The effect of the proteasome inhibitor bortezomib on acute myeloid leukemia cells and drug resistance associated with the CD34+ immature phenotype

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ABSTRACT

Background
Proteasome inhibition represents a promising novel anticancer therapy, and bortezomib is a highly selective reversible inhibitor of the proteasome complex. Acute myeloid leukemia (AML) is an immnunophenotypically heterogeneous group of diseases, with CD34+ cases being associated with drug resistance and poor outcome. We investigated the effects of bortezomib on the growth and survival of AML cells.

Design and Methods
We studied the *in vitro* activity and mechanism of action of bortezomib on both cell lines and fresh cells from 28 AML patients including CD34+ and CD34- cases.

Results
Bortezomib showed potent anti-AML activity (IC_{50} < 50 nM), which was greater than that of conventional agents (doxorubicin, cytarabine and fludarabine). Moreover, synergistic effects were observed when bortezomib was adminstered in combination with doxorubicin and cytarabine. Mechanistically, bortezomib induced accumulation of cells in the G_2/M phase, with up-regulation of p27, together with cell death through an increase in the mitochondrial outer membrane permeability involving caspase-dependent and -independent pathways. The apoptotic activity of bortezomib on fresh CD34+ blast cells from patients was similar to that observed on CD34- blast cells. Importantly, bortezomib was significantly more active than doxorubicin in the immature CD34+ cells, while there were no differences in its action on CD34- cells.

Conclusions
Bortezomib induces apoptosis in acute myeloid leukemia cells *in vitro*. Whether this drug might be useful in the treatment of patients with acute myeloid leukemia can be established only in *ad hoc* clinical trials.

Key words: bortezomib, acute myeloid leukemia, CD34.

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Introduction

The ubiquitin–proteasome pathway plays a fundamental role in cellular homeostasis as a critical regulator of cell proliferation and apoptosis. For this reason, the proteasome represents an attractive target for therapeutic intervention in cancer patients,[1] and this is supported by the results obtained in different malignancies with the proteasome inhibitor bortezomib (Velcade®, formerly PS-341), which is a highly selective, reversible inhibitor of the 26S subunit of the proteasome complex.[2] Studies on the mechanism of action of bortezomib have indicated that this drug stabilizes p21, p27 and p53, as well as the pro-apoptotic Bid and Bax proteins, caveolin-1 and IκB-α.[3,4] The last protein prevents activation of NFκB-induced cell survival pathways in several cellular systems, including a multiple myeloma model.[5] The anticancer effects of bortezomib have been demonstrated *in vitro* and *in vivo* for different malignancies such as multiple myeloma,[6,7] adult T-cell leukemia,[8] melanoma,[9] lung,[10,11] breast,[12] pancreatic,[13,14] prostate,[15,16] ovarian,[17] head and neck,[18] and colon cancer.[19] Moreover, several *in vitro* experiments have also shown that bortezomib enhances the antitumor properties of various antineoplastic drugs.[20-25]

Clinical investigations concerning the efficacy and safety of bortezomib alone or in combination with chemotherapy in multiple myeloma have been completed[26,27] and bortezomib was approved in 2003 for the treatment of relapsed and refractory multiple myeloma.[28] More recently, bortezomib was also approved for the treatment of mantle cell lymphoma. As far as concerns acute myeloid leukemia (AML), three small clinical trials have been conducted;[29-31] in two of them, bortezomib was combined with conventional agents,[30,31] and in one it was used as a single agent, but only modest and transient antileukemic activity was observed.[29]

In spite of these data, there is little information on the *in vitro* activity and mechanism of action of bortezomib in AML to support its clinical use. This is important, particularly due to the heterogeneity of AML, including a wide array of genetic lesions and immunophenotypic profiles. The CD34 antigen identifies early progenitor cells and, accordingly, AML can be divided into immature and mature forms ($CD34^+$ and $CD34^-$, respectively), the former subset associated with drug resistance and poorer outcome,[32-35] as compared to the more mature $CD34^-$ cases. Moreover, at relapse, blast cells usually display a more immature phenotype, as a reflection of drug resistance.[36,37] In fact, it has been suggested that the presence of an immature phenotype,[32,35] together with age and cytogenetics represent important prognostic factors in AML.[38] On this background, we carried out a detailed analysis of the *in vitro* activity and mechanism of action of bortezomib on AML cells using both cell lines and fresh cells from patients including $CD34^+$ and $CD34^-$ cases. In addition, we compared the activity of bortezomib with that of conventional agents used for the treatment of AML.

Design and Methods

Reagents and immunochemicals

Cell culture media, serum and penicillin-streptomycin were purchased from Invitrogen Corporation (Gaithersburg, MD, USA). Bortezomib (formerly known as PS-341; Millenium Pharmaceutics Inc. Cambridge, MA, USA) was dissolved in DMSO and stored at –20°C until use. Doxorubicin, cytarabine (ara-C) and fludarabine were purchased from Sigma (USA). Annexin V-FITC was obtained from Becton Dickinson (San Diego, CA, USA). Calpeptin and Z-VAD-FMK were from Calbiochem (San Diego, CA, USA). Other generic chemicals were purchased from Sigma Chemical Co., Roche Biochemicals (Mannheim, Germany), or Merck (Darmstadt, Germany). The origins of the different monoclonal antibodies employed in the western blotting analyses were as follows: the anti-p21, anti-pErk, anti-Erk1/2, and anti-caspase-3, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Apaf-1, anti-caspase-8, anti-caspase-9, anti-AIF, anti-Bcl-X, anti-PARP, anti-Bcl-2, anti-Cdk4 and anti-cyclin D1 antibodies were from Becton Dickinson, anti-p53 antibody was from Calbiochem Science, and the HRP-conjugated secondary antibodies were from Bio-Rad.

