DNMT1*, and bortezomib negatively affects Sp1 expression, Sp1/NF- κ B physical interaction, and Sp1 binding to the *DNMT1* gene promoter, we next postulated whether bortezomib down-regulates *DNMT1* expression and induces DNA hypomethylation. MV4-11 cells were incubated with different concentrations of bortezomib (0, 1, 6, 20, 60, or 100 nM) for 24 hours, or with a fixed concentration (60 nM) for variable periods of time, and *DNMT1* mRNA levels were measured by real-time RT-PCR. We observed that bortezomib treatment significantly reduced *DNMT1* mRNA expression in a dose- and time-dependent fashion (Figure 5A,B left panels). To demonstrate whether a decrease in the DNMT1 protein corresponded to the lower levels of *DNMT1* transcription induced by bortezomib, we examined protein lysate from bortezomib-treated MV4-11 cells by Western blot and observed that DNMT1 protein levels were

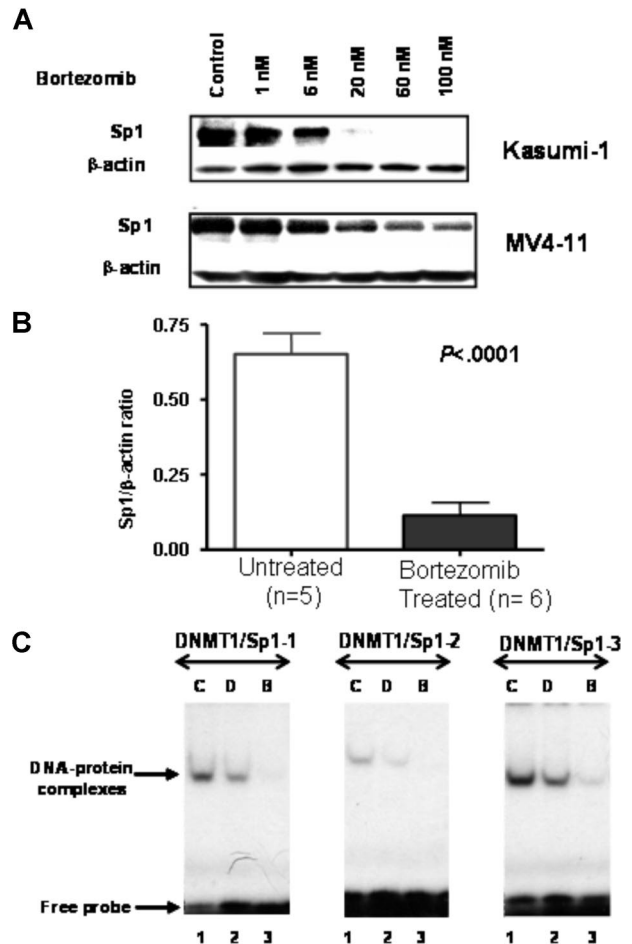


Figure 4. Bortezomib inhibits Sp1 protein expression and function. (A) Reduced Sp1 protein expression in Kasumi-1 and MV4-11 cells treated with indicated dosage of bortezomib for 24 hours. (B) Reduced Sp1 protein level in MV4-11 xenografts following 48-hour exposure to a single dose (2 mg/kg) of bortezomib (intravenous bolus). Error bars represent SD. (C) Bortezomib abolished Sp1 and NF- κ B binding to *DNMT1* promoter. EMSA was performed with nuclear extracts prepared from MV4-11 cells untreated or treated with bortezomib or decitabine. The *DNMT1*/Sp1 probes used are shown on the top of each panel. C indicates untreated; D: decitabine (2.5 μ M); and B: bortezomib (60 nM).

significantly decreased in a dose- and time-dependent fashion (Figure 5A,B right panels). In contrast, we did not observe significant changes in the levels of control proteins (ie, HDAC1), supporting a specific activity of bortezomib on *DNMT1* expression. *DNMT1* mRNA and protein levels were also decreased in other bortezomib-treated hematopoietic and nonhematopoietic cell lines (ie, Kasumi-1, K562, THP-1, Jurkat, Eo1-1, NB4, ML-1, and HCT116; data not shown).

To further explore the potential clinical relevance of our findings, primary bone marrow mononuclear cells from AML patients were cultured and treated *in vitro* with 20 nM or 60 nM bortezomib for 48 hours. Following treatment, whole cell extracts were analyzed for DNMT1 expression by Western blot. Similar to the effects observed in cell lines, bortezomib induced a reduction of the DNMT1 protein in AML primary cells compared with untreated controls, while no significant changes in HDAC1 were observed (Figure 5C).

