

ORIGINAL ARTICLE

Rationale and efficacy of proteasome inhibitor combined with arsenic trioxide in the treatment of acute promyelocytic leukemia

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Arsenic trioxide (ATO) mediates PML-RARA (promyelocytic leukemia–retinoic acid receptor- α) oncoprotein degradation via the proteasome pathway and this degradation appears to be critical for achieving cure in acute promyelocytic leukemia (APL). We have previously demonstrated significant micro-environment-mediated drug resistance (EMDR) to ATO in APL. Here we demonstrate that this EMDR could be effectively overcome by combining a proteasome inhibitor (bortezomib) with ATO. A synergistic effect on combining these two agents *in vitro* was noted in both ATO-sensitive and ATO-resistant APL cell lines. The mechanism of this synergy involved downregulation of the nuclear factor- κ B pathway, increase in unfolded protein response (UPR) and an increase in reactive oxygen species generation in the malignant cell. We also noted that PML-RARA oncoprotein is effectively cleared with this combination in spite of proteasome inhibition by bortezomib, and that this clearance is mediated through a p62-dependent autophagy pathway. We further demonstrated that proteasome inhibition along with ATO had an additive effect in inducing autophagy. The beneficial effect of this combination was further validated in an animal model and in an on-going clinical trial. This study raises the potential of a non-myelotoxic proteasome inhibitor replacing anthracyclines in the management of high-risk and relapsed APL.

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INTRODUCTION

Acute promyelocytic leukemia (APL) is characterized by a reciprocal translocation, t(15;17)(q22;q21), that results in a novel PML-RARA (promyelocytic leukemia–retinoic acid receptor- α) oncogene.¹ Arsenic trioxide (ATO) has proven efficacy as first-line therapy in the treatment of APL.² There has been a recent concern of ATO resistance in patients treated with upfront ATO.³ The focus of ATO resistance has centered on mutations in PML-RARA gene,^{3–5} specifically missense or point mutations in the B2 domain of the PML gene that results in the inability of ATO to directly bind to the PML and PML-RARA oncoprotein, leading to resistance.⁵ Although additional mutations have been noted in up to a third of relapsed APL patients in the PML-RARA gene, it is not clear whether such mutations are associated with secondary ATO resistance as described for those in the PML B2 domain.³ However, the published data suggest that patients with such mutations have an unfavorable clinical outcome.^{3–5}

The relative specificity of ATO in the treatment of APL results from the ability of ATO to bind directly to the PML and chimeric PML-RARA protein that in turn leads to sumoylation of the PML portion followed by polyubiquitination and subsequent proteasomal degradation.^{6,7} Based on the current understanding of the mechanism of action of ATO in APL, proteasomal inhibition would be antagonistic.⁸ We had previously reported in an *in vitro* model that there was evidence of significant *de novo* micro-environment-mediated drug resistance (EMDR) to ATO.⁹ Recently published

data indirectly validate our preliminary observation by demonstrating that stromal cell and malignant promyelocyte interaction, mediated by VLA-4 (very late antigen-4) and VCAM-1 (vascular cell adhesion molecule-1) interaction, upregulates the nuclear factor (NF)- κ B pathway in both the stromal and malignant cell and in turn mediates chemoresistance.¹⁰

The direct cytotoxic effect of bortezomib on malignant promyelocytes has been previously reported.^{11,12} Similarly, the synergistic effect of ATO and bortezomib on non-APL leukemic cells has been previously reported.¹³ Preliminary *in vitro* observations from our laboratory suggest that proteasome inhibition can overcome EMDR to ATO. We also noted a possible synergistic cytotoxic effect of combining bortezomib (a proteasome inhibitor) and ATO on malignant promyelocyte in a stromal co-culture system. These preliminary observations were contradictory to existing dogma on the mechanism of action of ATO. The mechanism of such a synergy has not been previously evaluated and the theoretical antagonism of combining these two agents on PML and PML-RARA degradation, which is central to clearance of the leukemia-initiating compartment in APL and achieving cure,^{5,8} has not been addressed. In this study we evaluated the mechanism of bortezomib (BTZ)-induced cytotoxicity against malignant promyelocytes, its potential mechanism of synergy with ATO and the fate of PML-RARA when ATO was combined with BTZ.

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MATERIALS AND METHODS

Cell lines and primary cells

The human APL cell line NB4^(ref. 14) (kind gift from Dr Harry Iland, RPAH, Sydney, Australia, with permission from Dr Michel Lanotte) and an ATO-resistant NB4 cell line NB4 EVAsR1 (generated in-house and derived from NB4) was used for some of the experiments (detailed characterization of this cell line is provided in the Supplementary Methods section). Bone marrow samples from APL patients were collected during diagnosis before treatment and at hematological relapse after obtaining a written informed consent. Mesenchymal stromal cells were expanded *in vitro* using well-established protocols. HS-5 cell line was obtained from ATCC (Manassas, VA, USA). Additional details of cell culture techniques and other cell lines used are provided in the Supplementary Materials and methods. The study was approved by the institutional review board (IRB, Min. No. 7826 dated 18.04.2012).

In vitro cytotoxicity assay

The *in vitro* cytotoxicity of drugs were determined at 48 h using the MTT assay as described previously.¹⁵ The half-maximal inhibitory concentration (IC₅₀) values were generated using Graph Pad Prism5 software (La Jolla, CA, USA). Combination index between drugs was calculated using Calcsyn software (Biosoft, Cambridge, UK).

Assays for apoptosis

Leukemic cell lines or primary APL cells were added (1×10^5 cells/well) on a layer of primary mesenchymal stromal cells or HS-5 stromal cell line in 24-well plates. The co-cultured cells were incubated overnight and then exposed to ATO (2 μ M) with and without BTZ (200 nM) along with appropriate controls. After 48 h of incubation at 37 °C CO₂ incubator, the leukemic cells were carefully pipetted out and their viability was measured using Annexin V/7-aminoactinomycin D (7AAD) apoptosis assay kit (BD Pharmingen, San Diego, CA, USA) as per the manufacturer's protocol. CD105 staining was used to exclude stromal cells if present during acquisition and analysis. The flow data were analyzed using Cell Quest pro software (BD Biosciences, San Jose, CA, USA).

Measurement of ROS production

Levels of reactive oxygen species (ROS) were determined by dihydrorhodamine123 (DHR123; Life Technologies, Carlsbad, CA, USA) fluorescence as previously described.¹⁶

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi_m$) of the cells treated with drugs was measured using JC-1 dye (Life Technologies, Carlsbad, CA, USA) as previously reported.¹⁷ The fluorescence intensity was measured using Spectramax M4 (Molecular Devices, Sunnyvale, CA, USA) (green channel: excitation: 485 nm; emission: 530 nm; cutoff 515 nm; red channel: excitation: 485 nm; emission: 590 nm; cutoff 570 nm). The ratio of red to green fluorescence (590/530) was calculated and the $\Delta\psi_m$ of treated cells was compared with the untreated cells.