Cell lines: cell proliferation, cell cycle and apoptosis assays

All AML cell lines (HEL, KG-1, MV4-11 and HL-60) were cultured in RPMI 1640 containing 10% fetal-bovine serum (Gibco), $2x10^{-3}$ M glutamine, 100 units/mL penicillin and 100 ∝g/mL streptomycin at 37°C in a humidified atmosphere in the presence of 5% CO_2-95% air. HL60 cells were derived from a patient with FAB M2 AML, the HEL and KG-1 cell lines were derived from patients with erythroid leukemia (FAB M6), while the source of MV4-11 was a patient with myelomonocytic leukemia (FAB M4). The proliferation of AML cells was examined using MTT colorimetric assays as described elsewhere.[39,40] Pilot studies were conducted on all the AML cell lines to optimize cell concentrations and incubation times with the different drugs. Interactions between bortezomib and other anti-AML drugs were analyzed using the Calcusyn software program (Biosoft, Ferguson, MO, USA). Data from cell viability assays (MTT) are expressed as a fraction of cells with growth affected (FA) in drug-treated versus untreated cells. This program is based upon the Chou and Talalay method.[41,42] For flow cytometric evaluation of apoptosis, 1 10^6 of HEL cells were washed with phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM

Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were incubated with 5 μL of annexin-V-FITC for 15 min at room temperature in the dark, and then 10 μL of propidium iodide (PI) were added.

To obtain a quantitative evaluation of the mitochondrial transmembrane potential (Ψ_m), cells were incubated in PBS with 20 nM 3,3´-dihexyloxacarbocyanine iodide [DiOC6(3)] (Molecular Probes, Leiden, The Netherlands) for 20 min at 37°C in the dark, washed with PBS and, then, following addition of 10 μL PI (Calbiochem, San Diego, CA, USA) underwent flow activated cell sorting (FACS) on a FACScalibur flow cytometer (BD Biosciences) and analysis with the Paint-a-gate program.

To analyze the cell cycle distribution, cells were made permeable by the addition of 70% ethanol for 4 h at 4°C and stained with PI in the presence of 5 μg/mL RNAse (Sigma). Ten thousand events were acquired on a FACScalibur flow cytometer (BD Biosciences) and analyzed with the Paint-a-Gate program.

Western blotting

Cell lines were treated with 50 nM of Bortezomib and were collected and centrifuged at 10,000 × *g* for 2 min. The cells were then washed with PBS and lysed in ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris (pH 7.0), 1 μM pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM sodium orthovanadate). Samples were centrifuged at 10,000 × g at 4°C for 10 min and supernatants were transferred to new tubes.

Subcellular fractionation

HEL cells were harvested in isotonic mitochondrial buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μM pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM sodium orthovanadate) and Dounce homogenized by 60-70 strokes. Samples were transferred to Eppendorf tubes and centrifuged at 770 × *g* for 10 min at 4°C to separate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × *g* for 25 min at 4°C to obtain the mitochondrial pellet. The supernatant was further centrifuged at 100,000 × *g* for 1 hour at 4°C to yield the final soluble cytosolic fraction.

Patients' samples and apoptosis assays

For cytometric analyses of apoptosis in bone marrow (BM), cell subpopulations from 29 AML patients, excluding those with acute promyelocytic leukemia, were obtained at diagnosis before any treatment. Both $CD34^+$ and $CD34^-$ cells co-existed in nine cases. In seven cases, all blast cells were $CD34^+$, while in the other 13 samples, they were all $CD34^-$. Accordingly, a total of 16 samples had a significant $CD34^+$ population, either as a pure population or in a mixture, and in 22 samples a $CD34^-$ population was identified and available for investigation of drug-induced antitumor activity. The multiparametric flow cytometry analysis of the $CD34^-$ populations showed that in all cases, these populations were inmunophenotypically more mature cells based on the expression of different maturation antigens (CD15, CD11b, CD64, CD14, CD65, cMPO, $CD45^{++}$). The average age of the patients was 64±12 years (mean±SD). Cytogenetic information was available for 22 samples (7 complex karyotype or 11q23; 15 normal risk karyotypes), the remaining seven patients had no mitoses. According to the FAB classification, the distribution of cases was as follows: two M0, (7%); eight M1, (28%); five M2, (17%); four M4, (14%); eight M5 (28%); one M6, (4%) and one case was considered not classifiable. Patients were treated according to the Spanish Cooperative PETHEMA group's protocols LAM99 <65 (n=19), and LAM99>65 (n=1), and 68% (n=14) of 20 evaluable patients achieved morphological complete remission. The remaining patients (n=9) were considered to have received only supportive care, due to older age.