The ability of bortezomib to down-regulate *DNMT1* expression was also evaluated *in vivo* in nude mice engrafted with MV4-11 or K562 cells. The tumor-bearing mice were treated with a single injection of 2.0 mg/kg bortezomib or vehicle alone (intravenous

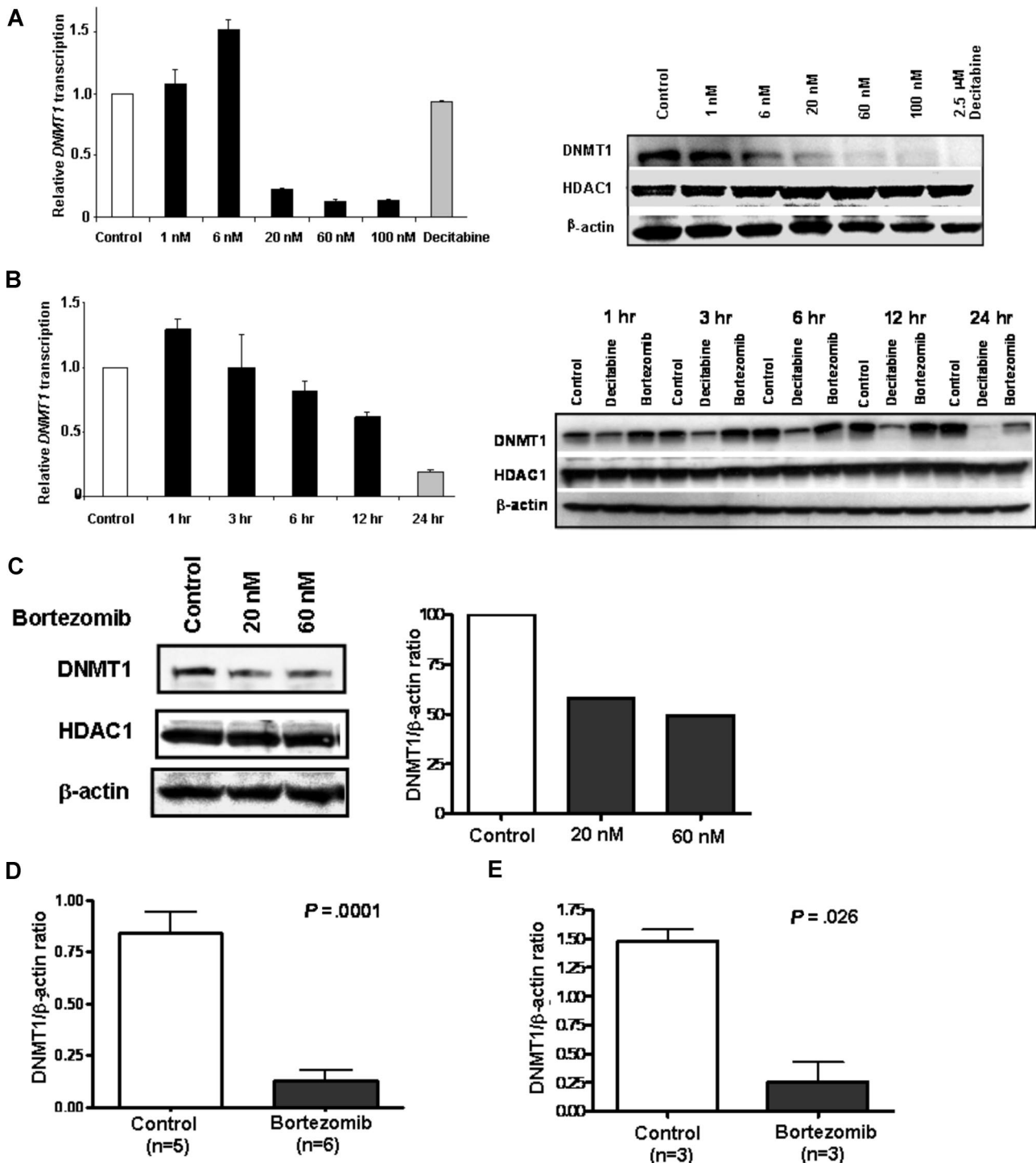


Figure 5. Bortezomib treatment down-regulates DNMT1 expression in vitro and in vivo. (A) Bortezomib decreased *DNMT1* mRNA transcription (left) and protein expression (right) in a dose-dependent manner. (B) Bortezomib decreased *DNMT1* mRNA (left) and protein (right) in a time-dependent fashion. MV4-11 cells were incubated with different concentration of bortezomib (0, 1, 6, 20, 60, and 100 nM) for 24 hours or 60 nM bortezomib for indicated time periods. *DNMT1* mRNA levels were examined by quantitative real-time RT-PCR (A,B left panel), and DNMT1 protein levels were detected by Western blot (A,B right panel). (C) Down-regulation of DNMT1 protein expression was observed in AML patient primary blasts treated ex vivo with bortezomib. Primary cells were exposed to 20 nM or 60 nM bortezomib for 48 hours, and the cell lysates were applied to Western blot for DNMT1 protein (left panel). Quantification of DNMT1 protein level normalized by β -actin is shown (right panel). (D,E) DNMT1 protein expression was repressed in tumors from MV4-11 (D) and K562 (E) xenograft mice following treatment with a single dose (2 mg/kg) of bortezomib (intravenous bolus). Error bars represent SD.

