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Antitumor Activity of Bortezomib Alone and in Combination with Trail in Human Acute Myeloid Leukemia

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Key Words

Acute myeloid leukemia · Apoptosis · Bortezomib · TRAIL

Abstract

Acute myeloid leukemia (AML) is a malignant disease characterized by abnormal proliferation of clonal precursor cells. Although different strategies have been adopted to obtain complete remission, the disease actually progresses in about 60–70% of patients. Bortezomib has been used in multiple myeloma and other lymphoid malignancies because of its antitumor activity. Here we examined the sensitivity of bone marrow cells from AML patients to bortezomib alone or in combination with TRAIL, a member of the TNF family that induces apoptosis in tumor cells while sparing normal cells (34 patients: 25 newly diagnosed, 4 relapsed, 5 refractory). Bortezomib induced cell death in blasts from each patient sample. The cytotoxic effect was dose- and time-dependent (concentration from 0.001 to 10 μM for 24 and 48 h) and was associated with a downregulation of Bcl-xL and Mcl-1, an upregulation of TRAIL-R1, TRAIL-R2, p21, activation of executioner caspases and a loss of the mitochondrial membrane potential. Moreover, low doses of bortezomib primed TRAIL-resistant AML cells for enhanced TRAIL-mediated killing. These results suggest that a combination of proteasome inhibitors and TRAIL could be effective for treating AML patients, even patients who are refractory to conventional chemotherapy.

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Introduction

Acute myeloid leukemia (AML) is a malignant neoplasm of hematopoietic cells characterized by abnormal proliferation of clonal neoplastic myeloid precursor cells and an impairment of normal hematopoiesis. Although significant advances in understanding the molecular pathogenesis of AML have been made in recent decades, and more specifically targeted agents have been developed for treating AML, only about 20–30% of patients actually enjoy long-term disease survival and the outcome of AML is frequently fatal [1, 2]. Such a severe outcome demands the development of novel treatment strategies against this disease.

Because of the pivotal role of proteasome inhibitors in apoptosis, bortezomib, either alone or in combination with other apoptosis-inducing compounds, seems to have potential for treating AML. In particular, the proteasome regulates the levels of cyclins and cyclin-dependent kinase inhibitors, and of pro- and antiapoptotic proteins such as Bcl-2 family members. It also controls the levels of tumor suppressor genes such as p53, oncogenes, and the activities of signal transduction pathways such as the NF-κB pathway by degrading the inhibitor of nuclear factor-κB (IκB) [3]. Proteasome inhibitors can induce misregulation of numerous proteins leading to cell death, and this occurs preferentially in transformed cells. Recently, it has been shown in several tumor models that proteasome inhibitors can block proliferation and induce apoptosis in tumor cells both in vitro and in vivo; therefore, molecules such as bortezomib represent a potentially powerful anticancer therapy [4]. These compounds can also induce tumor regression, alone or in combination with cytotoxic drugs, in xenograft murine models of human lymphoma, myeloma [5] and different carcinomas [6–12]. The antitumor effect against leukemic cells of a combination of proteasome inhibitors with other drugs has already been described by Guzman et al. [13] and Tan [14] on T-ALL and primary AML cells. Remarkably, bortezomib has recently been approved for therapeutic use in progressive multiple myeloma [15], and large-scale phase I [16, 17] and II programs for various neoplastic diseases are currently in progress [18]. Since modulation of proteasome function can enhance the efficacy of chemotherapeutics, proteasome inhibition by bortezomib is a rational target for chemosensitization.

Another new candidate for AML treatment is tumor necrosis factor-related apoptosis-inducing ligand or Apo2 ligand (TRAIL/Apo2L). TRAIL is a member of the TNF family that has recently been proposed as a novel anticancer agent because of its ability to induce apoptosis in malignant cells while sparing normal cells. TRAIL acts by interacting with its surface death receptors, TRAIL-R1 (also known as DR4) and TRAIL-R2 (also known as DR5), which contain a conserved death domain (DD) motif. After binding to TRAIL ligand, these receptors trimerize and cluster, leading to the formation of the death-inducing signaling complex and recruitment of the adaptor molecule FADD, which in turn recruits caspase-8 and activates apoptotic signaling. While TRAIL/Apo2L is not toxic to most normal human cells in vitro or to TRAIL-treated animals, it induces apoptosis in tumor cells of diverse origins both in vitro and in vivo and in various in vivo tumor models [19, 20]. Although previous studies have shown that AML blasts are resistant to TRAIL, recent reports indicate that bortezomib sensitizes cancer cells to this ligand by inducing the expression of TRAIL receptors in solid tumor cell lines [21–24].