Proteasome activity assay

1×10^5 NB4 cells were lysed in RIPA buffer (containing protease inhibitor cocktail; Sigma, St Louis, MO, USA) on ice for 30 min. The cell lysates were collected by centrifugation and incubated with proteasome substrate, Z-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-Gly-Gly-Leu-AMC) (Sigma) for 60 min at 37 °C. After incubation, the fluorescence intensity was measured using Spectramax M4 (Molecular Devices) (excitation: 380 nm emission: 460 nm). The activity of untreated cells was normalized to 100% and the treated cell activity was compared with that of the untreated cells.

Co-immunoprecipitations and immunoblots

NB4 homogenates were obtained by cell lysis in RIPA buffer (Sigma), with complete protease inhibitors (Roche, Basel, Switzerland). Co-immunoprecipitation was performed using Co-IP kit (Thermo Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Nuclear extracts were taken from cells using NE-PER kit (Thermo Pierce) according to the manufacturer's protocol. The lysates and elutes were analyzed in SDS-polyacrylamide gel electrophoresis. After protein transfer to nitrocellulose membrane, membranes were blocked with non-fat dry milk (5%, 2 h) followed by incubation with primary antibodies overnight. The

details of antibodies used are given in the Supplementary Methods. The protein bands were detected by standard chemiluminescence method (Thermo Pierce Femto, Rockford, IL, USA).

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then, 500 ng of the extracted RNA was converted into cDNA using superscript II cDNA kit (Invitrogen). Quantification of the *PML-RARA* transcripts was done using EAC (Europe against Cancer) program protocols.¹⁸ The reverse transcriptase-PCR and real-time quantitative PCR sensitivity was assessed in-house using methodology as reported previously by us.¹⁹ The expression of other genes was studied using SYBR green method (Finnzymes F410L, Thermo Scientific, Rockford, IL, USA) (primer sequence given in Supplementary Table S1). The Ct values were normalized with *GAPDH* or *ACTB* and the fold differences were calculated using $2^{-\Delta\Delta Ct}$ method. The NF- κ B array (RT² profiler PCR array human NF- κ B signaling target; Qiagen, Hilden, Germany, Catalog no: PAHS-225z) was performed according to the manufacturer's instructions.

Knockdown experiment

The knockdown of p65 and p62 transcripts were performed by MISSION endoribonuclease-prepared short interfering RNA (esiRNA) purchased from Sigma (catalog numbers: p62- EHU027651, p65- EHU141461). Then, 30 nm of esiRNA was electroporated into NB4 cells using Amexa electroporator unit (Lonza Nucleofecto, Cologne, Germany; Program NB4 X-01) and recovered in complete media for 24 h.

Confocal microscopy analyses

NB4 cells were treated with ATO, BTZ or combination and cytospin slides were made after 24 h of treatment. The cells were fixed in 4% paraformaldehyde followed by blocking using 5% goat serum. It was further incubated with primary antibodies such as PML (Santa Cruz, Dallas, TX, USA), LC3, P62 and Ubiquitin (Abcam, Cambridge, UK) overnight at 4 °C. The slides were rinsed with phosphate-buffered saline three times and incubated with secondary antibodies (anti-mouse and/or anti-rabbit) conjugated with Alexafluor 594 and Alexafluor 488 (Invitrogen) for 1 h. The slides were again washed, air dried and counterstained with DAPI (4',6'-diamidino-2-phenylindole) containing mountant (Vectashield, Burlingame, CA, USA). The images were acquired in confocal microscope (Olympus FV1000, Melville, NY, USA) at $\times 20$ and $\times 100$ with oil immersion and the images were analyzed using Fluoview software version 3.1b.

Mouse model and drug treatments

FVB/N mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice at 6 to 8 weeks of age were used in all the experiments. The animal study design and euthanasia protocols were approved by the institutional animal ethics committee (IAEC approval number 17/2012). APL cells from the spleen of MRP8-PML-RAR transgenic mice²⁰ (FVB/N) were harvested and cryopreserved (a kind gift from Dr Scott Kogan). APL cells (5×10^4 cells/mouse) were injected intravenously via the tail vein into genetically compatible FVB/N recipients, without conditioning with either radiation or chemotherapy. ATO was given intraperitoneally at the concentration of 5 mg/kg of mice starting on day 7 post injection of malignant cells and continued for 28 days, whereas BTZ was given subcutaneously at a dose of 0.5 mg/kg of mice on days 8, 12, 16 and 20. Details of methodology used for secondary transplantation are provided in Supplementary Methods.

RESULTS

Malignant promyelocytes are protected from ATO-induced apoptosis by stromal cells through upregulation of NF- κ B pathway. We had previously reported that NB4 cells (APL cell line) or the primary blasts from APL patients had a significant survival advantage against ATO when co-cultured with either primary stromal cells or stromal cell line.⁹ A global gene expression array for differential gene expression in NB4 cells with and without HS-5 cell co-culture was performed. The Gene Set Enrichment Analysis (GSEA) revealed an enrichment of NF- κ B pathway in the malignant cell line upon co-culture (Figure 1a and Supplementary Figure S1). This was further validated by real-time PCR array where an