bolus). The tumors were excised after 48 hours and analyzed by Western blot. DNMT1 protein levels were found significantly decreased in tumors of mice engrafted with MV4-11 ($P = .001$; pairwise t test) or K562 ($P = .026$; pairwise t test) cells and treated

with bortezomib compared with untreated controls (Figure 5D,E). No significant alterations occurred in the levels of other proteins (ie, HDAC1), suggesting a specific activity of bortezomib on DNMT1 both in vitro and in vivo.

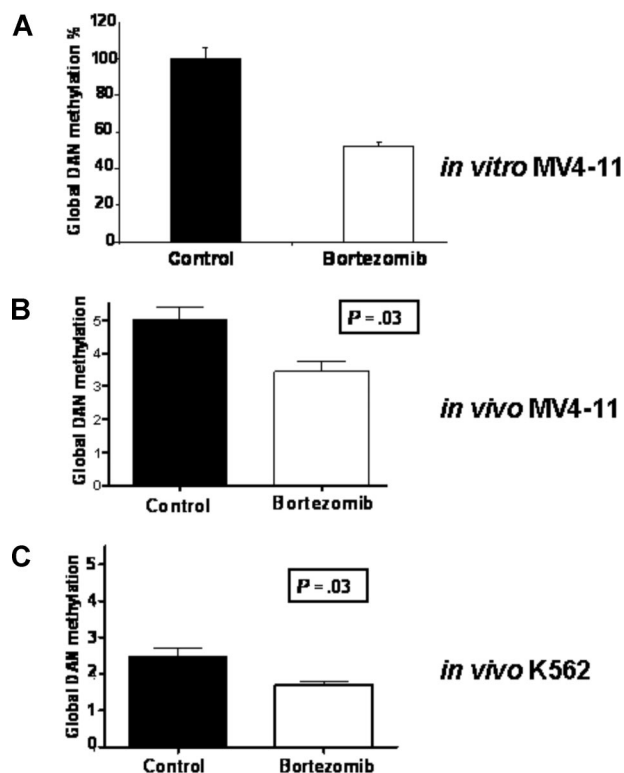


Figure 6. Bortezomib induces global DNA hypomethylation in vitro and in vivo. (A) Global DNA methylation was found to be decreased in MV4-11 cells treated in vitro with 20 nM bortezomib for 24 hours. (B,C) Global DNA hypomethylation was promoted in tumors from MV4-11(B) or K562 (C) xenograft mice following treatment with a single dose (2 mg/kg) of bortezomib (intravenous bolus). DNA (500 ng) was hydrolyzed and subjected to LC-MS/MS assay. Error bars represent SD.

A decrease in *DNMT1* transcription correlates with a decrease in global genomic DNA methylation in vitro and in vivo

Given the inhibitory effects of bortezomib on *DNMT1* transcription, we next investigated whether this compound also induced DNA hypomethylation. MV4-11 cells were treated with 20 nM bortezomib for 24 hours. Genomic DNA was isolated and hydrolyzed and assessed for levels of global DNA methylation by LC-MS/MS.⁴⁷ Approximately 50% reduction in global DNA methylation was observed in bortezomib-treated cells compared with untreated controls (Figure 6A). To test whether this effect could be reproduced in vivo, DNA samples from MV4-11 and K562 xenograft tumors were analyzed by LC-MS/MS.⁴⁷ We observed DNA hypomethylation in the tumor cells from bortezomib-treated animals compared with untreated controls (Figure 6B,C). This effect correlated with the aforementioned changes in *DNMT1* protein levels, suggesting that bortezomib acted as a hypomethylator both in vitro and in vivo through decreasing *DNMT1* expression. Very similar results were observed in other leukemia cell lines (ie, Kasumi-1 and THP-1; data not shown). Other anticancer drugs with different mechanism of action, such as depsipeptide, trichostatin A (TSA), and 17-allylaminodemethoxygeldanamycin (17AAG), failed to induce global DNA hypomethylation, supporting a specific effect of bortezomib on cellular epigenetic profiles.