Mononuclear cells (MNC) were isolated by a Ficoll-Hipaque density sedimentation and maintained in IMDM containing 15% FCS; the percentage of blasts after purification was 88±9%. To consider a MNC sample as valid, it had to have less that 5% trypan blue-positive cells at arrival at our laboratory, and, after incubation for 18 h with drugs, there had to be less than 40% annexin V positive events in the control. In order to analyze the apoptotic activity of bortezomib and to compare it with that of doxorubicin and cytarabine, 1×10^6 BM cells were incubated in six-well plates with bortezomib (50 nM), doxorubicin (1 μM) or cytarabine (1 μM), or without any drug (control) for 18 h at 37 °C in a humidified atmosphere in the presence of 5% CO_2-95% air. The drug concentrations were selected based on the median plasma levels achieved in patients for these drugs and our results in cell lines. Subsequently, cells were incubated for 15 min at room temperature in the dark with 5 μL annexin-V-FITC (Bender MedSystems, Burlingame, CA, USA) together with a combination of monoclonal antibodies: anti-CD33-PE, anti-CD34-PerCP, anti-CD45-APC (BD Biosciences). A total of 50,000 cells were acquired on a FACScalibur flow cytometer (BD Biosciences) and analyzed with the Paint-a-Gate program. Using quadruple staining (annexin V/CD33/CD34/CD45), we were able to identify and distinguish the most immature blast cell population ($CD34^+$, $CD45^{dim}$) from the more mature blast cell population ($CD33^+$, $CD34^-$) and normal residual lymphocytes ($CD45^+$, SSC^{lo}). The number of apoptotic cells was measured in each cell population. The percentage of apoptotic events was corrected according to the proportion of apoptotic cells in the control tube (to which no drug was added).

Statistical analysis

The percentage of apoptotic cells referred to the viable fraction of cells, which was calculated using the control tube, for normalization in order to reduce the variability among samples. Induction of apoptosis (annexin V^+

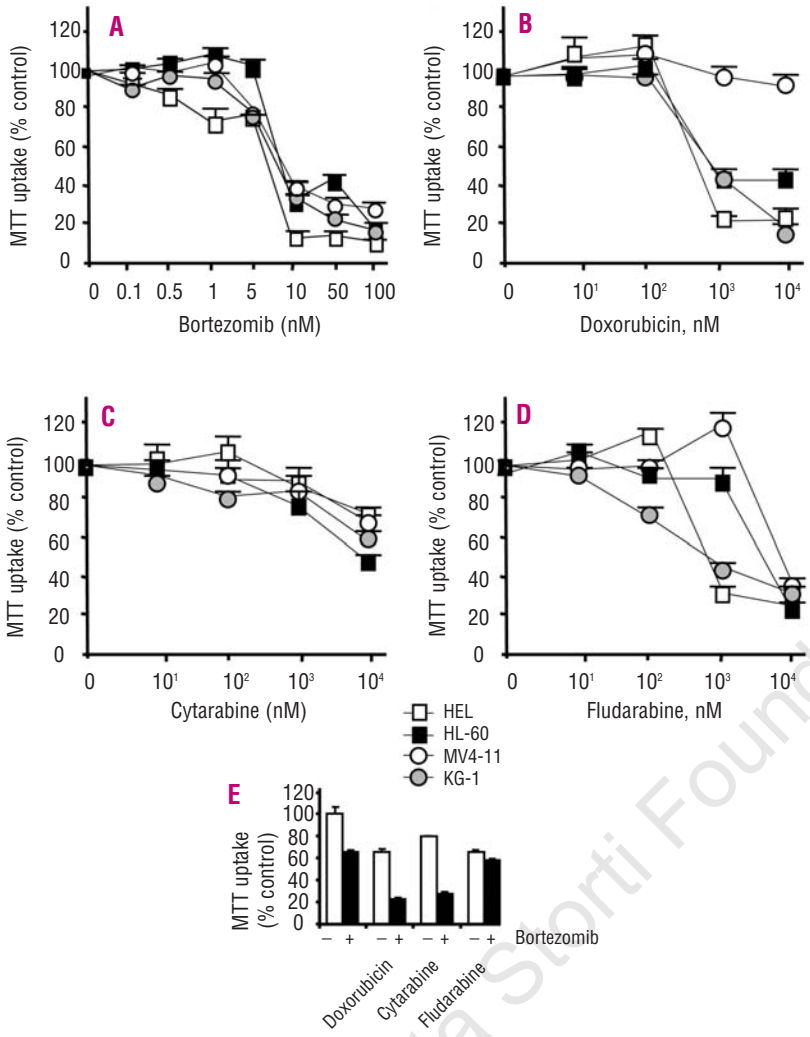


Figure 1. Effect of bortezomib on the proliferation of acute myeloid leukemia cells. MTT uptakes of acute myeloid leukemia cell lines incubated with different doses of bortezomib (A), doxorubicin (B), cytarabine (C), and fludarabine (D). Cells were plated at identical densities in 96-well dishes. Bortezomib, doxorubicin, cytarabine and fludarabine were added at the indicated concentrations. MTT uptake assays were performed 48 hours later as described in the *Design and Methods* section. The average proliferation values of control untreated samples were taken as 100%. Data are represented as the mean ± SD of quadruplicates of an experiment that was repeated at least twice. (E) Bortezomib (15 nM) was combined with doxorubicin (250 nM), cytarabine (250 nM) or fludarabine (250 nM) and 48 hours later, MTT assays were done in HEL cells.

events) was calculated on the total blast cell population, $CD34^+$ blast cells, $CD34^-$ blast cells and normal residual lymphocytes. Statistical analyses were performed using the SPSS 11.0 statistical package.