In this study we have examined the effects of bortezomib alone or in combination with TRAIL on AML primary cells. We found that bortezomib has a consistent cytotoxic effect on AML cells, higher than that of standard chemotherapeutic agents currently used to treat AML patients. Moreover, this cytotoxic effect increases significantly when bortezomib is combined with TRAIL, probably because of the modulation of TRAIL receptors after proteasome inhibition.

Materials and Methods

Primary Blast Cells

Fresh leukemic blasts from 34 AML patients diagnosed and treated at the Division of Hematology, Ospedale Ferrarotto, University of Catania were obtained after informed consent during routine examination. Bone marrow (BM) cells were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were maintained in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, Calif., USA). Cells were kept in a 5% CO_2 atmosphere at a density of 5×10^5 cells/ml. Blasts constituted >70% for all samples (from 70 to 100%). A BM biopsy for histological diagnosis was obtained from all AML patients at presentation.

Reagents

Antineoplastic agents were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and resuspended in DMSO (etoposide) or in water (cytarabine and daunorubicin). Bortezomib (also known as PS-341) was purchased from Millennium Pharmaceuticals (Cambridge, Mass., USA). Bortezomib (0.1 μM), cytarabine (1 μg/ml), doxorubicin (5 μM) and etoposide (0.5 μM) were used in vitro at doses compatible with the plasma levels reached in vivo during cancer treatment. Human recombinant LZ-TRAIL was purchased from Alexis (Lausen, Switzerland) and the caspase inhibitor z-VAD-fmk from Bachem.

Cell Viability Assay

Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, Wisc., USA) according to the manufacturer's instructions. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), to a colored formazan product. The formazan is measured by the absorbance at 490 nm, which is directly proportional to the number of living cells. Briefly, cells were seeded into 3 wells of 96-well plates at 5×10^3 cells/200 μl/well and treated with different combinations of the chemotherapeutic drugs tested. After 24 and 48 h of incubation the culture medium was removed and the cells were washed with PBS (pH 7.4); 100 μl of fresh

culture medium without drugs and 20 μl of MTS were added to each well and the cells were incubated for 3 h. The plates were read on a Microplate Reader (Synergy HT, BIO-TEK). Survival was expressed as the percentage of viable cells in the treated sample relative to the untreated control cells.

HALO/COMET Assay

Genomic DNA fragmentation was detected by the HALO/COMET assay according to Godard et al. [25]. Cells were collected by scraping, washed in PBS, and resuspended as 10×10^5 cells/10 μl of PBS. The samples were analyzed in duplicate: one for the HALO assay and one for the COMET assay. The first group of samples was subjected to denaturation with high pH buffer (300 mM NaOH, 1 mM EDTA, pH 12.8) for 20 min followed by neutralization (0.4 M Tris-HCl, pH 7.5) for 5 min. Staining with ethidium bromide (2 μg/ml) and analysis with a Leica fluorescence microscope were performed. The second series of samples, after denaturation, was electrophoresed and treated as well as the HALO samples. The run was performed in the same denaturation buffer for 30 min at 20 V on ice bath under semidark conditions. For each slide in the two types of assays, 50 images (corresponding to 50 cells) were analyzed.

Flow Cytometry

1×10^5 cells were used for flow cytometry. Cells were washed with cold PBS containing 1% bovine serum albumin (BSA) and incubated for 1 h at 4°C with control or specific primary antibodies: goat anti-human TRAIL-R1 and TRAIL-R2 (both 1:50, R&D Systems, Minneapolis, Minn., USA). After washing, the cells were incubated for 40 min at 4°C with phycoerythrin-conjugated anti-goat secondary antibodies (1:100, Jackson Laboratories, West Grove, Pa., USA). Labeled cells were washed twice with PBS/BSA and the fluorescence intensity was evaluated by a FACScan (Beckman Coulter, Fullerton, Calif., USA).