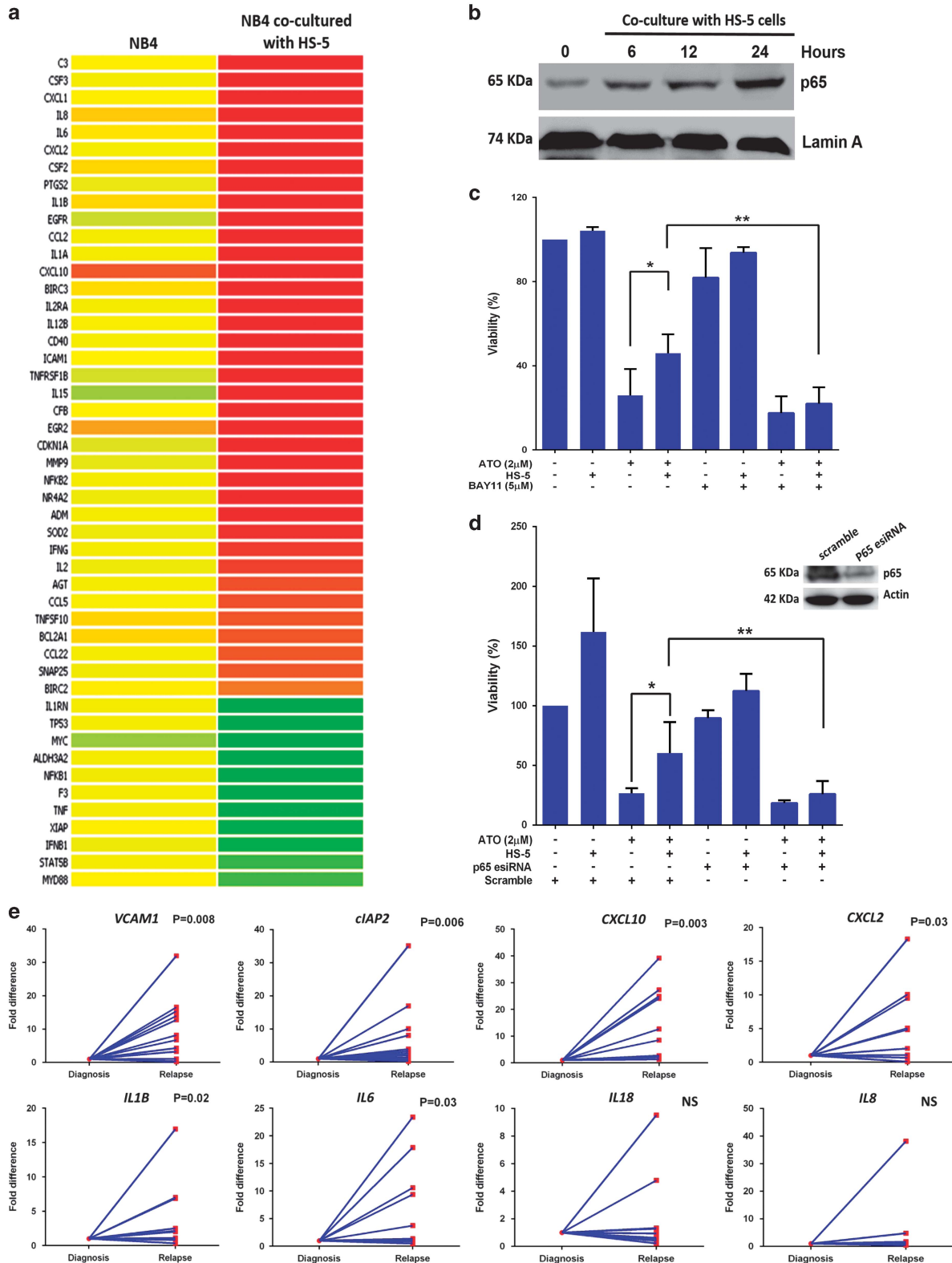


Figure 1. Stromal cells protect malignant promyelocytes against ATO *in vitro* through upregulation of NF- κ B pathway. **(a)** Heat map showing differential regulation of NF- κ B pathway in leukemic cells in co-culture in comparison with NB4 cells alone. **(b)** Activation of NF- κ B pathway in the malignant promyelocytes (NB4 cells) co-cultured with HS-5 cells for 6, 12 and 24 h shows increased translocation of p65 subunit in the nuclear compartment over time ($n=3$) on co-culture. **(c)** NF- κ B inhibitor (Bay11-7082-5 μ M) was able to overcome the protective effect mediated by the HS-5 cells along with ATO (2 μ M); assays were done using apoptosis assay (Annexin V/7AAD measured after 48 h of exposure; $n=5$). **(d)** Knockdown of p65 was able to overcome the protective effect against ATO (2 μ M) mediated by the HS-5 cell co-culture (Annexin V/7AAD measured after 48 h of exposure; $n=5$). **(e)** Expression of NF- κ B target genes *VCAM1*, *CIAP2*, *CXCL10*, *CXCL2*, *IL1B*, *IL6*, *IL18* and *IL8* in matched samples of newly diagnosed ($n=13$) and relapsed ($n=13$) APL blasts. Statistical significance was calculated using Wilcoxon matched pairs signed rank test (paired two tailed) and Student's *t*-test (two tailed *t*-test) and the *P*-values are denoted as * $P=0.02$ and ** $P=0.001$.

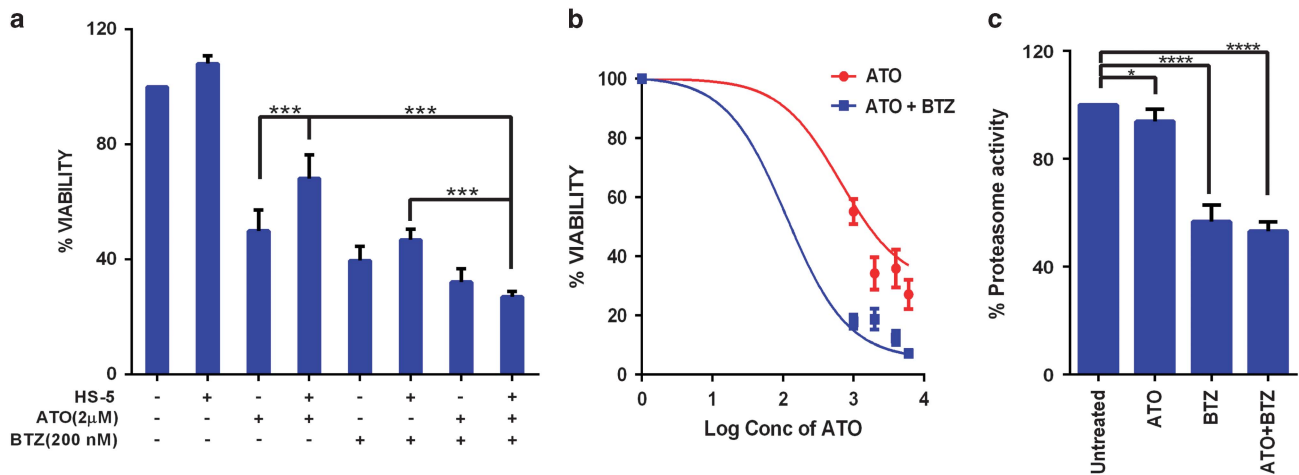


Figure 2. Bortezomib overcomes the stromal cell-mediated drug resistance and has a synergistic effect with ATO. (a) Bortezomib (BTZ) at pharmacologically relevant concentrations (200 nM) restores the sensitivity of malignant promyelocytes to ATO (2 μ M) in NB4 cells (Annexin V/7AAD measured after 48 h of exposure; $n = 8$) even in the presence of HS-5 cells; the viability of untreated cells was normalized to 100% and the treated cell viability was compared with normalized untreated cells. (b) The combination of ATO and BTZ had a combination index (CI) of 0.7 calculated using Calcsyn software ($n = 12$), where at a dose of 200 nM BTZ significantly reduced the IC_{50} of ATO on NB4 cells (measured by an MTT assay after 48 h of drug exposure). (c) Proteasome activity of NB4 cell lysates 6 h after treatment with either ATO (2 μ M), BTZ (200 nM) or both. The activity was measured by the ability of the lysate to hydrolyze Z-Gly-Gly-Leu-AMC peptide ($n = 5$). Statistical significance was calculated using Student's *t*-test (two-tailed *t*-test) and the *P*-values are denoted as * $P = 0.02$, *** $P = 0.0001$ and **** $P < 0.0001$.