Results

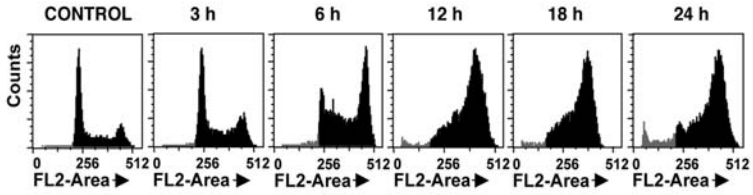
Activity of bortezomib in AML cell lines

To investigate the effect of bortezomib on the growth and survival of AML cells, we first used MTT assays on four different representative AML cell lines. Treatment with increasing doses of bortezomib (0.1-100 nM) for 48 hours potently suppressed MTT uptake (Figure 1A), with IC_{50} values between 5 nM and 10 nM for all four cell lines used. Comparisons of the IC_{50} values of bortezomib with those of other drugs commonly used in AML indicated that bortezomib was clearly more potent than doxorubicin (Figure 1B), cytarabine (Figure 1C) and fludarabine (Figure1D). MV4-11 was resistant to doxorubicin, and the IC_{50} value for doxorubicin for HEL, HL60 and KG-1 were 500 nM, 1 μM and 1 μM, respectively. All cell lines were resistant to pharmacological doses of cytarabine, and cell growth inhibition was only observed with micromolar concentrations. MV4-11 was resistant to fludarabine and growth inhibition appeared only at 10 μM, while the IC_{50} values for HEL and KG-1 were 500 nM, and that for the HL60 cell line, 3 μM.

Bortezomib increases the action of doxorubicin and cytarabine

In order to investigate whether bortezomib could increase the activity of conventional drugs used in AML treatment, HEL cells were treated with several combinations of bortezomib and conventional drugs (doxorubicin, fludarabine and cytarabine). For these experiments, we used suboptimal doses of the compounds, and evaluated their combined effect by MTT absorbance assays, then analyzed the data using the Calcusyn program. As shown in Figure 1E, bortezomib was found to synergistically increase the anti-AML effect of doxorubicin (CI: 0.17) and cytarabine (CI: 0.51). However, bortezomib did not enhance the ability of fludarabine to inhibit the proliferation of HEL cells.

A



	CONTROL	3 h	6 h	12 h	18 h	24 h
G_0/G_1	53.0	50.0	21.0	11.5	5.0	7.5
S	24.0	23.0	35.0	13.5	15.0	7.5
G_2/M	23.0	27.0	44.0	75.0	80.0	85.0

B

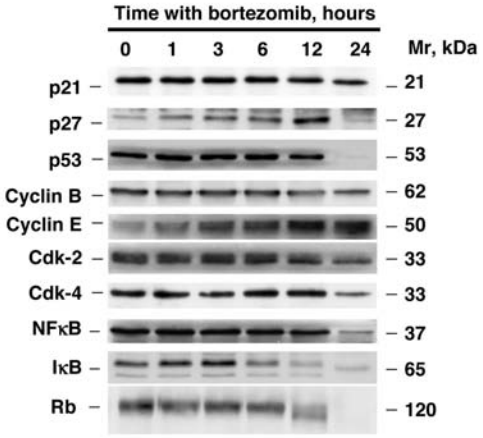


Figure 2. Bortezomib provokes G_2/M cell cycle arrest. (A) HEL cells were incubated with bortezomib (50 nM) for 3, 6, 12, 18 and 24 hours, and examined for cell cycle profiles using propidium iodide staining. The table shows the percentages of cells in the different phases of the cell cycle. (B) HEL cells were treated with bortezomib (50 nM) for the indicated times, and the expression of cell cycle-related proteins was analyzed by western blotting. The position of the Mr marker is shown at the right.

Bortezomib provokes cell cycle arrest in AML cells

We next evaluated whether the reduced MTT uptake observed in AML cell lines treated with bortezomib was due to stimulation of cell death or cell cycle arrest. HEL cells were cultured with bortezomib 50 nM for 0, 3, 6, 12, 18 and 24 hours and then cell cycle profile was analyzed by PI staining. As shown in Figure 2A, bortezomib caused an increase in G_2/M and a marked decrease in G_0/G_1 and S phases in a time-dependent manner. Analyses of several proteins implicated in cell cycle progression indicated that bortezomib decreased the levels of pRb, but rapidly increased the levels of p27 and cyclin E. Bortezomib also decreased the amount of IκB, and provoked a shift in the molecular weight towards a faster migrating form, likely representing dephosphorylated or underphosphorylated IκB. No major changes in p21, cyclin B, CDK2, CDK4, NFκB or p53 levels were observed, except at longer incubation times after which a decrease in most of these proteins was detected, probably due to massive protein degradation.