Analysis of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) of intact cells was measured by flow cytometry with the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Molecular Probes, Leiden, The Netherlands). JC-1 was added directly to the cell culture medium (1 μM final concentration) including cells (0.5×10^6/ml) and incubated for 10 min at 22°C. The medium was then replaced with PBS, and the cells were quantitated for J-aggregate fluorescence intensity by using a FACScan flow cytometer (Beckman Coulter).

Immunostaining Procedure

Immunofluorescence staining was performed on cells seeded on glass slides. Cells were washed in PIPES buffer (80 mM PIPES pH 6.8, 5 mM EGTA and 2 mM MgCl₂) and fixed with 4% paraformaldehyde for 10 min. This step was followed by block-permeabilization in PBS containing 0.2% BSA and 0.1% Triton X-100 for 10 min, followed by exposure to PBS containing 1% BSA for 1 h in order to reduce nonspecific staining. The cells were then incubated with rabbit anti-human NF-κB/p65 (1:100, Santa Cruz) or goat anti-human TRAIL-R1 or TRAIL-R2 (both 1:50, R&D Systems) for 1 h at room temperature. After three washes in PBS the cells were incubated with rhodamine-conjugated mouse anti-rabbit immunoglobulins (1:500 Jackson Laboratories) or donkey anti-goat immunoglobulins (1:400, Jackson Laboratories). Nuclei

were stained with PI (Sigma). Immunohistochemical staining was performed on 2-μm-thick paraffin-embedded AML BM and colon cancer sections (used as positive control). After a deparaffination-hydration step, sections were permeabilized with PBS containing 0.4% Triton X-100 for 30 min and nonspecific staining was blocked with PBS containing 5% BSA for 30 min. Specimens were incubated for 1 h with goat anti-human TRAIL-R1 and TRAIL-R2 (both 1:50, R&D Systems). Washed sections were treated with biotinylated anti-goat immunoglobulins and then incubated in streptavidin conjugated with peroxidase (Dako, Carpintera, Calif., USA). Staining was detected using diaminobenzidine (DAB). Sections were counterstained with hematoxylin, then dehydrated and mounted in permanent nonaqueous mounting medium.

Caspase Assay

Apo-ONE Homogeneous Caspase-3/7 buffer and substrate (Promega) were mixed and then added to cultured cells, untreated or treated with bortezomib and TRAIL. Sequential cleavage of the substrate by caspase-3/7 activity in the sample yields an intensely fluorescent product, the amount of which is proportional to the amount of caspase-3/7 activity in the sample. The fluorescence was measured spectrophotometrically at 485 nm.

Western Blotting

Cell pellets were washed twice with cold PBS and lysed on ice for 10 min in JS buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 5 mM EGTA, 1% glycerol, 1% Triton X-100, 1.5 mM MgCl₂) supplemented with protease inhibitors: 0.1 mg/ml phenylmethylsulfonyl fluoride and 1 μl/ml each of aprotinin, leupeptin and pepstatin. After centrifugation at 2,000 rpm for 15 min at 4°C to remove cell debris, the protein content was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif., USA). After denaturation, aliquots of 30 μl total protein were loaded on to 10 or 12% SDS-polyacrylamide gel. The gels were electroblotted onto nitrocellulose membranes (Hybond C-extra, Amersham Biosciences, Little Chalfont, UK). Filters were blocked for 1 h in 5% nonfat dry milk in TBS-T (2.5 mM Tris-HCl, 15 mM NaCl, 0.05% Tween 20), then incubated for 1 h with the following primary antibodies: mouse monoclonals anti-Bcl-xL (1:100, Santa Cruz Biotechnology), anti-Smac (1:1,000, Santa Cruz Biotechnology), anti-actin (1:10,000, Sigma), anti-caspase-7 (1:1,000, Pharmingen, San Diego, Calif., USA), hamster anti-Bcl2 (1:100, Pharmingen, San Diego, Calif., USA), and the polyclonals goat anti-IAP1 (1:50, Santa Cruz Biotechnology), rabbit anti-P21 (1:500; Santa Cruz Biotechnology), rat anti-FLIP (1:15, Alexis, San Diego, Calif., USA), anti-Mcl-1 (1:500; Santa Cruz Biotechnology), anti-caspase-3 (1:500, Santa Cruz Biotechnology). Filters were then rinsed with TBS-T buffer and incubated for 1 h with the corresponding secondary antibody conjugated with peroxidase (Amersham Biosciences). After washing, proteins were detected by the enhanced chemiluminescent method (ECL Plus; Amersham Biosciences).

