Bortezomib Sensitizes Human Acute Myeloid Leukemia Cells to All-Trans-Retinoic Acid–Induced Differentiation by Modifying the RARα/STAT1 Axis

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Abstract

All-trans-retinoic acid (ATRA) has held great promise for differentiation-based therapy but reportedly downregulates retinoid acid receptor-α (RARα) in a proteasome-dependent manner, which leads to decreased acute myeloid leukemia (AML) cell differentiation efficiency. Therefore, research strategies that seek to further sensitize cells to retinoids and extend the range of retinoid-affected myeloid malignancies beyond acute promyelocytic leukemia (APL) are key investigative avenues. Here, we show that bortezomib, the first proteasome inhibitor approved for newly diagnosed and relapsed multiple myeloma, exhibited strong synergism with ATRA to promote HL60 and NB4 AML cell differentiation. We observed that bortezomib sensitized AML cells to ATRA-induced morphologic, biochemical, and functional changes, indicative of myeloid differentiation without cell death. In addition, treatment of human leukemia HL60 xenografts with bortezomib and ATRA together did not increase bortezomib-induced progressive weight loss but resulted in significant tumor growth inhibition in addition to increased differentiation (P < 0.05). These enhanced differentiation effects were accompanied by RARα stabilization and STAT1 activation. Taken together, our study was the first to evaluate bortezomib and ATRA synergy in AML cell differentiation and to assess new opportunities for bortezomib and ATRA combination as a promising approach for future differentiation therapy. Mol Cancer Ther; 12(2); 195–206. ©2012 AACR.

Introduction

A breakthrough in clinical oncology was achieved as all-trans-retinoic acid (ATRA; Fig. 1A) sparked intensive differentiation therapy research (1). However, differentiation therapy is limited because ATRA is the sole efficient agent for differentiation-based therapy. Acute promyelocytic leukemia (APL), a distinct subtype of acute myeloid leukemia (AML), is the only subtype of leukemia to respond to differentiation therapy and has not been effective in patients with non-APL AMLs. ATRA has had great promise in both cancer treatment and prevention (2), and research strategies that seek to further sensitize cells to retinoids and extend the range of myeloid malignancies that respond to retinoids beyond APLs are key avenues of investigation (3).

The biologic effects of ATRA are mediated by 2 families of nuclear receptors, the retinoic acid receptors (RAR) and the retinoid X receptors (RXR), which work as RAR/RXR heterodimers and bind to retinoic acid response elements (RARE) in the promoter region of retinoid-responsive genes (4). Pharmacologic concentrations of ATRA cause a conformational change in the leukemia-generating fusion protein, PML-RARα molecular complex. Corepressors are released, normal retinoic acid receptor-α (RARα)-responsive gene regulation is restored, and terminal APL cell differentiation is induced (5). However, previous studies showed that RARs and their fusion proteins are degraded by the ubiquitin/proteasome pathway (UPP; ref. 6), which may limit ATRA-mediated myeloid cell differentiation. Moreover, our previous study showed that RARα accumulation enhanced target gene transcription, which may also enhance leukemia cell differentiation (7). Therefore, combining ATRA with proteasome inhibitors may result in more profound clinical remission than other treatments in RAR-positive cells.

Bortezomib (PS-341 or Velcade; Fig. 1A) is a highly specific proteasome inhibitor with potent anti-cancer activity, which has been approved by the U.S. Food and Drug Administration for multiple myeloma and mantle
cell lymphoma treatment. Many preclinical studies have shown that bortezomib alone or in combination with other chemotherapeutic agents induces apoptosis in a variety of human cancer cells (8–10). However, bortezomib-mediated differentiation has rarely been reported. Our recent study reported that MG132, another proteasome inhibitor, increased cellular sensitivity to ATRA by inhibiting RARα catabolism and amplification of RARα-mediated terminal differentiation (7). Coincident with these results, bortezomib effectively promoted ATRA-induced neuroblastoma terminal differentiation also via influencing RAR expression (9). In addition, we recently reported that bortezomib promotes myeloid differentiation by activating the MEK/ERK and STAT1 cascades (11). Given the importance of proteasome inhibitors in RAR accumulation, bortezomib may further sensitize cells to retinoid action and increase the range of myeloid malignancies beyond APLs that are affected by retinoids. We therefore wanted to test bortezomib and ATRA in combination for activity in AML and APL differentiation therapy.