increase in NF- κ B target genes was observed (Supplementary Figure S2). Western blotting analysis also showed an increased translocation of NF- κ B subunit p65 into the nuclear compartment of NB4 cells co-cultured with HS-5 stromal cells (Figure 1b). Inhibiting this pathway using a chemical inhibitor (Bay11-7082) or knockdown of p65 subunit using esiRNA in NB4 cells resulted in reversion of resistance to ATO upon co-culture (Figures 1c and d).

We also observed an upregulation of this pathway target genes in relapsed compared with newly diagnosed (13 matched newly diagnosed and relapsed) APL patient blasts (Figure 1e) even in the absence of co-culture with stroma. This suggests that the NF- κ B pathway can potentially play an important role in the mechanism of EMDR in APL, especially in relapsed APL.

Proteasome inhibitor overcomes the bone marrow micro-environment-mediated drug resistance through downregulation of NF- κ B pathway

To overcome the ATO resistance of NB4 cells on co-culture we screened various molecules (data not shown). We observed that proteasome inhibitors (MG-132 and bortezomib) when combined with ATO were able to overcome the protective effect mediated by stromal cell (HS-5 and primary mesenchymal stromal cells) co-culture to NB4 cells (Figure 2a) and to primary APL cells (Supplementary Figure S3). Bortezomib, a well-known inhibitor of the NF- κ B pathway, was able to reverse the effect of stromal culture-induced activation of the NF- κ B pathway (validated by documenting downregulation of NF- κ B target genes that had previously been upregulated on co-culture; Supplementary Figure S4).

Direct cytotoxicity of bortezomib on malignant promyelocytes and its synergy with ATO

The IC_{50} for BTZ on NB4 cell line was 14 nM (Supplementary Figure S5), whereas for in-house-generated ATO-resistant cell line (NB4 EV-AsR1) the IC_{50} value was 17 nM. For primary APL blasts the median IC_{50} was 12 nM. These values are comparable to its effect on myeloma cell lines.^{21,22} BTZ was able to synergize with ATO (Figure 2b) as demonstrated by a combination index value (0.7).

Combining ATO and BTZ does not alter the efficacy of BTZ in inhibiting proteasome complex (Figure 2c).

Mechanism of synergy of combining ATO with bortezomib in APL
Next, the potential mechanism of synergism between these two drugs was evaluated. As a result of proteasome complex inhibition a significant accumulation of ubiquitinated proteins at 24 h in NB4 cells treated with BTZ or a combination ATO and BTZ was observed (Figure 3a). A similar observation was noted *in-vivo* in mice treated with ATO and BTZ and *in vitro* in primary human APL cells; Supplementary Figure S6). As a result, this combination induced upregulation of the UPR pathway more than either ATO or BTZ could as single agents, as evidenced by significant upregulation of *CHOP* and *ATF4* (UPR pathway genes involved in apoptosis; Figures 3b and c and Supplementary Figure S7). The combination of ATO and BTZ also significantly induced ROS generation (Supplementary Figure S8) and decreased the mitochondrial membrane potential of NB4 cells when compared with ATO or BTZ alone (Figure 3d). Inhibiting ROS by *N*-acetyl cysteine followed by treatment with ATO and BTZ significantly rescued the cells from apoptosis induced by ATO and ATO+BTZ but not BTZ alone (Figure 3e). The net effect of the above changes led to a more effective induction of apoptosis by the combination of these drugs than either drug alone (Figure 3f). Although it has been previously reported that BTZ induces apoptosis in malignant promyelocytes through UPR pathway and endoplasmic reticulum stress,¹² these data demonstrate the additive effect of ATO on these effects along with an increased ROS activity and decreased mitochondrial membrane potential that accounts for the documented synergy of these two agents.

Proteasome inhibition is dispensable for the degradation of PML-RARA

Degradation of PML-RARA was observed through nuclear body reformation in the ATO+BTZ-treated NB4 cells that were similar to ATO-alone-treated cells, whereas a micro-speckled pattern was observed in the untreated cells (Supplementary Figure S10). This was further validated by immunoblot, where the degradation was seen at 48 h (Figure 4a). BTZ and the combination of ATO+BTZ

resulted in initial stabilization of PML-RARA but subsequent degradation beyond 24 h was observed; these data correlated with the clearance of ubiquitinated proteins at the same time

points (Figure 3a and Supplementary Figure S11). As ATO is known to induce ubiquitination of PML-RARA, an immunofluorescence assay for ubiquitin and PML was performed. On combining ATO