Statistical Analysis

A paired t test was used to analyze the statistical significance of the results. Values of $p < 0.05$ were considered significant. Data are presented as mean values ± SD of the mean.

Results

Samples

A total of 34 samples from patients with AML at diagnosis or resistant/refractory AML were examined. Patients' characteristics such as FAB classification, cytogenetic features, blast percentage and prior therapies are detailed in table 1.

AML Blasts Exhibit a Greater Dose-Dependent Sensitivity to Bortezomib than to Conventional Chemotherapy

Since previous studies on multiple myeloma have shown that the mean concentration of bortezomib in the plasma after a standard dose (1.3 mg/m^2) reaches 1.8 μM (509 ng/ml), with a range of 0.3–3.4 μM (109–1,300 ng/ml) [26], we evaluated the sensitivity to bortezomib-mediated cell death in AML blasts derived from the 34 patients using a low concentration of bortezomib (0.1 μM). Cell viability was greatly reduced in all AML blasts without correlation with the status of the patients from whom the blast cells were derived (at diagnosis, resistant or relapsed) (fig. 1a). In addition, most AML cells showed consistent reduction in cell viability in a dose- and time-dependent manner (fig. 1b). COMET assay evidenced an increased COMET score in cells from patients treated for 24 h with 0.1 μM bortezomib indicating that bortezomib is able to induce apoptosis in AML blasts (fig. 1c). Furthermore, we examined MMP on 3 samples from AML patients at diagnosis and we observed loss of MMP after bortezomib treatment (fig. 1d). These biologically active concentrations are fully compatible with those achieved in vivo in the clinical setting, suggesting that clinical use of bortezomib for treating AML may be possible. In a recent report [27] AML blasts were subdivided into two subgroups according to their sensitivity to bortezomib [low-sensitivity group with <50% dying cells and high-sensitivity group (HSG) with >50% dying cells]. The sensitivity to bortezomib-mediated apoptosis correlated with the differentiation properties of AML blasts: the majority of the HSG cases corresponded to the M4 and M5 FAB subtypes, while the majority of the low-sensitivity group corresponded to M1 and M2 FAB subtypes. Similarly, we have found that the majority (8/34) of AML blasts that belong to HSG cases have morphologic and immunophenotypic features of the M4 FAB subtype while neither leukocyte cell count, nor specific karyotype or mutations, such as FLT3, are correlated with bortezomib sensitivity.

Since cytarabine, doxorubicin and etoposide are currently used for treating AML, we compared the in vitro efficacy of high concentrations of these cytotoxic drugs (comparable with the highest levels of achievable plasma peaks) with low doses of bortezomib. AML cells were exposed to bortezomib (0.1 μM), cytarabine (1 μg/ml), doxorubicin (5 μM), and etoposide (0.5 μM) for 48 h. Cell viability was assessed by trypan blue exclusion (data not shown) and MTS assays. After 48 h treatment, most AML cells exposed to bortezomib showed reduction in cell viability, while all the other drugs showed only minor activity (fig. 1e). Many AML cells from relapsed or refractory patients were resistant to all the compounds tested with the exception of bortezomib. These results suggest that AML blasts have considerable sensitivity to bortezomib, even when they are resistant to high doses of conventional chemotherapeutic drugs. AML cells are characterized by constitutive abnormal activation of NF-κB and this activation is commonly associated with resistance to spontaneous apoptosis, thus favoring the survival and growth of AML blasts [28]. NF-κB has been shown to be constitutively active only when it resides in the nucleus. Since proteasome inhibition interferes with NF-κB status and we have demonstrated a high sensitivity of blast cells to bortezomib-induced cell death, we ex-