In the present study, using both in vitro and in vivo models, we observed significant synergism of bortezomib and ATRA in combination against human AML cells. The enhanced differentiation might be associated with RARα stabilization and STAT1 activation. Collectively, this study evaluated the ability of bortezomib to synergize with ATRA in AML cells and induce differentiation, thus suggesting combination therapy as a promising approach for future differentiation therapy.

Materials and Methods

Cells and reagents

Human myeloid leukemia HL60 and NB4 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Following receipt, cells were grown and were frozen as a seed stock as they were available. Both cell lines were passaged for a maximum of 2 months, after which new seed stocks were thawed. Both of the cell lines were authenticated using DNA fingerprinting (variable number of tandem repeats), confirming that no cross-contamination occurred during this study. Both of the cell lines were tested for mycoplasma contamination at least every month. HL60 and NB4 cell lines were cultured in Iscove’s Modified Dulbecco’s Media (IMDM) or RPMI-1640 media, respectively (Gibco BRL). All of the media were supplemented with 10% fetal calf serum (Gibco BRL) and 1% penicillin/streptomycin. Both of the cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO2.

ATRA was purchased from Sigma and dissolved in ethanol. Bortezomib was a kind gift from Dr. Wei Lv (East China Normal University, Shanghai, China) and was dissolved in dimethyl sulfoxide (DMSO).

Cellular proliferation and apoptosis analysis

Total cell number and viability were determined by trypan blue exclusion with manual counting in Burker chambers. Apoptosis was detected with the Annexin V–FITC Apoptosis Detection Kit according to the manufacturer’s instructions (BD Biosciences).

Real-time PCR

Total RNA was extracted from 1 × 10^6 cells with TRIzol reagent (Bio Basic, Inc.), and cDNA was synthesized using 2 μg of total RNA with RevertAid M-MulV Reverse Transcriptase (Fermentas International, Inc.). Equal amounts of cDNA were taken for transcript PCR amplification, which was carried out using Quantitect SYBR Green PCR Kits (Qiagen, Inc.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers used for PCR were as follows: RARα: forward 5’-ACCCCTCTACCCCGCATCTACAAG-3’, reverse 5’-CATGCCCACTTAAAGCGACT TCTGC-3’; STAT1: forward 5’-GGAGGCGAACCTGAC TTCCA-3’, reverse 5’-CTTGGTGCTTCTCTTTGGTCT-3’; GAPDH: forward 5’-GTCATCATGACAACTTGGG-3’, reverse 5’-GACCTTGACAAGTGTCTGCT-3’; PU.1: forward 5’-ATGTGCCTCCAGTACCCATC-3’, reverse 5’-TCTTCTGTAGTTGTCATCTC-3’; C/EBPA: forward 5’-AACGTCTGGACGCTGACACAG-3’, reverse 5’-AAT CCTAGTCTGGTCCTTG-3’; C/EBPB: forward 5’-ACAGCGACGAGTACAAGATCC-3’, reverse 5’-GAC CGTCTGGAAACAGTCC-3’; C/EBPE: forward 5’-GAC ACTATATAGTGGACGCTC-3’, reverse 5’-ACACCCCTTGAAGGATAGCAG-3’; and e-Myc: forward 5’-GCCACGTCCCTCACACATG-3’, reverse 5’-TCTTGCGAGGATAGTCTT-3’. Instrumentation settings are detailed in Supplementary Information.

Differentiation and proteasome activity detection

Cell differentiation induction was determined by assessing morphologic changes, CD11b expression, and an nitro blue tetrazolium (NBT) reduction assay (11).

To assess CD11b expression, cells (1 × 10^6) were harvested and washed with PBS, blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes, and incubated with CD11b-PE for 30 minutes on ice. After incubation, CD11b expression levels were analyzed with a FACScalibur flow cytometer (BD Biosciences).

To assess NBT reduction, cells (5 × 10^6) were harvested and incubated with PBS containing NBT (1 μg/mL) at 37°C for 30 minutes. Cytospin slides were prepared and examined for cells containing precipitated formazan particles. At least 200 cells were assessed for each experiment.

Cell morphology was evaluated by Wright–Giemsa staining. Cytospin preparations were fixed with methanol and air dried. The slides were then stained with Wright–Giemsa solution, examined with a Leica microscope, and images were captured with a Leica DFC300 FX charge-coupled device camera.