Bortezomib treatment increases expression of epigenetically silenced genes

A recent report showed that *RIL*, a LIM domain gene mapping to 5q31.1, is frequently methylated and silenced in several cancer and

leukemia cell lines, and decitabine treatment restores *RIL* expression.⁵¹ Therefore, to assess whether bortezomib could reactivate methylation-silenced genes, we used HCT116 and ML-1 cells, in which the *RIL* gene is highly methylated.⁵¹ In both cell lines, *RIL* expression was significantly increased by bortezomib exposure (ie, ~2-fold at 3 hours and >5-fold at 12 hours in HCT116 cells [Figure 7A left panel] and ~4-fold at 24 hours and >15-fold at 48 hours in ML-1 cells [Figure 7A right panel]) compared with untreated controls. *DNMT1* protein levels were concomitantly down-regulated by bortezomib in both cell lines (Figure 7B). To investigate the methylation status of *RIL* gene promoter after bortezomib treatment, genomic DNA from bortezomib-treated ML-1 cells was digested with the restriction enzyme *HpaII* cutting only nonmethylated sites or *BstUI* cutting only methylated sites. Following digestion, DNA was analyzed by RT-PCR using primers specific to the *RIL* gene promoter. Efficient digestion by *HpaII* or *BstUI* leads to weaker amplification bands in hypomethylated or hypermethylated *RIL* promoters, respectively, compared with undigested controls. Consistent with the re-expression data and similar to decitabine-treated controls, these data provide the evidence that the *RIL* gene promoter is hypomethylated in ML-1 cells following bortezomib treatment (Figure 7C).

Discussion

The ubiquitin-proteasome system regulates several fundamental cellular processes, such as cell-cycle progression, differentiation, and apoptosis.²² The proteasome inhibitor bortezomib inhibits the 26S proteasome machinery via the formation of a pseudotetrahedral complex and is postulated to influence gene expression by affecting the proteasome-mediated degradation of transcription factors such as NF- κ B.²² Recent studies indicated that in the mouse *DNMT1* is a target for the zinc finger protein Sp1,¹⁵ which also serves as a broad transcriptional coregulator with NF- κ B.^{18,19} Since NF- κ B is constitutively activated^{20,21} and *DNMT1* is frequently overexpressed in AML cells,^{3,4} we hypothesized that epigenetic regulatory pathways dependent on *DNMT1* in cancer cells could be modulated by bortezomib-induced inhibition of the 26S proteasome, possibly by interfering with Sp-1/NF- κ B-mediated transactivation.²²

To test our hypothesis, we first studied the effect of bortezomib treatment on Sp1. We found that inhibition of 26S proteasome by bortezomib leads to depletion of Sp1 in vitro and in vivo, disruption of the Sp1/NF- κ B (p65) physical interaction, and inhibition of Sp1 DNA-binding activity. Notably, levels of NF- κ B (p65) are not obviously altered by bortezomib treatment, although drug exposure affects the activity of this protein as supported by a decrease in DNA binding and disruption of the Sp1/NF- κ B coimmunoprecipitation complex. It has previously been reported that the expression and functional activity of Sp1 is regulated by posttranscriptional modifications and at least in part by proteasomal degradation.^{16,17} To our knowledge, however, this is the first report that bortezomib treatment induces Sp1 depletion. Whether this is a direct result of bortezomib-mediated inhibition of the 26S proteasome or relates to indirect interference of this drug on specific phases of the cell cycle that are preferentially associated with Sp1 expression remains to be fully elucidated. Nevertheless, our data add another target to a long list of proteins whose changes in expression or activity mediate bortezomib antitumor effect.⁵²

We next demonstrated that bortezomib induces a marked decrease in *DNMT1* transcription, and this in turn leads to global