Bortezomib causes apoptosis in AML cells

We then investigated whether bortezomib caused apoptotic cell death. A significant, time-dependent induction of annexin V-positive cells was observed in HEL-cells after treatment with bortezomib (Figure 3A). Treatment with bortezomib also caused internucleosomal DNA fragmentation indicative of cell death (Figure 3B). As mitochondria appear to be organelles critically involved in the triggering of apoptotic cell death, we explored whether bortezomib altered mitochondrial membrane potential (Ψ_m). Analysis of Ψ_m by the use of the mitochondrial membrane potential probe $DioC_6(3)$ showed a decrease in Ψ_m in cells treated with bortezomib, suggesting that mitochondria were indeed affected in HEL cells treated with this compound (Figure 3C).

We next evaluated the biochemical parameters that are affected upon apoptotic cell death. Apoptosis triggered by bortezomib provoked cleavage of PARP, caspase-3, caspase-8 and caspase-9, with the generation of active low M_r cleaved fragments, (Figure 4A), suggesting that bortezomib exerts its effect by activating both the intrinsic and extrinsic caspase pathways. To investigate the importance of caspases in the anti-leukemic action of bortezomib, the ability of the caspase-3 inhibitor Z-VAD-FMK to rescue from cells from bortezomib-induced death was evaluated. HEL cells were preincubated for 60 minutes with Z-VAD-FMK, then bortezomib was added, and the incubation continued for 24 hours. As shown in Figure 4B, preincubation with the caspase-3 inhibitor blocked bortezomib-induced cell death. These results indicate that bortezomib activated the caspase-dependent apoptotic pathway, and that this activation was the main executor of cell death caused by this compound in

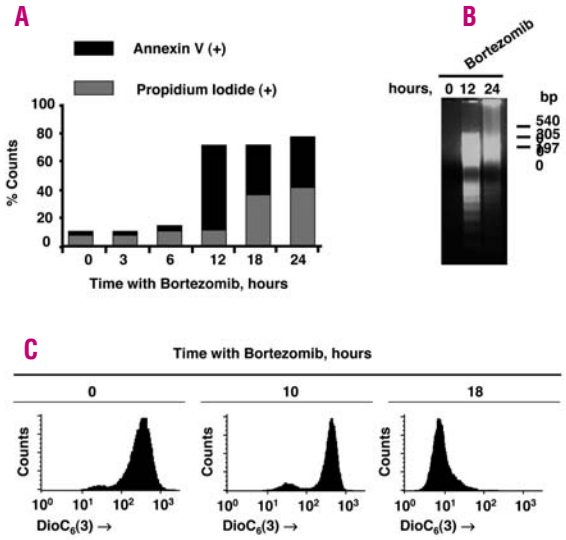


Figure 3. Bortezomib causes apoptotic cell death in an acute myeloid leukemia cell line. (A) Time-course of the effect of bortezomib on HEL cells. Cells were plated in 6-well plates, treated with bortezomib (10 nM), and 18 hours later, stained with annexin V-FITC and propidium iodide. (B) Bortezomib provokes internucleosomal DNA fragmentation, HEL cells were treated with bortezomib for the indicated times and DNA was isolated and analyzed by agarose gel electrophoresis. The position of the Mr markers is shown at the right. (C) Bortezomib induces $\Delta\Psi$m disruption. HEL cells were treated with bortezomib (50 nM), and the $\Delta\Psi$m analyses performed with [$DiOC_6^{(3)}$] by flow cytometry.

HEL cells. Loss of Ψ_m often reflects increases in mitochondrial outer membrane permeability. Bcl-2 family members act as important regulators of mitochondrial outer membrane permeability. Western blot analyses indicated that bortezomib down-regulated the Bcl-2 family member BCLX, but not BCL2, and slightly increased MCL1 levels within the first 12 hours of treatment. (Figure 4C). The increase in mitochondrial outer membrane permeability also favors the release of AIF, a mediator of caspase-independent cell death.[43] Subcellular fractionation of HEL cells treated for 18 hours with bortezomib showed that this drug caused a translocation of AIF from the mitochondrial to the cytosolic fraction after 18 hours of treatment (Figure 4D).

Bortezomib induces apoptosis in CD34⁺ and CD34⁻ cell populations from fresh AML samples

We used a multiparametric flow cytometry method to discriminate blast cells from normal residual lymphocytes, and, more interestingly, to discriminate between immature and more mature leukemic cell populations. The average percentage of apoptosis induced by bortezomib in the total blast cell of the whole series of 28 patients was 48±22% (mean±SD). In 14 samples, bortezomib induced apoptosis in ≥50% of leukemic cells. In 12 samples, between 50 and 20% of leukemic cells became apoptotic, and only in two samples was the level of apoptotic leukemic cells less than 20%. The apoptotic activity of bortezomib on CD34⁺ blast cells was similar to that observed in CD34⁻ blast cells (48±22% versus 57±27%, p=0.86; Figure 5A)

Bortezomib induces apoptosis more efficiently than doxorubicin and cytarabine in AML samples from patients

In a set of seven samples from AML patients, cytarabine (mean apoptosis induction±SD: 10±6%) was shown to be less cytotoxic than either bortezomib or doxorubicin (37±16% and 21±14%, respectively). For this reason, we decided to continue our experiments using only doxorubicin as the reference drug. When the effect of bortezomib was compared with that induced by doxorubicin, using both drugs at their optimal con-