Fig. 1. Cytotoxic effect of bortezomib on AML blasts from 34 patients (at diagnosis, resistant or relapsed). **a** AML blasts were exposed to 0.1 μM bortezomib for 48 h. Cell viability was measured by MTS and is expressed as percentage of the corresponding untreated sample. The results represent the mean average of 3 independent experiments carried on each AML sample of 34 patients. **b** AML blasts were incubated in vitro for 24 (□) and 48 h (■) in the presence of increasing concentrations (0.01–10 μM) of bortezomib. Cell viability was measured by MTS. The results represent the mean ± SD of 34 independent experiments for each AML sample. **c** AML blasts from newly diagnosed patients were stained by the COMET assay after exposure to 0.1 μM bortezomib for 24 h. Two representative experiments are shown. **d** Flow cytometry analysis showed loss of MMP in blasts from 3 AML patients at diagnosis after 0.1 μM bortezomib treatment for 24 h. Mitochondrial depolarization is indicated by the increase in green fluorescence after bortezomib treatment (red). One of three experiments is shown. **e** Percentage of cell viability of myeloid blasts treated for 48 h with different compounds. AML cells were treated for 48 h with bortezomib (Bort; 0.1 μM), cytarabine (Ara; 1 μg/ml), doxorubicin (Doxo; 5 μM) or etoposide (VP-16; 0.5 μM). The percentage of apoptotic cells was determined using the MTS assay. The results show the mean ± SD of four independent experiments. **f** Immunofluorescence analysis of 10 AML samples untreated (control) or treated with 0.1 μM bortezomib. Cells were stained with anti-NF-κB (red). DAPI staining (blue) indicates the position of the nuclei in the corresponding view. Original magnification ×20. Two representative experiments of 10 AML patients at diagnosis are shown.
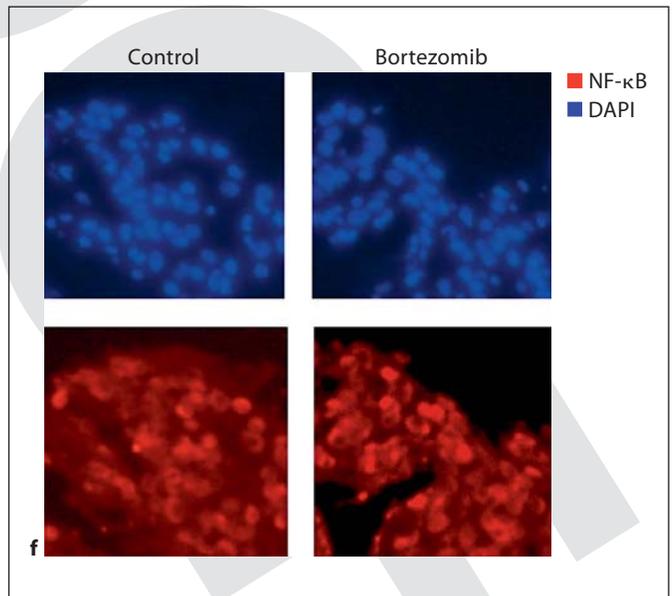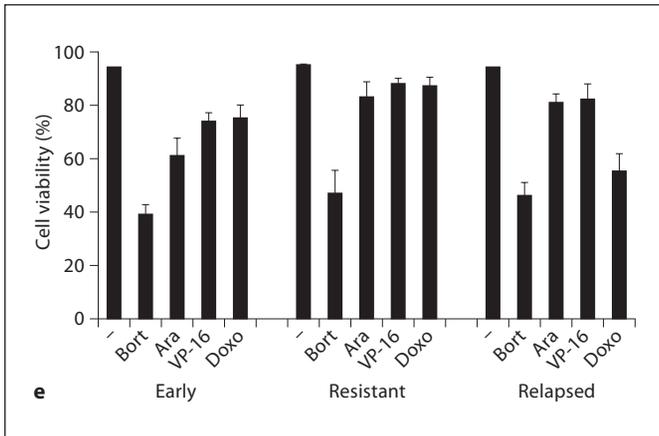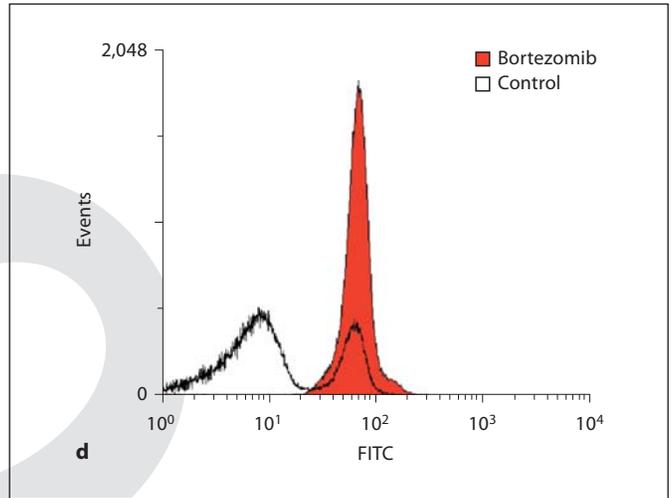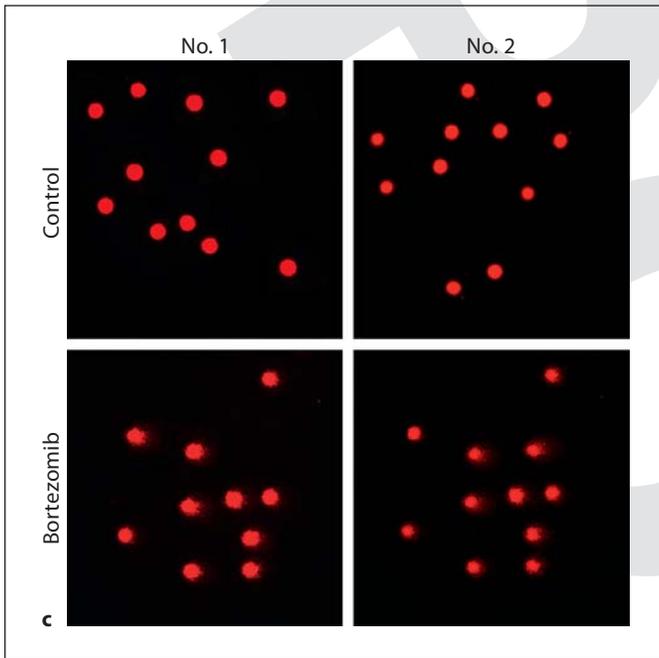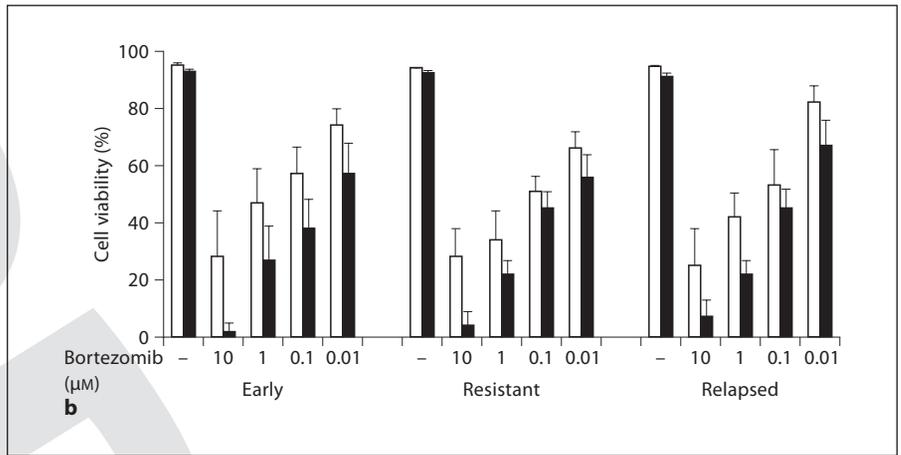
Table 1. Sample characteristics