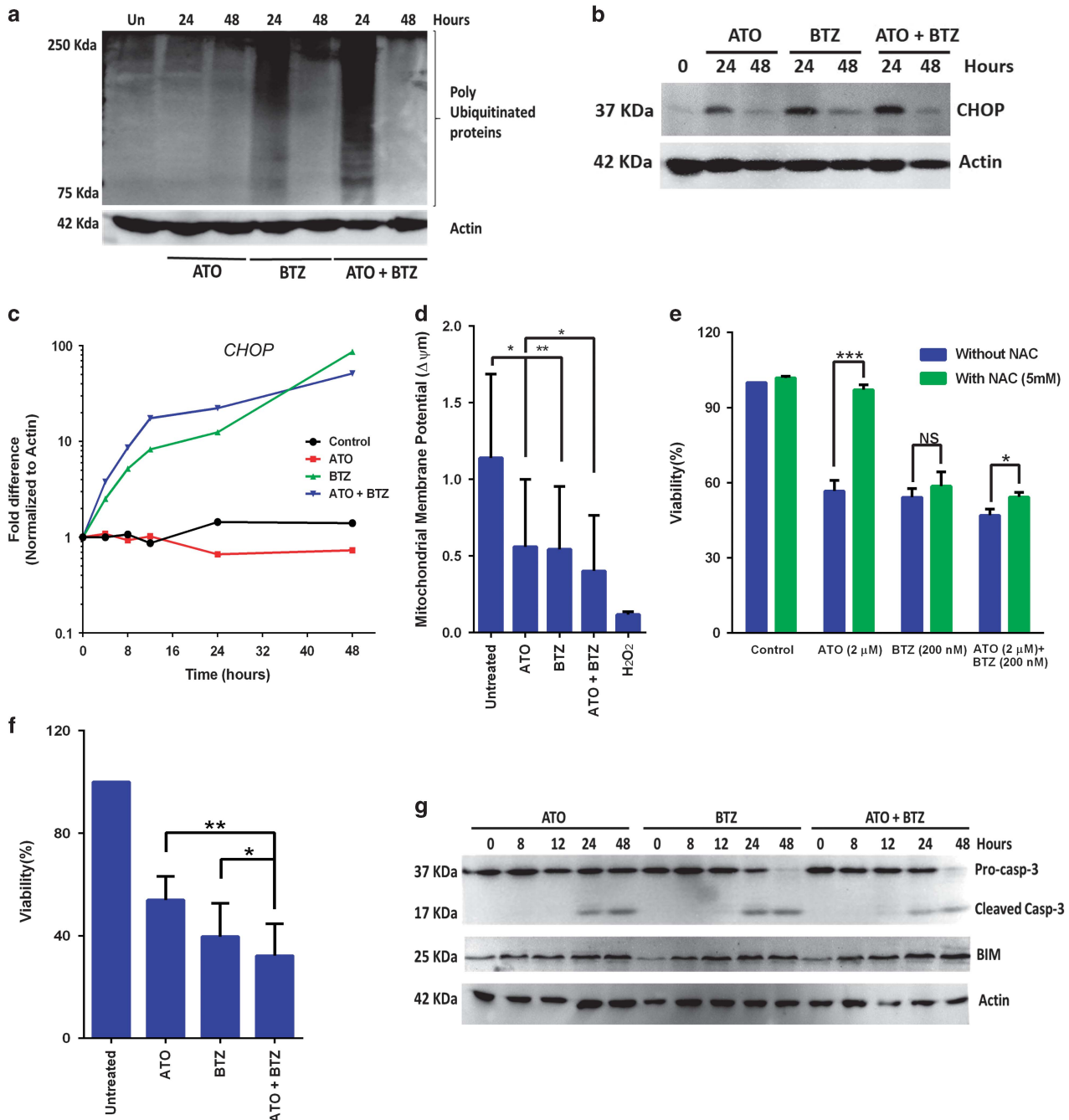


Figure 3. ATO and bortezomib synergizes by inducing apoptosis by UPR pathway. (a) Immunoblot shows accumulation of ubiquitinated proteins in ATO (2 μ M) and BTZ (200 nM) combination-treated NB4 cells at 24 h and disappearance of the same by 48 h ($n=3$). (b) Immunoblot shows upregulation of UPR gene *CHOP* at 24 h and subsequent reduction at 48 h after drug exposure at same concentration as mentioned above in NB4 cells ($n=5$). (c) Upregulation of UPR gene *CHOP* was also observed by real-time PCR that increased over time to a maximum at 48 h after drug treatment, at the same concentrations as mentioned above ($n=3$) in NB4 cells. (d) The combination of ATO (2 μ M) and BTZ (200 nM) decreased mitochondrial membrane potential (MMP) in NB4 cells (MMP was measured 6 h post drug treatment; $n=6$). (e) Pretreatment of NB4 cells with ROS inhibitor, *N*-acetyl cysteine (NAC, 5 mM) significantly rescued the NB4 cells from ATO (2 μ M) and ATO (2 μ M) plus BTZ (200 nM) induced apoptosis but not from BTZ-alone-treated cells (viability was assessed 48 h post drug treatment using Annexin V/7AAD measurement; $n=3$). (f) Viability assay demonstrating ATO (2 μ M) and BTZ (200 nM) combination induces a significant greater cell kill than either drug alone (Annexin V/7AAD measured after 48 h of exposure; $n=6$). (g) Western blot demonstration of activation of caspase-3 and increased expression of pro-apoptotic protein BIM to induce apoptosis at different time points at drug concentrations as mentioned above. Statistical significance was calculated using Student's *t*-test (two-tailed *t*-test) and the *P*-values are denoted as NS, not significant, * $P=0.02$, ** $P=0.001$ and *** $P=0.0001$.