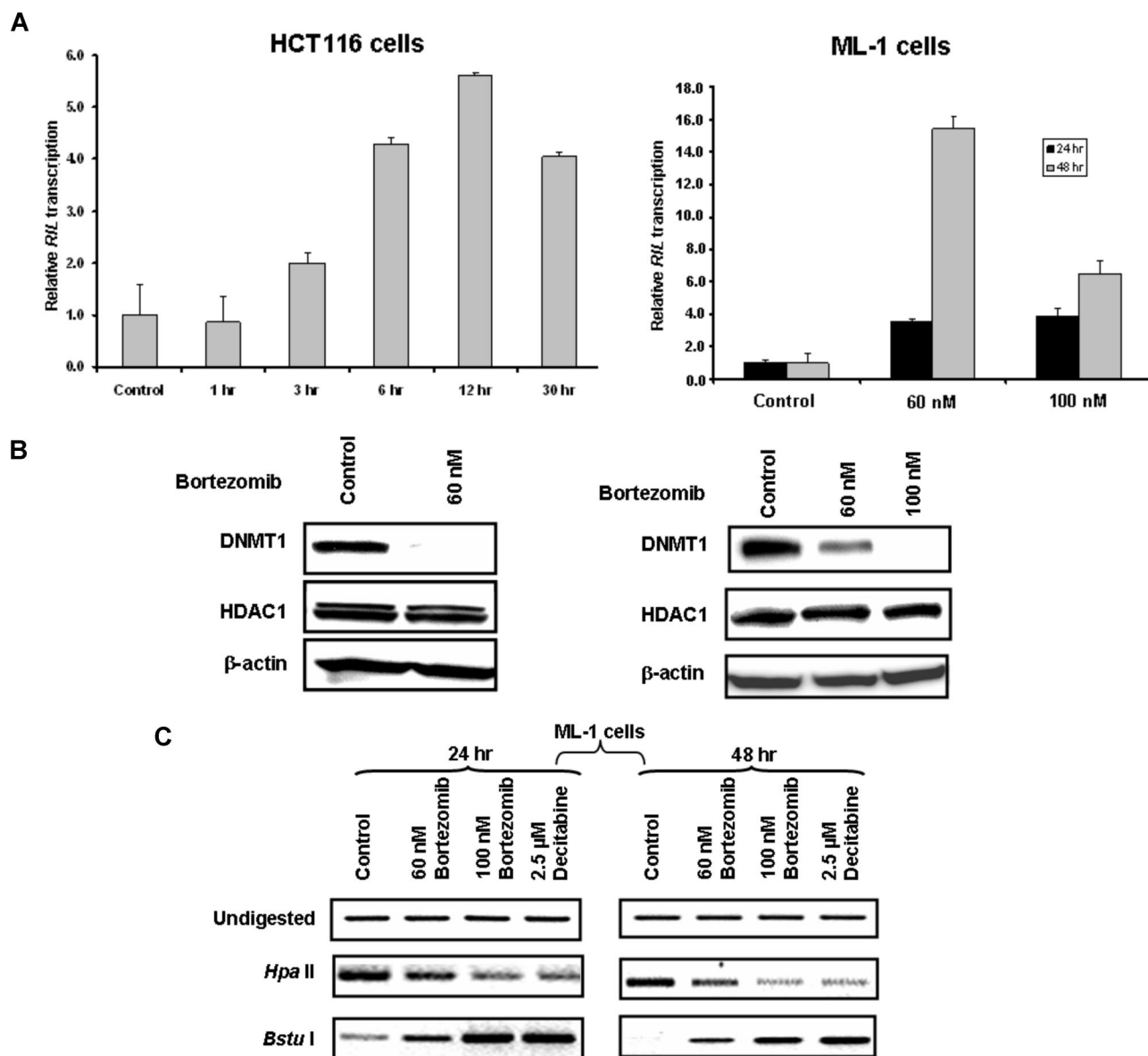


Figure 7. Bortezomib induces re-expression of methylation-silenced genes via promoter hypomethylation. (A) Bortezomib increased expression of *RIL* gene reportedly to be methylated and down-regulated in HCT116 and ML-1 cells. HCT116 and ML-1 cells were treated with bortezomib for indicated time points and dosage; *RIL* gene expression was measured by real-time RT-PCR. Error bars represent SD. (B) Bortezomib induced DNMT1 down-regulation in HCT116 and ML-1 cells. HCT116 and ML-1 cells were treated with bortezomib, and Western blot was performed using antibodies against DNMT1 or HDAC1 (control). (C) Hypomethylation of *RIL* promoter by bortezomib treatment in ML-1 cells. DNA (1 μ g) from bortezomib-treated or untreated ML-1 cells were digested by *Hpa*II or *Bst*uI, and PCR was performed using primers specific for *RIL* gene promoter. *Hpa*II indicates no digestion, then hypermethylated; *Bst*uI, no digestion, then hypomethylated.