Proteasome activity measurements were conducted as previously described (12).
Animal studies

HL60 xenografts were established by injecting $1 \times 10^6$ cells subcutaneously into 4- to 5-week-old nude mice (SLRC Laboratory Animal Inc.). When tumors reached a volume of 100 to 150 mm$^3$, mice were randomized into control and treated groups. The mice then received vehicle, intraperitoneal (i.p.) bortezomib administration (0.1 mg/kg, 1:99, v/v DMSO:saline) once per week, intragastric (i.g.) ATRA administration (5.0 mg/kg, 1:9, v/v ethanol:CMC-Na) 5 times per week, or bortezomib and ATRA in combination. Detailed animal studies, including CD11b expression, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining are described in Supplementary Materials.

Western blotting and immunofluorescence

Western blotting was conducted as reported previously (13). Briefly, protein extracts were resolved by 8%-15% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with primary antibodies. Antibodies against RAR$\alpha$ (C-20),
C/EBPε (C-22), STAT1 (E23), p-STAT1 (Tyr701), p-IκB-α (39A1431), IκB-α, p50 (4D1), p65 (6D889), phospho-MEK (Thr291), MEK, p38, JNK2, c-Myc (3G32), p27, GAPDH, and β-actin were purchased from Santa Cruz Biotechnology. Antibodies for p-MAPK/JNK (Thr183/Tyr185) and phospho-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology; and PML-RARα (ab43152) was purchased from Abcam. Western blots were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies.

Figure 2. Effect of bortezomib (BTZ) on ATRA-induced AML cell differentiation. A, CD11b expression in HL60 cells. B, HL60 cell NBT-reducing activity. C, CD11b expression in NB4 cells. D, NB4 cell NBT-reducing activity. E and F, HL60 and NB4 cell morphologic differentiation. The data are representative of 1 independent experiment. G, PU.1, C/EBPA, C/EBPB, C/EBPE, and c-Myc mRNA levels in HL60 and NB4 cells as determined by real-time PCR. GAPDH expression was used as an internal control gene. A–D and G, the data are presented as the mean ± SD of 3 independent experiments. A–G, cells were treated with the indicated BTZ concentrations in the presence of vehicle or ATRA for 3 days.
Combination Differentiation Therapy of Bortezomib and ATRA

First, to determine whether there are synergistic effects of bortezomib and ATRA (Fig. 1A) on AML cell proliferation, we set up serial concentrations of 2 drugs based on clinical pharmacokinetic studies (bortezomib < 100 nmol/L, ATRA ≤ 1 μmol/L; refs. 15–17) and determined that bortezomib showed distinct synergy with ATRA in HL60 and NB4 cell lines, with most combination index (CI) values below 0.8 (Supplementary Fig. S1A and S1B). We chose the ATRA therapeutic concentration that had been used in most in vitro studies in combination with serial dilutions of bortezomib and determined the synergistic effects of bortezomib and ATRA on human AML cell proliferation. As shown in Fig. 1B, bortezomib alone decreased cell proliferation both in HL60 and NB4 cells in a dose-dependent manner; moreover, bortezomib and ATRA combination treatment further enhanced this inhibition. In HL60 cells, 1.0 to 10.0 nmol/L bortezomib with ATRA in combination significantly inhibited cell proliferation (Fig. 1B, left), and bortezomib at concentrations less than 5.0 nmol/L together with ATRA did not cause cell death, as evaluated by trypan blue staining (Fig. 1C, left). In NB4 cells, the same treatment combinations significantly inhibited cell proliferation (Fig. 1B, right panel), but at 7.5 nmol/L bortezomib and ATRA treatment, there was evidence of cell death (Fig. 1C, right). These results revealed that bortezomib and ATRA combination treatment inhibited AML cell proliferation without inducing cell death.

Annexin V-propidium iodide (PI) analysis further confirmed a lack of apoptotic or necrotic cells in HL60 or NB4 cells after single or combination treatment with bortezomib and ATRA, whereas the positive control, 10.0 nmol/L bortezomib alone, significantly induced apoptosis (Fig. 1D). We measured growth curves and found that combination therapy with bortezomib and ATRA at the selected concentrations effectively inhibited cell proliferation in both cell lines in a time-dependent manner (Fig. 1E). Similar results were also obtained in three primary leukemia cell lines (Supplementary Fig. S1C), indicating that combination treatment significantly inhibited growth compared with monotreatment. These results indicate that combination therapy with bortezomib and ATRA synergistically inhibited proliferation of AML cells, which was not a result of bortezomib-induced apoptosis.