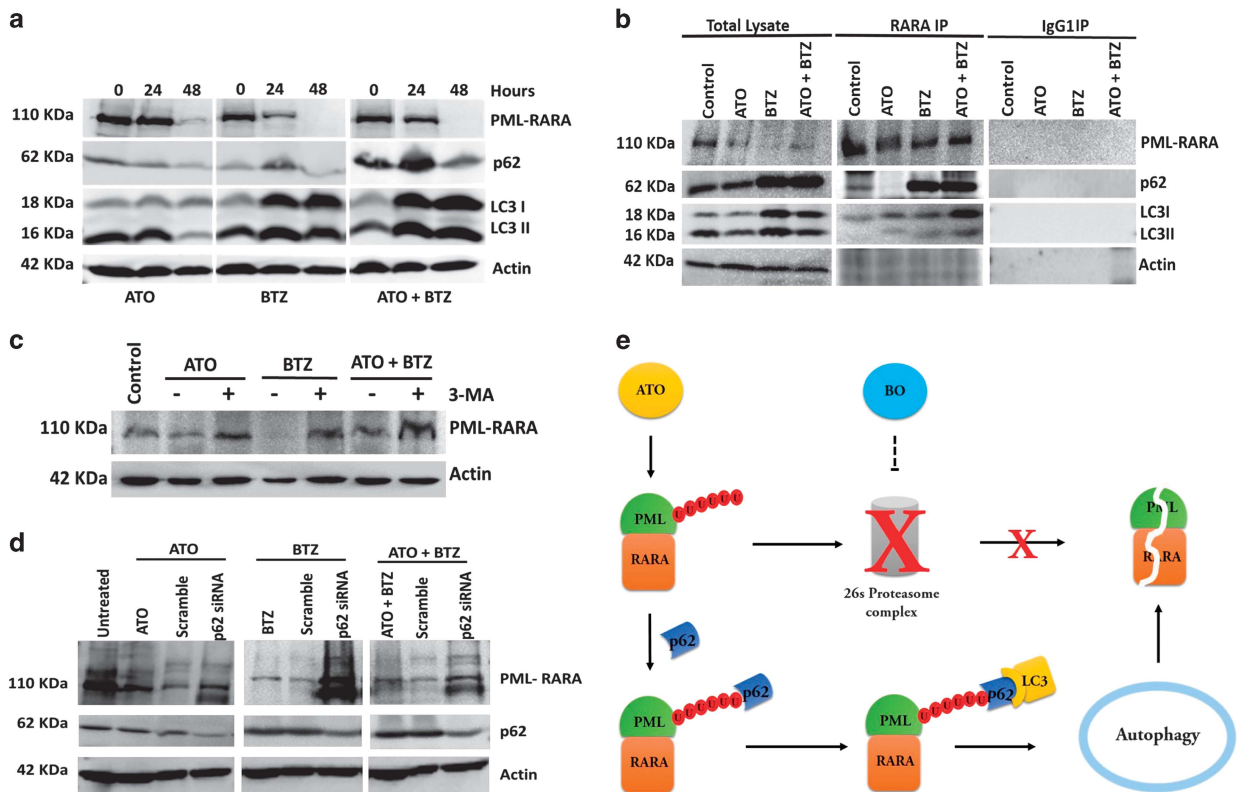


Figure 4. PML-RARA is degraded alternatively by autophagy pathway. **(a)** Immunoblot assay showing increased activation of autophagy pathway through increased conversion of LC3I to LC3II and p62 accumulation and degradation by 24 and 48 h corresponding to PML-RARA degradation (ATO (2 μ M) and BTZ (200 nM)). **(b)** Co-immunoprecipitation assays showing interaction between P62, LC3 and PML-RARA at 24 h post drug treatment. **(c)** Inhibition of autophagy by pretreating the cells with 3 methyl adenine (3 MA; 5 mM) inhibited the degradation of PML-RARA in cells treated with ATO (2 μ M) and BTZ (200 nM) for 24 h. **(d)** Knockdown of p62 by esiRNA inhibited the degradation of PML-RARA in cells treated with ATO (2 μ M) and BTZ (200 nM) for 24 h, $n = 3$. **(e)** Schematic representation of PML-RARA degradation by a combination of ATO and BTZ with proteasome inhibition.

and BTZ, we demonstrated that PML colocalized with ubiquitin even with this combination (Supplementary Figure S12). Together, these data suggest that PML-RARA is ubiquitinated and degraded by a proteasome-independent pathway when ATO is combined with BTZ.

Proteasome inhibition results in the induction of autophagy that degraded PML-RARA oncoprotein

Existing data suggested that induction of autophagy upon proteasomal inhibition can degrade the accumulated ubiquitinated proteins.²³ We hypothesized that PML-RARA (ubiquitinated upon ATO+BTZ treatment) must be degraded via the autophagy pathway when proteasome was inhibited. The analysis of autophagy genes expression in the combination-treated NB4 cells showed an additive induction of autophagy genes (such as LC3II, *ATG5* and *BECN1*; Figure 4a and Supplementary Figure S13). With the combination of ATO+BTZ we also observed a time-dependent induction and subsequent degradation of p62 protein (adaptor protein involved in degradation of PML-RARA during myeloid differentiation²⁴ by all-*trans* retinoic acid (ATRA)) correlated with ubiquitinated protein and PML-RARA levels at these time points (Figure 4a and Supplementary Figure S14 with extended time points). On immunofluorescence analysis, PML proteins colocalized with LC3 and P62 upon treatment with this combination (Supplementary Figure S12), and this was further validated by co-immunoprecipitation assay (Figure 4b). However, the same combination treatment (ATO+BTZ) of NB4 cells along with inhibition of autophagy with a small-molecule inhibitor 3-methyl adenine (3MA) or transient knockdown of p62 by esiRNA resulted

in the accumulation of PML-RARA (Figures 4c and d). Induction of autophagy in NB4 cells by starvation also degraded PML-RARA (data not shown). These results indicate that autophagy degrades PML-RARA, that this process involves the p62 cargo binding protein and that the combination of ATO and BTZ has an additive effect in inducing autophagy.

ATO and bortezomib combination is effective in ATO-resistant NB4 cells

We next assessed the efficacy of this combination on an in-house-generated ATO-resistant NB4 cell line (NB4-EVAsR1: harboring A216V mutation in PML B2 domain; data provided in Supplementary Methods). These resistant cell lines were sensitive to BTZ. Treating the resistant cells with ATO and bortezomib resulted in a synergistic effect (combination index=0.02; Supplementary Figure S15). The mechanism of action of combination drugs was similar to NB4-naive cells (data not shown). Bortezomib was also able to synergize with other drugs such as ATRA and anthracyclines and this effect was seen in both naive NB4 cell line as well as resistant NB4 cell line. The effect of ATO with ATRA and BTZ on the viability was comparable with that of ATO with ATRA and anthracycline in both naive and ATO-resistant NB4 cells (Supplementary Figure S16).

ATO and bortezomib combination is effective in reducing leukemic burden and inducing long-term survival in an APL mouse model

We further evaluated the combination's efficacy *in vivo* using a transplantable APL mouse model (Figure 5a). The APL mice

treated with a combination of ATO and BTZ showed a significant reduction in the tumor burden on day 20, as evidenced by reduction in spleen size (Figure 5b), decreased PML-RARA copy numbers (Figure 5c) and decreased bone marrow blasts analyzed by flow cytometry (Figure 5d) and immunohistochemistry (hematoxylin and eosin staining; Figure 5e) compared with ATO- or BTZ-alone-treated mice. The combination therapy was also associated with a significantly superior survival compared with mice treated with either agent alone (Figure 5f).

Combination of ATO and bortezomib reduces leukemia-initiating cells in APL

We further evaluated the combination's effect on leukemia-initiating cells in APL. In the mouse model, secondary transplantation of bone marrow cells of leukemic mice harvested on day 20 after treatment with placebo, ATO alone or ATO+BTZ demonstrated a significantly superior survival of secondary recipients that received bone marrow cells from the ATO combined with BTZ-treated mice (Figures 5g and h). In the mice that had long-term survival (surviving > 250 days; only seen in ATO+BTZ group) we failed to observe an expression of PML-RARA transcript in all the organs tested (data not shown). We also observed a prolonged survival of the mice that had been serially transplanted with the bone marrow cells from the long-term surviving mice (previously treated with ATO and BTZ; Supplementary Figure S17). Together, these data suggest that this combination is able to eliminate the leukemia-initiating cell compartment in a mouse model of APL.