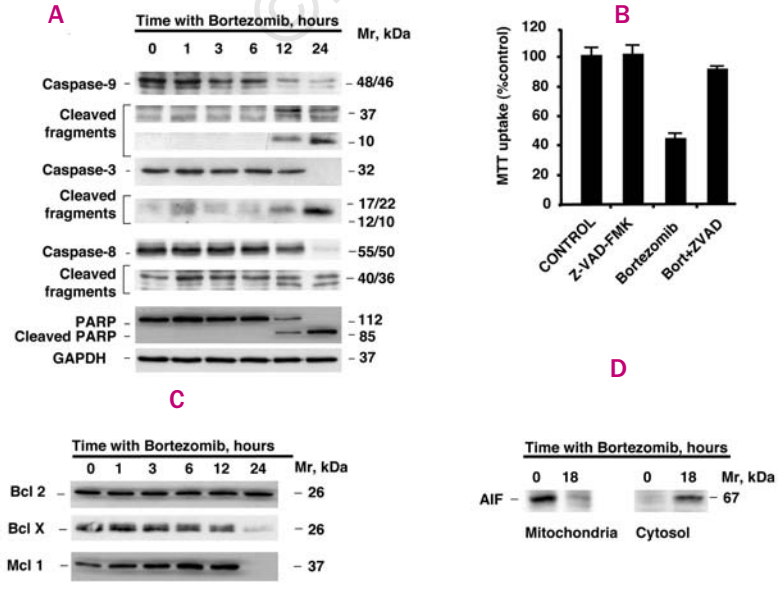


Figure 4. Effect of bortezomib on apoptotic pathways. (A) HEL cells were treated with bortezomib (50 nM) for the indicated times, and the expression of PARP, caspase-3, caspase-8 and caspase-9 proteins analyzed by western blotting. GAPDH was used as the loading control. (B) Effect of the pan-caspase inhibitor, Z-VAD-FMK, on bortezomib-induced cell death. HEL cells were plated and pre-treated, where indicated, with Z-VAD-FMK (20 μM) for 60 minutes. Bortezomib (50 nM) was added to the corresponding samples, and the experiment continued for 24 hours. MTT absorbance tests were carried out as described above. (C) The action of bortezomib on the levels of Bcl-2, Bcl-X and MCL-1 proteins. HEL cells were treated with bortezomib (50 nM) for the indicated times, and cell extracts were used for western blotting with anti-Bcl-2, anti- Bcl-X or anti-MCL-1 antibodies. (D) HEL cells were treated for the indicated times with bortezomib (50 nM), and the subcellular distribution of AIF in mitochondrial and cytosolic fractions was analyzed by western blotting.