DNA hypomethylation and re-expression of epigenetically silenced genes. Transactivation of *DNMT1* by Sp1 has previously been reported. Kishikawa et al showed that Sp1 binds to the *DNMT1* gene promoter and forced Sp1 expression in *Drosophila* SL2 cells, which lack the Sp family of transcription factors, enhances transcription of *DNMT1* gene promoter.¹⁵ We reported here that in human cancer cells, *DNMT1* is coregulated through both Sp1 and NF- κ B (p65). Sp1 down-regulation by mithramycin A or shRNA leads to DNMT1 depletion via transcriptional inhibition, while forced expression of *Sp1* and NF- κ B (p65) in 293T cells results in DNMT1 up-regulation. As bortezomib reduces Sp1 expression levels, Sp1 and NF- κ B physical interaction, and Sp1/NF- κ B promoter-binding activity, we concluded that bortezomib acts as a DNA hypomethylator through interference with Sp1-mediated *DNMT1* transactivation. However, inhibition of *DNMT1* transcription by bortezomib could also occur through alternative mecha-

nisms. It has been shown that the 26S complex has a broad impact on gene transcription by regulating levels of RNA polymerase II⁵³ and its recruitment on transcriptionally activated genes.^{26,54} Moreover, inhibition of the 19S moiety and 20S complex decreases RNA elongation and termination, respectively.⁵³ This suggests that interference with 26S complex activity by bortezomib could result in generalized transcriptional down-regulation. In our experiments, however, the effect of bortezomib on *DNMT1* expression appeared specific, as levels of other proteins (ie, HDAC1) remain unchanged following drug exposure. Interestingly, a paradoxical increase in DNMT1 expression was observed in cells exposed to bortezomib at very low doses (ie, 1 and 6 nM) or for very short time (ie, 1 hour) (Figure 5A,B). Although this observation remains to be fully elucidated from a mechanistic standpoint, it is possible that at very low doses or short duration of bortezomib exposure, mechanisms that interfere with the hypomethylating effects of this drug might

be activated (eg, transient increase in ubiquitinated, but active Sp1). Furthermore, these data underscore the importance for dose optimization and selection of optimal time points for correlative studies that intend to demonstrate the hypomethylation activity of bortezomib in upcoming clinical trials.

Bortezomib treatment ultimately caused global DNA hypomethylation, both in vitro and in vivo, and restored the expression of the *RIL* gene in HCT116 and ML-1 cells, where this gene is silenced via hypermethylation.⁵¹ Consistent with these results, we also showed that bortezomib induces hypomethylation of the *RIL* gene promoter. Our study suggests that bortezomib induces hypomethylation through pathways of DNMT1 depletion that are different from those of azanucleosides. Ghoshal et al showed that one of the possible hypomethylating mechanisms of the azanucleosides is DNMT1 depletion via covalent trapping of the protein on DNA followed by selective proteasomal degradation.⁵⁵ In contrast, we showed that bortezomib as a single agent induces DNMT1 depletion by interfering with *DNMT1* gene transactivation as supported by a decrease of *DNMT1* mRNA in cells treated with bortezomib, and not decitabine (Figure 5A). Altogether, these data support bortezomib as an effective nonazanucleoside hypomethylator and a novel strategy for broadening epigenetic-targeting approaches to azanucleoside-resistant malignancies. Of note, the development of bortezomib as an epigenetic-targeting agent in human cancer will be facilitated by the fact that this is an already FDA-approved agent with a known and relatively benign toxicity profile.

Finally, we showed here that bortezomib has hypomethylating activity in AML, a disease where hypermethylation-mediated gene silencing is a well-characterized event and found to be a suitable target for therapeutic intervention. Cortes et al recently reported limited clinical responses in a phase I study of bortezomib as a single agent in patients with refractory or relapsed acute leukemia.⁵⁶ Data from this study, however, should not discourage additional trials testing the hypomethylating activity and clinical effectiveness of bortezomib in AML. In fact, like decitabine, which is shown to be most effective as a hypomethylating agent when used at doses at least one log lower than those maximally tolerated, it is possible that to effectively elicit hypomethylating and anticancer activities of bortezomib in AML, treatment doses and schedules different from those currently recommended by the "package insert" of this commercially available drug may be required.

In summary, we have demonstrated that bortezomib causes down-regulation of *DNMT1* via the Sp1/NF- κ B pathway and induces global DNA hypomethylation in human leukemia cells,

both in vitro and in vivo. Importantly, bortezomib appears to induce DNA hypomethylation with mechanisms different from those provided by currently available hypomethylating azanucleosides. Therefore, based on our results, we propose testing response to bortezomib in clinical trials rationally designed to target DNA hypomethylation and reverse expression of epigenetically silenced genes in cancer. Because of the difference in mechanisms of activity, it would not be unreasonable to test treatment with bortezomib in combination with azanucleosides for synergistic antileukemic activity in hematologic malignancies where myelotoxicity does not constitute dose-limiting toxicity (ie, acute leukemia and high-risk MDS).

Acknowledgments

We thank Dr Christopher J. Hickey for the technical support.

This work was supported in part by CA90469, CA102031, and CA095512 grants from the National Cancer Institute (Bethesda, MD).