Pa-tient	FAB type	Blasts %	Cytogenetics	Status
1	M2	90	46, XX	n.d.
2	M1	90	47, XY, + 15	first relapse
3	M2	50	not determined	n.d.
4	therapy-related/AML	80	not determined	n.d.
5	M4	60	46, XY, i (16)(q11)	n.d.
6	MDS/AML	60	not determined	n.d.
7	M2	42	46, XY	n.d.
8	M4 Eo	70–80	not determined	n.d.
9	MDS/AML	70	46, XY	n.d.
10	MDS/AML	20	not determined	n.d.
11	M4	90	46, XX, inv (16)	n.d.
12	MDS/AML	67	45, XX, der (3)t (3;?), del (5); -7	n.d.
13	MDS/AML	80	not determined	n.d.
14	MO	50	not determined	n.d.
15	M2	90	47, XY, +4	n.d.
16	M4	40	46, XY, t (6;9)	n.d.
17	M4	90	not determined	n.d.
18	M4	99	46, XY	n.d.
19	M2	78	47, XY, t (8;21); +13	n.d.
20	M1	90	not determined	n.d.
21	M2	98	not determined	n.d.
22	M2	60	46, XX	n.d.
23	M4	50	46, XY	n.d.
24	M0	90	not determined	n.d.
25	M4	95	46, XX	n.d.
26	M2	73	46, XY, del (1), del (3), −7, +8; 46 XY dic (1;1)	refractory
27	M6	76	not determined	refractory
28	M2	90	not determined	n.d.
29	M2	65	46, XY	first relapse
30	M2	40	46, XX, del (5)	first relapse
31	M2	95	not determined	refractory
32	M0	80	46, XY, del (9)(q22)	refractory
33	M1	70	45, XX, t(3;3), -7	refractory
34	MDS/AML	89	46, XY	first relapse