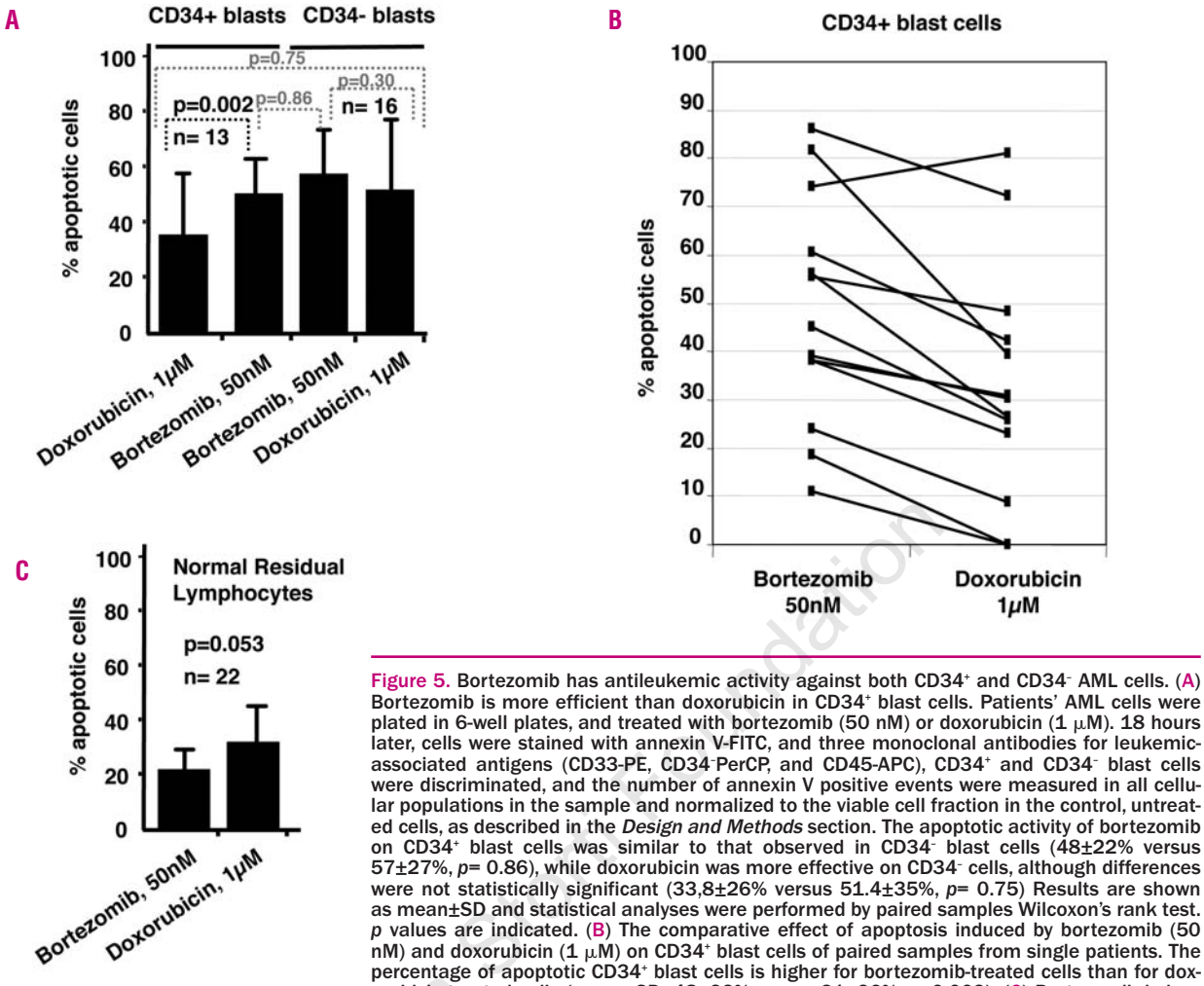


Figure 5. Bortezomib has antileukemic activity against both CD34⁺ and CD34⁻ AML cells. (A) Bortezomib is more efficient than doxorubicin in CD34⁺ blast cells. Patients' AML cells were plated in 6-well plates, and treated with bortezomib (50 nM) or doxorubicin (1 μM). 18 hours later, cells were stained with annexin V-FITC, and three monoclonal antibodies for leukemic-associated antigens (CD33-PE, CD34-PerCP, and CD45-APC), CD34⁺ and CD34⁻ blast cells were discriminated, and the number of annexin V positive events were measured in all cellular populations in the sample and normalized to the viable cell fraction in the control, untreated cells, as described in the *Design and Methods* section. The apoptotic activity of bortezomib on CD34⁺ blast cells was similar to that observed in CD34⁻ blast cells (48±22% versus 57±27%, p= 0.86), while doxorubicin was more effective on CD34⁻ cells, although differences were not statistically significant (33,8±26% versus 51.4±35%, p= 0.75) Results are shown as mean±SD and statistical analyses were performed by paired samples Wilcoxon's rank test. p values are indicated. (B) The comparative effect of apoptosis induced by bortezomib (50 nM) and doxorubicin (1 μM) on CD34⁺ blast cells of paired samples from single patients. The percentage of apoptotic CD34⁺ blast cells is higher for bortezomib-treated cells than for doxorubicin-treated cells (mean±SD: 48±22% versus 31±26%, p=0.002). (C) Bortezomib is less toxic to normal residual lymphocytes. In 22 samples normal residual lymphocytes were identified and the percentage of apoptotic events induced by either bortezomib or doxorubicin was measured (18±12% versus 27±22%, p=0.053).

centrations, we observed that doxorubicin was slightly less cytotoxic on the total blast cell population (39±33% versus 48±22%, p=0.30 *data not shown*). More interesting was the difference between the two compounds' activities on CD34⁺ and CD34⁻ blast cell subsets. Bortezomib was significantly more active than doxorubicin in the immature CD34⁺ cells (48±22% versus 31±26%, p=0.002). (Figure 5A) In contrast, the activity of the two drugs on the CD34⁻ subset did not differ significantly (57±27% versus 47±35%, p=0.17). Considering paired samples from single cases, we observed that the effect of bortezomib on CD34⁺ cells was greater than that of doxorubicin, suggesting that bortezomib may overcome drug resistance associated with the immature phenotype (Figure 5B).

Bortezomib is less toxic than doxorubicin to normal residual lymphocytes

Within the same samples we analyzed the toxicity to residual normal lymphocytes. Our results demonstrate that bortezomib is highly specific for leukemic cells, since the toxicity to residual normal lymphocytes was low (18±12%). Moreover, this toxicity was lower than that observed for doxorubicin (Figure 5C). Finally, we analyzed the toxicity of bortezomib against normal CD34⁺ cells from four normal bone marrow samples, and found that bortezomib is highly specific for CD34 leukemic cells, since toxicity to normal CD34⁺ cells was low (5±5.2%, *data not shown*).

Discussion

Nearly 80% of patients with AML achieve a complete remission with induction chemotherapy. However, a high proportion relapse, and eventually die of their disease.[44,45] Recent studies have shown that proteasome inhibitors represent a valuable novel anticancer therapy. These agents inhibit the degradation of multiubiquitinated target proteins, i.e., cell cycle regu-