Authorship

Contribution: S.L. designed all the experiments, performed bench work, and wrote the paper; Z.L. designed and performed the assays testing changes in the DNA methylation and wrote the paper; Z.X., J.P., L.H., T.V., and M.T. performed bench work and critically reviewed the paper; J.Y., R.B.K., R.A.B., P.P., R.G., and D.P. designed experiments and critically reviewed the paper; E.L. performed EMSA experiments and critically reviewed the paper; W.B., J.C.B., and M.A.C. provided patients' samples and critically reviewed the paper; K.K.C. designed the global methylation assay and critically reviewed the paper; L.-C.W. designed EMSA experiments and critically reviewed the paper; G.M. designed all the experiments, wrote the paper, and provided administrative and financial support.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415-428.
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349:2042-2054.
- Toyota M, Koyanagi K, Toyota MO, Jairo KW, Willman CL, Issa JP. Methylation profiling in acute myeloid leukemia. *Blood*. 2001;97:2823-2829.
- Mizuno S, Chijiwa T, Okamura T, et al. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood*. 2001;97:1172-1179.
- Herman JG, Jen J, Merlo A, Baylin SB. Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. *Cancer Res*. 1996;56:722-727.
- Issa JP, Zehnbaumer BA, Civin CI, et al. The estrogen receptor CpG island is methylated in most hematopoietic neoplasms. *Cancer Res*. 1996;56:973-977.
- Baylin SB, Fearon ER, Vogelstein B, et al. Hypermethylation of the 5' region of the calcitonin gene is a property of human lymphoid and acute myeloid malignancies. *Blood*. 1987;70:412-417.
- Issa JP, Baylin SB, Herman JG. DNA methylation changes in hematologic malignancies: biologic and clinical implications. *Leukemia*. 1997;11(suppl 1):S7-S11.
- Yu L, Liu C, Vandeusen J, et al. Global assessment of promoter methylation in a mouse model of cancer identifies ID4 as a putative tumor-suppressor gene in human leukemia. *Nat Genet*. 2005;37:265-274.
- Leonhardt H, Page AW, Weier HU, Bestor TH. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*. 1992;71:865-873.
- Robert MF, Morin S, Beaulieu N, et al. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet*. 2003;33:61-65.
- Oki Y, Aoki E, Issa JP. Decitabine: bedside to bench. *Crit Rev Oncol Hematol*. 2007;61:140-152.
- Issa JP, Kantarjian HM, Kirkpatrick P. Azacitidine. *Nat Rev Drug Discov*. 2005;4:275-276.
- Muller CI, Ruter B, Koeffler HP, Lubbert M. DNA hypermethylation of myeloid cells, a novel therapeutic target in MDS and AML. *Curr Pharm Biotechnol*. 2006;7:315-321.
- Kishikawa S, Murata T, Kimura H, Shiota K, Yokoyama KK. Regulation of transcription of the *Dnmt1* gene by Sp1 and Sp3 zinc finger proteins. *Eur J Biochem*. 2002;269:2961-2970.
- Black AR, Black JD, Azizkhan-Clifford J. Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol*. 2001;188:143-160.
- Bouwman P, Philippsen S. Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol*. 2002;195:27-38.
- Hirano F, Tanaka H, Hirano Y, et al. Functional interference of Sp1 and NF-kappaB through the