Table 1. Effects of bortezomib in combination with ATRA on mice tumor size and tumor weight at pre- and post-dose

<table>
<thead>
<tr>
<th>Groups</th>
<th>Start</th>
<th>End</th>
<th>Tumor weight, g</th>
<th>Inhibition rate, %</th>
<th>RTV</th>
<th>T/C, %</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>19.7 ± 2.0</td>
<td>20.4 ± 3.0</td>
<td>1.94 ± 0.89</td>
<td>-</td>
<td>6.5</td>
<td>-</td>
</tr>
<tr>
<td>ATRA (5.0 mg/kg)</td>
<td>18.9 ± 1.2</td>
<td>15.6 ± 1.9</td>
<td>1.17 ± 0.64*</td>
<td>40.0</td>
<td>3.8</td>
<td>58.0</td>
</tr>
<tr>
<td>BTZ (0.1 mg/kg)</td>
<td>17.7 ± 0.8</td>
<td>17.5 ± 1.5</td>
<td>1.55 ± 0.89</td>
<td>20.0</td>
<td>4.0</td>
<td>62.0</td>
</tr>
<tr>
<td>ATRA + BTZ</td>
<td>19.7 ± 2.9</td>
<td>16.5 ± 1.3</td>
<td>0.80 ± 0.37*</td>
<td>58.9</td>
<td>2.9</td>
<td>44.5</td>
</tr>
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</table>

NOTE: Nude mice with HL60 transplant tumor were treated with bortezomib 0.1 mg/kg (i.p.) once a week, ATRA 5 mg/kg (i.g.) 5 times per week, or bortezomib 0.1 mg/kg (i.p.) once a week in combination with ATRA 5 mg/kg (i.g.) 5 times per week for a period of 14 days. Criteria for therapeutic activity: T/C (%), optimal growth inhibition < 25 = good and optimal growth inhibition < 50 = moderate. Abbreviations: BTZ, bortezomib; RTV, relative tumor volume; T/C(%) = RTV_treatment/RTV_control × 100.

*P < 0.01 versus control.
the 0.01 μmol/L ATRA-treated group to 51.5% ± 4.5% in the ATRA and bortezomib combination group (P < 0.001 vs. the ATRA group), and from 43.1% ± 16.1% in the 1 μmol/L ATRA-treated group to 78.9% ± 4.6% in the combination group (P < 0.05 vs. the ATRA group). When combined with bortezomib, ATRA is 100 times more powerful than on its own to induce HL60 cell differentiation because 0.01 μmol/L ATRA and bortezomib combination treatment was as efficient as 1 μmol/L ATRA alone. Enhanced differentiation was also observed by NBT reduction assay (Fig. 2B). Similar observations were made in NB4 cells, as shown in Fig. 2C and D. Bortezomib potentiated ATRA-induced differentiation in a dose-dependent manner, as assessed by CD11b expression and NBT assay. It is noteworthy that 0.5 nmol/L bortezomib and 1.0 nmol/L ATRA in combination showed similar differentiation as 100 nmol/L ATRA alone. The augmented differentiation noted with combination treatment in both HL60 and NB4 cell lines was not simply an additive effect because at the concentrations used, bortezomib alone did not induce differentiation.

The ability of bortezomib to enhance ATRA-induced AML cell differentiation was evaluated by morphologic analysis using Wright–Giemsa staining. Compared with untreated controls, ATRA-treated cells had modestly decreased nucleus to cytoplasmic ratios, whereas cells treated with ATRA and bortezomib in combination had more mature morphology, with an increased cytoplasmic to nuclear ratio and obvious nuclear segmentation (Fig. 2E and F). We further analyzed changes in RARα target gene expression in HL60 and NB4 cells, and real-time PCR results showed that whereas the proto-oncogene c-Myc decreased after combination ATRA and bortezomib treatment, myeloid regulator PU.1 and C/EBP (A/B/E) expression increased (Fig. 2G). Taken together, the CD11b expression, NBT reduction tests, morphologic changes, and RARα target gene expression results clearly showed that bortezomib and ATRA combination treatment enhanced ATRA-induced myeloid differentiation.