Bortezomib and ATO combination is effective in relapsed APL patients

In 2011, based on preliminary *in vitro* laboratory data, two patients (RS and TK) with second relapse, both following an autologous stem cell transplant after their first relapse, were administered a combination of ATO (conventional doses as reported previously;² INTAS Pharmaceuticals, Ahmedabad, India) with bortezomib (1.4 mg/m²/weekly × 4 doses; NATCO, Hyderabad, India) in induction and consolidation on a compassionate basis after getting written and informed consent. TK also received two doses of mitoxantrone (Neon Laboratories Ltd, Mumbai, India) in induction. RS subsequently received only maintenance therapy with ATO and ATRA (Roche Pharmaceuticals) as he did not have an allogeneic stem cell donor, whereas TK underwent a matched unrelated donor stem cell transplantation in molecular remission. The combination therapy was well tolerated with no significant Grade III/IV nonhematological toxicity. Both patients are currently in continuous molecular remission at 61 and 60 months since second relapse, respectively. An additional three patients received this combination on a compassionate basis after getting written and informed consent. One patient (SS) with multiple relapses had a transient hematological remission and relapsed and went on palliation, whereas two other patients in first relapse (BJ and AA) received a similar ATO and bortezomib combination in induction and consolidation. BJ had an autologous stem cell transplant, whereas AA received maintenance therapy with ATO and ATRA, and both remain in continuous molecular remission at 60 and 42 months (data summarized in Supplementary Table S2).

Based on this preliminary favorable experience, a phase II clinical trial for patients with APL in first relapse was initiated in 2013 after getting institutional IRB approval (IRB Min 8225 dated 27 February, 2013). The study was registered in the public domain (NCT01950611). The study continues to enroll patients at our center. Sixteen patients diagnosed to have relapsed APL have been enrolled in this study. All patients achieved hematological remission. The combination of ATO with bortezomib was well tolerated. There were no induction deaths. With the exception of one patient who developed grade III peripheral neuropathy requiring treatment, none of the other patients had any grade III/

IV nonhematological toxicity. All patients remain in continuous molecular remission at a median follow-up of 447 days. Longer follow-up is required to validate the impact of this regimen on relapse-free and overall survival but one can conclude that the combination is safe and well tolerated.

DISCUSSION

Bone marrow microenvironment acts as a sanctuary for the leukemic cells to establish a niche and protect themselves from chemotherapeutic agents.²⁵ There are numerous reports on EMDR in leukemia.^{26–31} We had previously reported, for the first time, that there was significant EMDR to ATO in APL.⁹ We observed this protective effect with both primary stromal cells and stromal cell line but not with human umbilical vein endothelial cells, COS-7 or peripheral blood mononuclear cells (data not shown). We did not find any difference in the ATO concentration levels or in the intracellular ATO levels achieved in the malignant cells with and without co-culture, suggesting that the cross-talk with the stroma resulted in cell-intrinsic changes that mediated resistance. A global gene expression array was performed and the differentially regulated genes were subjected to GSEA where we could observe a significant enrichment of the NF-κB pathway. These findings were consistent with recently published data that demonstrated the important role of the NF-κB pathway in inducing this resistance to chemotherapy on stromal co-culture.¹⁰ We screened a number of small molecules and antibodies based on the data we had for their ability to overcome EMDR to ATO. We were specifically interested in the beneficial effect of proteasome inhibitor bortezomib that is a known inhibitor of NF-κB, and also because it is an FDA (Food and Drug Administration)-approved drug that has been widely used in the clinic in the treatment of myeloma^{32–34} and other hematological malignancies.^{35–37}

We observed a significant cytotoxicity of bortezomib as a single agent on malignant promyelocytes (at concentrations evaluated there was no significant cytotoxicity on stromal cells, peripheral blood mononuclear cells or CD34⁺ cells). In contrast to existing dogma that suggested that bortezomib and ATO were likely to be antagonistic, we demonstrated *in vitro* synergy between these two agents. The cytotoxicity on malignant promyelocytes was significantly superior to either agent alone. Although there was a trend to an increase in apoptotic proteins such as BIM on an immunoblot (Figure 3g) and a similar trend to an decrease in expression of anti-apoptotic genes such as *BCL2* and *cIAP2* on real-time PCR (Supplementary Figure S9), we could not demonstrate a similar increase in some apoptotic proteins such as Cleaved caspase 3 (Figure 3g).

We noted an induction of UPR on combining ATO and BTZ as demonstrated by significant increase in expression of *ATF4* and *CHOP*. For *CHOP* proteins, this was seen only until 24 h and at 48 h that there was a reduction in levels as seen on an immunoblot (Figure 3b). However, the real-time PCR values continued to be significantly increased even at 48 h (Figure 3c). We suspect that this is because of *CHOP* being a target of the ubiquitin proteasome system as has been reported previously³⁸ and subsequent clearance of *CHOP* along with other ubiquitinated proteins by autophagy as seen in Figure 3a.

Bortezomib has multiple mechanisms of action in cancer cells, and in combination with ATO we demonstrated the importance of multiple cellular processes in mediating the cytotoxicity in APL, in addition to NF-κB inhibition. A concern remained about the fate of the PML-RARA oncoprotein when this combination was used. Published data suggest that an intact proteasome was critical for ATO to mediate PML-RARA degradation and, in addition, clearance of the leukemia-initiating compartment in APL was dependent on degradation of this oncoprotein.^{6,8,39} In this study we demonstrated that even with very effective proteasome blockade using a