latory proteins such as cyclins and cyclin-dependent kinase inhibitors, and regulate cell cycle progression.[46] Bortezomib is the first proteasome inhibitor that has been introduced into clinical practice for the treatment of relapsed multiple myeloma,[2,28,47,48] and active clinical investigation is ongoing in other malignancies.[49-55] In this study we provide the framework for more intensive clinical investigation of bortezomib in AML. MTT uptake experiments on AML cell lines, sensitive and resistant to conventional chemotherapeutic agents, indicate that bortezomib is efficient at concentrations in the low nanomolar range, within pharmacologically achievable doses. Moreover, the *in vitro* activity of bortezomib appears to be clearly superior to that of conventional agents currently used for AML treatment such as doxorubicin, cytarabine and fludarabine. In addition, bortezomib showed a synergistic effect with doxorubicin and cytarabine against AML cells. This may be important since both conventional agents represent the backbone of AML treatment. Our studies on the mechanism of action of bortezomib indicate that this compound affects several pathways involved in the control of cell cycle progression and apoptosis. In HEL cells, bortezomib caused a progressive accumulation of cells in G_2/M with a decrease in the percentages of cells in G_0/G_1 and S phases. Induction of G_2/M arrest has previously been shown to occur in multiple myeloma,[56] non-small cell lung cancer,[10,11] and ovarian cancer[17] cells treated with bortezomib. Western blotting analyses indicated changes in the amounts of pRb, p27 and cyclin E. Furthermore, increased p27 levels have been reported in multiple myeloma cells[6] as well as in ovarian cancer cells[17] following treatment with bortezomib.

In addition to its effect on the cell cycle, bortezomib provoked cell death, as shown by annexin V positivity, loss of mitochondrial membrane potential, and DNA fragmentation. Analyses of the effect of bortezomib on AML cells indicated that this compound caused cleavage of the initiator caspases -8 and -9, effector caspase-3; and PARP. The cleavage of caspase-3 and PARP is consistent with results obtained in other types of tumor cells[6,10,11,17] treated with comparable exposure to bortezomib. Moreover, pretreatment with Z-VAD-FMK blocked bortezomib-induced cell death suggesting that the caspase-dependent apoptotic pathway is the main executor of cell death caused by this compound. In line with our observations, Hideshima *et al.*[4] also showed that caspase inhibitors were able to prevent bortezomib-induced apoptosis in multiple myeloma cells.

The progressive loss of mitochondrial membrane potential reflected an increase in the permeability of the outer mitochondrial membrane, which allowed release of pro-apoptotic proteins, such as AIF. The release of AIF is facilitated by decreased levels of Bcl-2 family members, which have been shown to regulate mitochondrial outer membrane permeability.[43,57] Western blot analyses indicated that bortezomib caused translocation of AIF from the mitochondrial to cytosolic fraction and down-regulated the antiapoptotic Bcl-2 family member Bcl-X. To the best of our knowledge, the contribution of AIF to bortezomib-induced cell death has not been previously reported. As AIF has been involved in caspase-independent cell death, our data both suggest a dual apoptotic mechanism induced by bortezomib in AML cells, involving both caspase-dependent and -independent pathways.

Finally, we had the opportunity to analyze the effect of bortezomib (compared to doxorubicin) on fresh cells obtained from a cohort of AML patients. As mentioned in the *Design and Methods* section, by using an appropriate triple antigen combination plus simultaneous staining with annexin V we were able to separate the blast cell population from the residual normal hematopoietic cells and to assess the proportion of apoptotic cells induced by the drug in each cell population. Moreover, even in cases with co-existence of $CD34^+$ and $CD34^-$ blast cells, our immunophenotypic approach clearly discriminated these two blast cell populations and enabled subsequent measurement of apoptosis induced in each subset. For this study, we used doxorubicin as a reference drug since it is a cornerstone of the treatment of AML. As observed in the cell lines, bortezomib showed greater antitumor activity than doxorubicin on patients' fresh blast cells, although the difference did not reach statistical significance. Interestingly, however, differences emerged when the $CD34^+$ and $CD34^-$ blast cell subsets were analyzed separately. Thus, while bortezomib had a similar pro-apoptotic effect on both cell populations, doxorubicin showed greater activity on the more mature cells ($CD34^-$). Moreover, bortezomib was significantly more active than doxorubicin on immature $CD34^+$ blast cells. This finding supports the belief that bortezomib could overcome the drug resistance associated with the immature phenotype.[34] Moreover, since the cell subset most commonly responsible for relapses is the $CD34^+$ subset, bortezomib may represent an ideal drug for the eradication of minimal residual disease, which is currently the major therapeutic challenge in the treatment of AML. Finally, we observed that the antileukemic effect of bortezomib was selective, since the toxicity to normal residual lymphocytes was low. Similarly, proteasome inhibition specifically provoked apoptosis in $CD34^+/CD38^-/CD123^+$ cells (leukemic stem cells) without significant toxicity to normal hematopoietic stem cells.[58,59]

In summary our study indicates that bortezomib has marked *in vitro* activity in both AML cell lines and fresh blast cells obtained from patients. Moreover, the similar antileukemic effect of bortezomib on $CD34^+$ and $CD34^-$ AML cells suggests that this agent may overcome the drug resistance associated with the immature $CD34^+$ phenotype. Collectively, these data open new pathways for the clinical development of bortezomib in the treatment of AML, and may add this already

approved drug to the therapeutic armamentarium against AML. For more than 30 years very few novel agents have been introduced to treat AML,[60] and yet many patients continue to relapse due to the persistence of residual resistant leukemic cells. Our data clearly support that further clinical investigation of bortezomib, particularly in combination with conventional agents, is warranted.

Authorship and Disclosures

EC: performed and designed the research, analyzed the data and wrote the paper; SÁ-F, JM-S, MG, EMO and JCM: performed research; PM: performed research and wrote the paper; MBV: designed the research, contributed analytical tools, and analyzed the data; AP and JFSM: designed the research and wrote the paper.

The authors reported no potential conflicts of interest.

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