Relapsed patients: relapsed after the first line of chemotherapy with cytarabine, doxorubicin and etoposide. Resistant or refractory patients: resistant to 2 or more lines of chemotherapy including cytarabine, doxorubicin and etoposide, high-dose cytarabine, mitoxantrone, and sequential doses of etoposide.

FAB = French-American-British classification; MDS = myelodysplastic syndrome; n.d. = newly diagnosed.
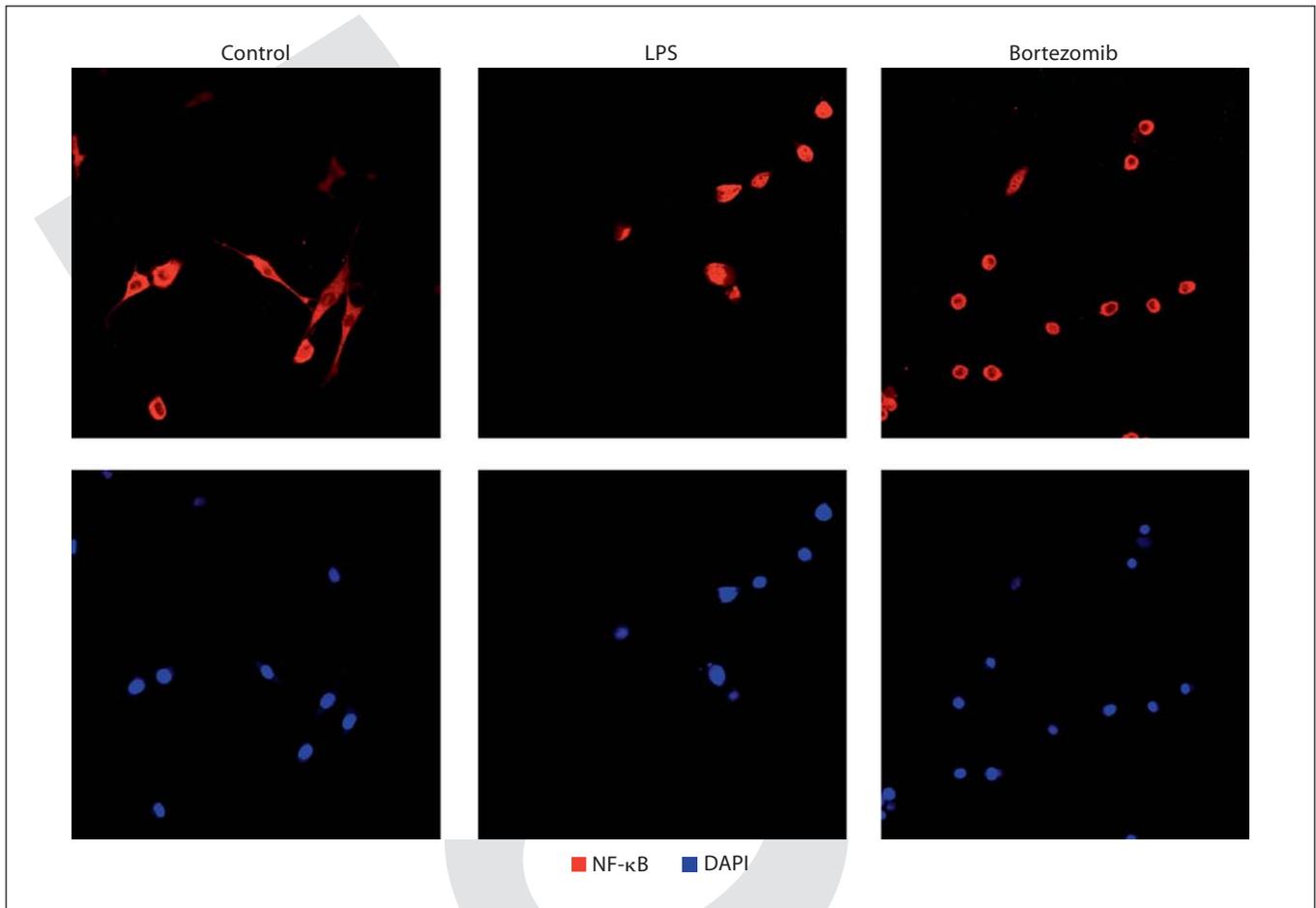
ment in myeloid blast cells (fig. 1f). After 24 h of bortezomib treatment we were unable to detect any cytosolic NF-κB/p65 by immunofluorescence or by Western blot by isolating cytoplasmic and nuclear lysates, suggesting that this drug acts independently of NF-κB status. Murine NIH 3T3 fibroblasts treated with 1 μM bortezomib for 24 h and then with lipopolysaccharide for 1 h were used as a positive control of NF-κB cytoplasmic and nuclear localization (fig. 2). These findings indicate that other effects of the drug might contribute to AML cell death.