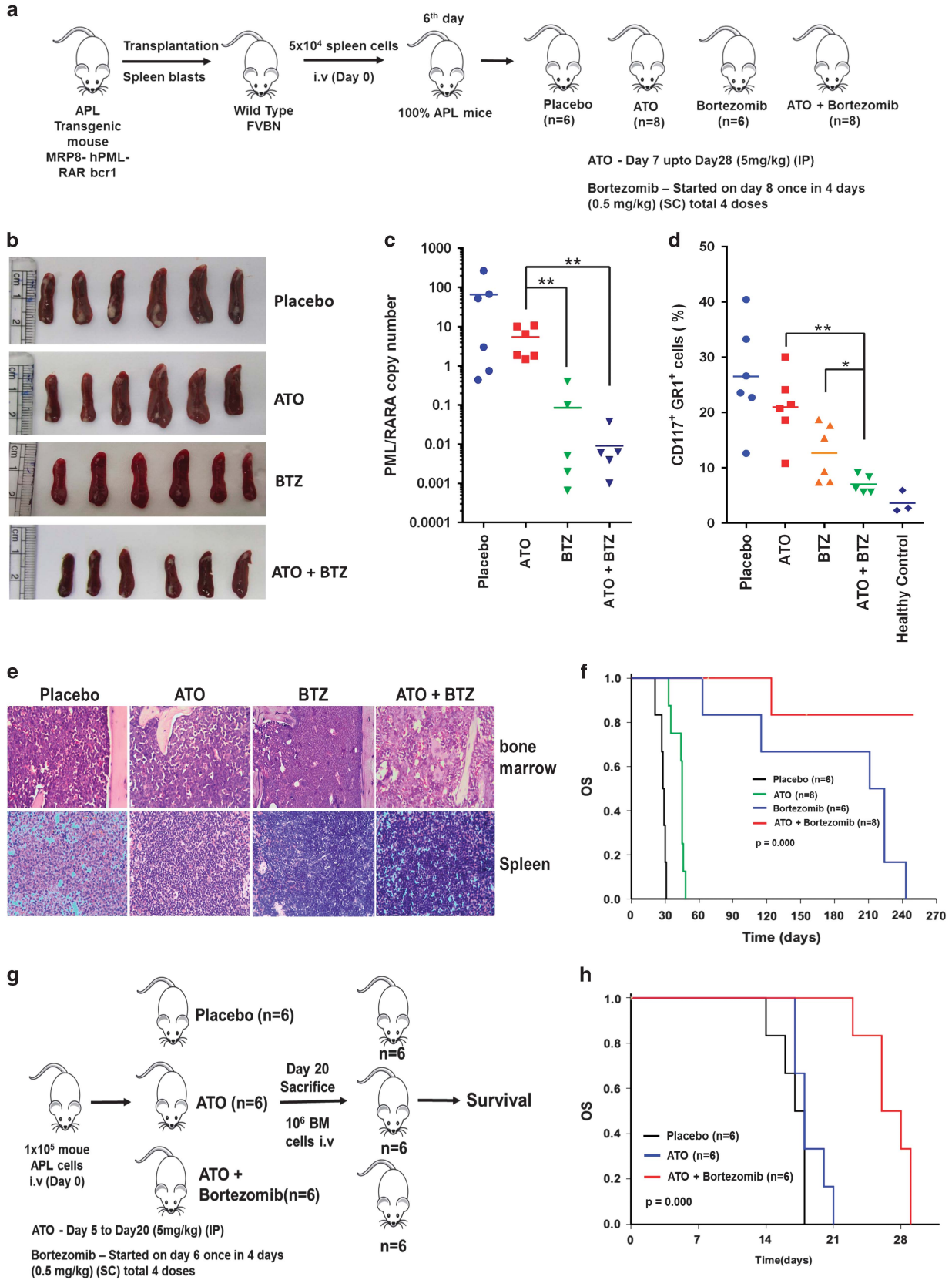


Figure 5. ATO and bortezomib combination reduces leukemia burden and leukemia-initiating compartment in an APL mouse model. (a) Schematic representation of transplantable APL mouse model and treatment plans. Demonstration of leukemic burden reduction in the combination treated arm through (b) reduced spleen size. (c) Reduced expression of PML-RARA transcript on day 20 in ATO- and bortezomib (BTZ)-treated group. (d) Flow cytometry analysis for tumor burden shown reduced leukemic cells (CD117+ and Gr1+ cells) in bone marrow of APL mice upon treatment with ATO and BTZ (e) Hematoxylin and eosin (H&E) staining of bone marrow and spleen from mice treated with Placebo, ATO alone, BTZ alone or ATO in combination with BTZ. Mice were killed on day 20 and examined for the presence of leukemic cells. In mice treated with placebo, there was a diffuse infiltrate of immature cells replacing the marrow and the spleen. In ATO-alone and BTZ-alone-treated mice, there was a reduction in the immature cell infiltrate in the bone marrow and the spleen. In mice treated with ATO+BTZ, scattered atypical cells were noted (less than with ATO or BTZ alone) and normal hematopoietic elements were seen in the marrow, and splenic architecture was preserved. (f) ATO and BTZ combination prolonged the median survival of APL mice (Placebo: 28 days; ATO: 45 days; BTZ: 211 days; ATO+BTZ, not reached). (g) Schematic representation of secondary transplantation experiment (not treated post transplantation) and followed them up to their death. (h) The survival curve of the secondary transplantation experiment shows that ATO and BTZ combined treated mice bone marrow cells upon secondary transplantation had a significant prolonged median survival (26 days) compared with placebo (17 days) or ATO-alone (18 days)-treated mice. Statistical significance was calculated using log rank test and nonparametric, unpaired, two-tailed, Mann-Whitney test. The *P*-values are denoted as **P*=0.02 and ***P*=0.004.

combination of ATO and bortezomib we could still demonstrate effective PML-RARA degradation and that this degradation was independent of the proteasome pathway. Previously reported data suggest that proteasome inhibition can induce the autophagy pathway for the degradation of ubiquitinated proteins^{23,40,41} and that PML-RARA can be degraded by the autophagy pathway during differentiation of NB4 cells treated with ATRA or ATO.^{24,42,43} Our data demonstrate that degradation of PML-RARA oncoprotein on combining bortezomib and ATO was mediated by autophagy that was additively induced with this combination. Reported data suggest that cargo-binding proteins such as P62, ALFY and NBR1 are involved in degrading ubiquitinated proteins.^{44–47} Previous reports suggest that p62 is involved in the degradation of PML-RARA by autophagy during myeloid differentiation²⁴ and ATO treatment.⁴² Consistent with these observations we have demonstrated that a combination of bortezomib and ATO induces autophagy and the PML-RARA oncoprotein is degraded by a p62-dependent autophagy pathway.

The efficacy of the combination of bortezomib and ATO was further validated by us in a transplantable mouse model of APL and in preliminary data from the clinic. Based on these encouraging results a phase II clinical trial was initiated and continues to recruit patients with relapsed APL. The preliminary phase II study data that are reported here suggest that this combination is well tolerated. In addition, we also demonstrate that this combination is effective in ATO-resistant cell lines.

We have also demonstrated (*in vitro* data) that the combination of bortezomib with ATO and ATRA is comparable to the effect of anthracycline with ATRA and ATO on malignant promyelocytes. In the evolving strategy of de-escalation of therapy in APL,⁴⁸ the addition of bortezomib with ATO along with ATRA has the potential to further de-escalate the therapy in high-risk and relapsed APL by replacing the myelotoxic anthracycline with a relatively non-myelotoxic proteasome inhibitor.

The microarray data discussed in this manuscript have been deposited in the NCBI Gene Expression Omnibus (GEO) under the GEO series accession number GSE73157.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SG: performed research, designed study, performed molecular tests, analyzed data and wrote paper; AAA: performed research, performed molecular tests, flow cytometry tests and analyzed data; EC, NB, HKP and SD: performed research, performed molecular tests and analyzed data; UK: performed research, clinical data accrual and analysis; MA and RM: performed microarray experiment and analyzed data; AK: performed research, clinical data accrual and analysis and histopathology analysis; AA: performed research, clinical data accrual and analysis; AS: performed research and administrative support; RAP and CC: performed research, mouse experiment design and analyzed data; BG: performed research, clinical data accrual, analysis and wrote paper; PB: performed research, performed molecular tests and analyzed data; VM: performed research, designed study, clinical data accrual, analyzed data and wrote paper.

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