- same DNA binding site. *Mol Cell Biol*. 1998;18:1266-1274.
19. Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. A cooperative interaction between NF- κ B and Sp1 is required for HIV-1 enhancer activation. *EMBO J*. 1993;12:3551-3558.
 20. Guzman ML, Neering SJ, Upchurch D, et al. Nuclear factor- κ B is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood*. 2001;98:2301-2307.
 21. Bueso-Ramos CE, Rocha FC, Shishodia S, et al. Expression of constitutively active nuclear- κ B RelA transcription factor in blasts of acute myeloid leukemia. *Hum Pathol*. 2004;35:246-253.
 22. Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer*. 2004;4:349-360.
 23. Ferdous A, Gonzalez F, Sun L, Kodadek T, Johnston SA. The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. *Mol Cell*. 2001;7:981-991.
 24. Ferdous A, Kodadek T, Johnston SA. A nonproteolytic function of the 19S regulatory subunit of the 26S proteasome is required for efficient activated transcription by human RNA polymerase II. *Biochemistry*. 2002;41:12798-12805.
 25. Glickman MH, Rubin DM, Fried VA, Finley D. The regulatory particle of the Saccharomyces cerevisiae proteasome. *Mol Cell Biol*. 1998;18:3149-3162.
 26. Gonzalez F, Delahodde A, Kodadek T, Johnston SA. Recruitment of a 19S proteasome subcomplex to an activated promoter. *Science*. 2002;296:548-550.
 27. Sulahian R, Sikder D, Johnston SA, Kodadek T. The proteasomal ATPase complex is required for stress-induced transcription in yeast. *Nucleic Acids Res*. 2006;34:1351-1357.
 28. Koepp DM, Harper JW, Elledge SJ. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell*. 1999;97:431-434.
 29. Ciechanover A. The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J*. 1998;17:7151-7160.
 30. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*. 1995;269:682-685.
 31. Karin M, Yamamoto Y, Wang QM. The IKK NF- κ B system: a treasure trove for drug development. *Nat Rev Drug Discov*. 2004;3:17-26.
 32. Collins GA, Tansey WP. The proteasome: a utility tool for transcription? *Curr Opin Genet Dev*. 2006;16:197-202.
 33. Spengler ML, Brattain MG. Sumoylation inhibits cleavage of Sp1 N-terminal negative regulatory domain and inhibits Sp1-dependent transcription. *J Biol Chem*. 2006;281:5567-5574.
 34. Schwartz AL, Ciechanover A. The ubiquitin-proteasome pathway and pathogenesis of human diseases. *Annu Rev Med*. 1999;50:57-74.
 35. Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol*. 2005;23:4776-4789.
 36. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res*. 2001;61:3071-3076.
 37. Hideshima T, Richardson PG, Anderson KC. Targeting proteasome inhibition in hematologic malignancies. *Rev Clin Exp Hematol*. 2003;7:191-204.
 38. Guzman ML, Swiderski CF, Howard DS, et al. Preferential induction of apoptosis for primary human leukemic stem cells. *Proc Natl Acad Sci U S A*. 2002;99:16220-16225.
 39. Hideshima T, Chauhan D, Richardson P, et al. NF- κ B as a therapeutic target in multiple myeloma. *J Biol Chem*. 2002;277:16639-16647.
 40. Yu J, Wei M, Boyd Z, et al. Transcriptional control of human T-BET expression: the role of Sp1. *Eur J Immunol*. 2007;37:2549-2561.
 41. Yu J, Wei M, Becknell B, et al. Pro- and anti-inflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity*. 2006;24:575-590.
 42. Becknell B, Trotta R, Yu J, et al. Efficient infection of human natural killer cells with an EBV/retroviral hybrid vector. *J Immunol Methods*. 2005;296:115-123.
 43. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell*. 2005;8:355-368.
 44. Trotta R, Ciariariello D, Dal Col J, et al. The PP2A inhibitor SET regulates natural killer cell IFN- γ production. *J Exp Med*. 2007;204:2397-2405.
 45. Lightcap ES, McCormack TA, Pien CS, Chau V, Adams J, Elliott PJ. Proteasome inhibition measurements: clinical application. *Clin Chem*. 2000;46:673-683.
 46. Song L, James SR, Kazim L, Karpf AR. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Chem*. 2005;77:504-510.
 47. Liu Z, Liu S, Xie Z, et al. Characterization of in vitro and in vivo hypomethylating effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. *Nucleic Acids Res* (<http://nar.oxfordjournals.org>). 2007;35:e31.
 48. Hong JW, Allen CE, Wu LC. Inhibition of NF- κ B by ZAS3, a zinc-finger protein that also binds to the κ B motif. *Proc Natl Acad Sci U S A*. 2003;100:12301-12306.
 49. Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2005;23:9234-9242.
 50. Ray R, Snyder RC, Thomas S, Koller CA, Miller DM. Mithramycin blocks protein binding and function of the SV40 early promoter. *J Clin Invest*. 1989;83:2003-2007.
 51. Bumber YA, Kondo Y, Chen X, et al. RIL, a LIM gene on 5q31, is silenced by methylation in cancer and sensitizes cancer cells to apoptosis. *Cancer Res*. 2007;67:1997-2005.
 52. Vink J, Cloos J, Kaspers GJ. Proteasome inhibition as novel treatment strategy in leukaemia. *Br J Haematol*. 2006;134:253-262.
 53. Gillette TG, Gonzalez F, Delahodde A, Johnston SA, Kodadek T. Physical and functional association of RNA polymerase II and the proteasome. *Proc Natl Acad Sci U S A*. 2004;101:5904-5909.
 54. Reid G, Hubner MR, Metivier R, et al. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol Cell*. 2003;11:695-707.
 55. Ghoshal K, Datta J, Majumder S, et al. 5-Azadeoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol*. 2005;25:4727-4741.
 56. Cortes J, Thomas D, Koller C, et al. Phase I study of bortezomib in refractory or relapsed acute leukemias. *Clin Cancer Res*. 2004;10:3371-3376.