Bortezomib and ATRA combination therapy arrested tumor growth and induced human AML xenograft differentiation

To determine whether bortezomib and ATRA synergized in vivo, we evaluated antitumor activity of bortezomib and ATRA in combination in an HL60 xenograft nude mouse model. Compared with the control group,
bortezomib or ATRA administration resulted in weak to moderate tumor weight inhibition (20.0% reduction for bortezomib, 40.0% reduction for ATRA), whereas simultaneous bortezomib and ATRA treatment significantly inhibited tumor growth by 58.9% (Table 1, Fig. 3A; ATRA, ATRA with bortezomib vs. control, \( P < 0.01 \)). Similarly, simultaneous bortezomib and ATRA treatment had moderate therapeutic activity, as indicated by a T/C value of 44.5%, compared with 58.0% or 62.0% in ATRA or bortezomib monotreatment groups (Table 1). Importantly, progressive weight loss was similar in mono- or combination treatment groups during the experiment (Table 1).

To ensure that the bortezomib and ATRA-mediated synergistic growth inhibition resulted in weak to moderate tumor weight inhibition (20.0% reduction for bortezomib, 40.0% reduction for ATRA), whereas simultaneous bortezomib and ATRA treatment significantly inhibited tumor growth by 58.9% (Table 1, Fig. 3A; ATRA, ATRA with bortezomib vs. control, \( P < 0.01 \)). Similarly, simultaneous bortezomib and ATRA treatment had moderate therapeutic activity, as indicated by a T/C value of 44.5%, compared with 58.0% or 62.0% in ATRA or bortezomib monotreatment groups (Table 1). Importantly, progressive weight loss was similar in mono- or combination treatment groups during the experiment (Table 1).

To ensure that the bortezomib and ATRA-mediated synergistic growth inhibition on AML xenografts may be from cytotoxicity, we first detected apoptosis in the HL60 xenografts by TUNEL staining. No TUNEL-positive cells were detected in any group (Fig. 3B), but significant TUNEL-positive cells were found in the positive control group (Fig. 3B–F). We next characterized the combined activity of bortezomib and ATRA-mediated differentiation in an animal model. As shown in Fig. 3C, we observed that almost 40% of the xenograft-derived HL60 cells were CD11b-positive following bortezomib and ATRA treatment, compared with monotreated controls (\( P < 0.05 \) vs. ATRA). CCAAT enhancer-binding protein-epsilon (C/EBP\( \epsilon \)) is reportedly a myeloid differentiation regulator and is a potential retinoid target gene (18–20). Thus, we investigated C/EBP\( \epsilon \) protein expression by immunohistochemistry in HL60 xenografts. The results showed that C/EBP\( \epsilon \) was diffusely distributed in bortezomib and ATRA-treated xenografts and was lower in monotreated xenografts, whereas C/EBP\( \epsilon \) expression in the control group was weak or undetectable (Fig. 3D).

Collectively, these data indicated that the therapeutic activity resulting from simultaneous bortezomib and ATRA treatment did not result from apoptosis, and involved differentiation. Thus, the synergistic effect of bortezomib and ATRA was validated by the data obtained from the in vivo assessment on HL60 xenograft models.

Bortezomib-inhibited proteasome activity correlates with suppression of ATRA-induced RAR\( \alpha \) degradation

To gain insight into the mechanisms underlying the synergism of bortezomib and ATRA, we first examined whether bortezomib-mediated proteasome inhibition contributed to combination treatment-mediated differentiation, as bortezomib is a specific proteasome inhibitor. Because the proteolytic core of the proteasome is the 20S proteasome (21), we measured proteasome activity in HL60 and NB4 cells. 20S proteasome activity was elevated by ATRA in both cell lines, which was in agreement with previously reported results (7, 12); however, bortezomib significantly blocked the ATRA-mediated increase in 20S proteasome activity (Fig. 4A and B). Thus, bortezomib-mediated proteasome inhibition is involved in bortezomib and ATRA-mediated differentiation.

Our previous studies reported that proteasome inhibitor-mediated RAR accumulation plays a critical role in myeloid differentiation (7, 9, 11); therefore, we assessed whether bortezomib and ATRA-induced proteasome inhibition altered RAR\( \alpha \) expression. As illustrated...
Figure 5. Bortezomib (BTZ) and ATRA-induced STAT1 activation is required for differentiation. A, STAT1 mRNA levels in HL60 and NB4 cells as assessed by real-time PCR. GAPDH was used as an internal control. B, STAT1 Western blot analysis in HL60 and NB4 cells after 3 days of treatment. C and D, Western blot analysis of p-STAT1(Tyr701) (C) and STAT1 MAPK signaling (D) in HL60 cells after 1 or 3 days of treatment. E, STAT1 expression in HL60 cells as assessed by immunofluorescence (green) after 24-hour treatment. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). F, DNA-binding activity...
in Fig. 4C, ATRA treatment alone significantly reduced RARα protein expression in both cell lines, which is consistent with our previous observations (7, 12), whereas bortezomib treatment alone increased RARα protein expression levels. A significant increase in RARα protein levels was observed in bortezomib and ATRA-treated cells in a dose-dependent manner compared with monotherapeutr controls. This effect correlated with the aforementioned proteasome activity changes (Fig. 4A and B), suggesting that bortezomib specifically affected RARα protein levels. Moreover, RARα mRNA levels were not different when comparing mono- and combination treatment (Fig. 4D). All of these results showed that bortezomib-induced inhibition of ATRA-mediated proteasome activation suppressed RARα degradation, which increased myeloid leukemia cell differentiation.

To test whether this effect could be reproduced in vivo, protein samples from HL60 xenograft tumors were analyzed for RARα expression. We observed enhanced RARα accumulation in tumor cells from bortezomib- and ATRA-treated animals compared with untreated or monotherapeutr controls (Fig. 4E), suggesting that bortezomib promoted differentiation both in vitro and in vivo by inhibiting the proteasome-dependent decrease in RARα expression. PML-RARα degradation is crucial for ATRA therapeutic activity in NB4 cells (22, 23); thus, we also determined PML-RARα stability after bortezomib and ATRA treatment. PML-RARα expression was moderately increased in the combination therapy group, indicating that bortezomib and ATRA combination treatment may rebalance the PML-RARα and RARα protein pools (Supplementary Fig. S2).

**Bortezomib and ATRA induce RARα-related STAT1 activation independent of mitogen-activated protein kinase activation**

The STAT1 promoter reportedly possesses a RARE (24–26). Recently, we reported that bortezomib promotes myeloid differentiation by activating STAT1 (11); thus, we asked whether STAT1 is involved in the proteasome inhibition–mediated RARα accumulation. We determined that ATRA treatment alone induced STAT1 both at the mRNA and protein levels in both cell lines in agreement with other reports (26), whereas bortezomib treatment alone mildly influenced STAT1 expression (Fig. 5A and B). In contrast, we observed a significant increase in STAT1 mRNA and protein expression levels in bortezomib- and ATRA-treated cells (Fig. 5A and B). Because bortezomib and ATRA treatment of HL60 cells significantly increased STAT1 expression, we questioned whether this STAT1 expression level increase resulted in upregulation of STAT1 tyrosine phosphorylation, which is important for the DNA-binding and transcriptional functions of this protein. As presented in Fig. 5C, STAT1 and p-STAT1 (Tyr701) levels were increased after bortezomib and ATRA treatment for up to 24 hours; however, the protein levels were markedly increased after 72 hours bortezomib and ATRA treatment compared with ATRA monotherapy. These data suggest that bortezomib and ATRA activate STAT1.

Given STAT1 was activated by bortezomib and ATRA, we also determined whether this activation was regulated by mitogen-activated protein kinase (MAPK; refs. 27, 28). In contrast to STAT1, which was activated in HL60 cells, p-MEK, p-JNK1/2, and p-p38 were not activated in bortezomib and ATRA-treated HL60 cells treated compared with monotherapy (Fig. 5D). Collectively, these results indicate that bortezomib and ATRA-induced STAT1 activation is independent of MAPK activation.

We further tested whether bortezomib and ATRA treatment affected STAT1 nuclear accumulation and DNA-binding activity. Whereas STAT1 was retained in the cytoplasm after bortezomib or ATRA single treatments, nuclear STAT1 was detected after 24-hour combination treatment as assessed with immunofluorescence (Fig. 5E). Gel retardation assays showed that combination treatment enhanced STAT1 DNA-binding activity (Fig. 5F). STAT1 target genes p27 and c-Myc (29, 30) were up- or downregulated in the combination treatment group compared with monotherapeutr cells, respectively (Fig. 5G). Hence, these results were consistent with specific STAT1 accumulation by Western blotting and PCR detection and indicated that STAT1 induction contributed to myeloid differentiation upon bortezomib and ATRA treatment.

**Bortezomib and ATRA-induced STAT1 activation is required for differentiation**

Finally, we investigated whether suppressing STAT1 expression inhibited bortezomib and ATRA-induced differentiation. To assess this, we knocked down STAT1 with shRNA. Of 5 STAT1 shRNA sequences, we chose the one with the strongest knockdown efficiency to develop an HL60-shRNA-STAT1 stable cell line. As shown in Fig. 5H, HL60-shRNA-STAT1 cells had a significant decrease in STAT1 expression compared with HL60-vector cells. These cells were then treated with different drugs, and proliferation was assessed. Consistently, bortezomib and ATRA combination treatment further decreased cell proliferation in HL60-vector cells, whereas HL60-shRNA-STAT1 cells had a less pronounced effect on proliferation after the same treatment. Next, we determined the effect of STAT1 activation on myeloid...
differentiation, as assessed by CD11b expression and the NBT reduction assay. As presented in Fig. 5I, only bortezomib and ATRA combination treatment altered CD11b expression between the shRNA-STAT1 and the vector group; the percentage of CD11b-positive cells decreased from 75.0% ± 13.2% to 47.5% ± 8.96% (shRNA-STAT1 vs. vector: P < 0.05), indicating that STAT1 silencing abolished the ability of bortezomib to enhance differentiation. Similar observations were also made with the NBT reduction assay, as illustrated in Fig. 5J and Supplementary Fig. S3B. Importantly, STAT1 silencing also affected CD11b expression and NBT reduction in the ATRA monotherapy group, but this effect was much less apparent than in the combination therapy group (Fig. 5I and J). Taken together, these results clearly indicate that STAT1 activation plays a key role in bortezomib-enhanced ATRA-induced myeloid differentiation.

Discussion

The success of ATRA-based differentiation therapy in APL has led researchers to expend great effort to apply differentiation-based approaches in other AML subtypes. However, a key barrier to implementing successful differentiation therapy in AML is that in contrast to APL, AML fails to respond to pharmacologic doses of ATRA (3). One potential reason that has been shown by our group and others is that RARs, nuclear retinoic acid receptors that control a complex network of differentiation genes, are degraded by UPP (6, 7, 12, 31, 32), thus decreasing differentiation efficiency in ATRA-treated AML cells. Bortezomib, which is the first proteasome inhibitor that is approved for clinical use, inhibits proteasomal activity and affects protein degradation. Because RAR degradation is inhibited after treatment with 26S proteasome inhibitors MG132 or lithium chloride and ATRA in leukemia cells (7, 33), we hypothesized that ATRA-mediated RAR degradation could be modulated by bortezomib-induced 26S proteasome inhibition to induce AML differentiation.

In our study, synergistic differentiation effects were achieved by bortezomib and ATRA treatment in HL60 and NB4 cells in vitro and in vivo. We observed that bortezomib sensitizes AML cells to ATRA-induced myeloid differentiation without cell death and that ATRA concentrations could be reduced 100-fold when combined with bortezomib (Figs. 1 and 2). In addition, treatment of leukemia HL60 xenografts with bortezomib and ATRA do not increase bortezomib-induced progressive weight loss but result in more significant tumor growth inhibition, accompanied by differentiation induction (Fig. 3, Table 1). This enhanced differentiation is accompanied by inhibition of RARα proteasomal degradation both in vitro and in vivo (Fig. 4). In addition, STAT1 activation is significantly greater with bortezomib and ATRA combination therapy than with either drug used alone (Fig. 5A–G). Further studies in HL60 cells show that STAT1 knockdown blocks bortezomib-induced differentiation (Fig. 5H–J). We also detected changes in NF-κB and MAPK signaling pathways in bortezomib and ATRA-treated HL60 cells. However, no synergism was observed in these signaling cascades (Fig. 5D, Supplementary Fig. S4A and S4B). Moreover, high bortezomib concentrations significantly increased p-JNK and decreased the p50–p65 complex (Supplementary Fig. S4C), further suggesting that bortezomib and ATRA combination therapy–induced differentiation is not NF-κB–dependent. Hence, these data suggest that bortezomib-dependent proteasome inhibition enhanced RARα and STAT1 activation, thus promoting ATRA-induced AML cell differentiation.

The proteasome is involved in intracellular protein turnover and is essential for many cellular processes, thus contributing to cellular homeostasis (34, 35). Previously, the proteasome was considered to be a promising target for inducing apoptosis (36–39), as shown by the successful introduction of bortezomib for relapsed multiple myeloma treatment. However, the role of proteasome in cell differentiation is not well characterized. Proteasome inhibitors induce osteoblast, oligodendroglia (40–42), and neuroblastoma cell differentiation (9). In contrast, proteasome activity is necessary for differentiation of some cell types, such as neuroblastoma, myoblasts, and lens cells (43–45). Thus, proteasome inhibition effects on cell differentiation are cell-type–specific. Our study shows that bortezomib monotherapy inhibits proteasome activity in both HL60 and NB4 cells, whereas ARTA monotherapy increases proteasome activity, and bortezomib prevents this increase (Fig. 4A and B). Thus, our data suggest that bortezomib enhances ATRA-mediated differentiation by preventing ATRA-induced proteasome activity. This is in agreement with previous work showing that treatment with the proteasome inhibitor lithium chloride in combination with ATRA increases leukemia cell differentiation (34), and MG132 enhanced the pharmacologic action of ATRA in AML cells (7). Thus, it is likely that proteasomal regulation is necessary for AML myeloid cell differentiation.

Recently, ATRA-mediated RARα downregulation reportedly occurred in APL by specifically targeting receptors for proteasome-dependent degradation (12, 31), suggesting that bortezomib targets the proteasome-dependent degradation system. Moreover, Gianni and group also reported that Pin1 or p38α pharmacologic inhibition stabilized RARα and PML-RARα by blocking their constitutive proteasomal degradation and sensitized myeloid leukemia cells to retinoids (46, 47). To this end, there is much evidence indicating that controlling RARα stability or activation may be an effective strategy to improve retinoid efficacy in AML. In our study, we observe that bortezomib and ATRA treatment enhanced RARα protein expression but did not upregulate RARα mRNA in AML cell lines and HL60 xenografts (Fig. 4C–E), indicating that bortezomib disrupted ATRA-induced RARα degradation through proteasomal inhibition. As previously mentioned, increasing RARα protein expression may be attributed to increased ATRA sensitivity in AML cells because 1 nmol/L ATRA and 5 nmol/L...
bortezomib treatment in combination induces proliferation (data not shown) and differentiation in NB4 cells to a similar extent as 0.1 μmol/L ATRA monotreatment (Fig. 2C and D), and the ATRA concentration when used in combination therapy is 100 times lower than in the single treatment group. Taken together, we conclude that bortezomib-mediated proteasome inhibition directly regulates the retinoid receptor, which is important for ATRA-induced terminal differentiation.

We finally show that STAT1 is activated by bortezomib and ATRA treatment and contributes to myeloid differentiation (Fig. 5). A RARE domain reportedly exists in the STAT1 promoter (24–26); however, whether STAT1 activation is a direct result of bortezomib-induced RARα accumulation or whether it is because of alternative mechanisms remains to be fully elucidated. For example, STAT1 dimers are negatively regulated by proteolytic degradation following ubiquitination (48, 49). Thus, STAT1 and p-STAT1 accumulation may be a direct result of bortezomib-induced proteasome inhibition. Nevertheless, it seems that bortezomib-induced STAT1 activity is likely to be involved in ATRA-induced myeloid AML cell differentiation. Similarly, STAT1 knockdown substantially decreased CD11b expression and NBT-positive cells levels after combination therapy (Fig. 5I and J) but did not abolish RARα accumulation (Supplementary Fig. S3A), further supporting the notion that proteasome inhibition may favor STAT1 activation leading to myeloid differentiation.

Although we observed RARα/STAT1 axis modification in both AML and APL upon bortezomib and ATRA combination therapy, an upregulation of PML- RARα was detected in NB4 cells (Supplementary Fig. S2), suggesting that combination therapy may induce alternate signaling pathways in AML that are different from ATRA-mediated PML-RARα degradation in APL. Whether and to what extent PML-RARα plays a role in bortezomib and ATRA-induced differentiation remain to be determined because the biologic importance of PML- RARα is controversial. However, our studies indicate that the synergistic effects of bortezomib and ATRA combination treatment are independent from PML-RARα degradation. Future studies focusing on this issue should provide new insights into differentiation therapy.

In conclusion, we present evidence showing better ATRA differentiation activity when combined with bortezomib both in vitro and in vivo. Our study also shows that bortezomib and ATRA combination treatment synergistically induces differentiation and inhibits proliferation by disrupting ATRA-induced RARα proteasomal degradation. Our findings support that proteasome inhibitor-mediated RARα accumulation will sensitize AML to ATRA-induced differentiation therapy and open the possibility that ATRA treatment in combination with bortezomib or other proteasome inhibitors may lead to new clinical applications of differentiation-based approaches for AML and other leukemia therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M. Ying, X. Zhou, B. Yang, Q. He
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