Proteasome Inhibitor-Induced Apoptosis Is Mostly Caspase Independent

Since bortezomib activates effector caspases in different tumor cells, we investigated whether this pathway was involved in the apoptosis induced by bortezomib in AML cells. The activity of the effector caspases was assessed 24 and 48 h after treatment with 0.1 μM bortezomib. Activation of caspase-3 and caspase-7 was measured by monitoring the cleavage of a fluorogenic caspase substrate, Ac-DEVD-AMC, using a fluorometric assay. Treatment of AML blasts from 10 patients (8 newly diagnosed, 1 relapsed and 1 refractory) with 0.1 μM bortezomib resulted in consistent caspase activation, as indicated by the significant formation of the fluorescent cleavage product AMC after 24 and 48 h (fig. 3a). A similar but less marked result was also obtained with 0.01 μM bortezomib (data not shown). To determine whether such activity involved the processing of executioner caspases, we evaluated the presence of the active forms of caspase-3 and caspase-7 by Western blotting, which confirmed that bortezomib activates the executioner caspases in myeloid blast cells (fig. 3b). These data suggest that bortezomib induces caspase activation in AML blasts.

Furthermore, to determine whether caspase activation contributes to bortezomib-induced decrease of viability of AML blasts, we treated the cells with 0.1 or 0.01 μM bortezomib in the presence or absence of the pan-caspase inhibitor zVAD-fmk. Although caspase-3 and 7 activation was found to be strongly inhibited by zVAD (fig. 3c), the cytotoxic activity of proteasome inhibition was only slightly reduced by this inhibitor (fig. 3d) and this minimal inhibition was not found to be statistically significant, suggesting that caspases contribute only minimally to the cytotoxic activity of bortezomib in AML cells and that other unidentified apoptotic mediators are involved. The Jurkat cell line was used as a known [29–31] positive control where bortezomib induces cell death and DEVDase activity and zVAD blocks this effect, showing a caspase-dependent death prevented by zVAD (fig. 4).

plored the NF-κB status in AML blasts from 10 patients (7 newly diagnosed, 2 relapsed, 1 refractory) before and after bortezomib treatment. We observed that NF-κB/p65 is constitutively expressed in the nuclei of all samples but its localization is not affected by bortezomib treat-

Fig. 2. Murine NIH 3T3 fibroblasts treated with 1 μM bortezomib for 24 h and then with lipopolysaccharide for 1 h. Original magnification ×20.

Bortezomib Alters the Expression Levels of Apoptotic and Cell Cycle Regulatory Proteins

In order to evaluate the possible contributions of different apoptosis-related proteins to bortezomib-induced apoptosis, we determined the expression of a number of proteins involved in cell growth and survival. Western blot analysis of apoptosis-related proteins was performed on blasts derived from 8 patients (5 newly diagnosed, 2 relapsed and 1 refractory) untreated or treated with 0.1 μM bortezomib for 24 h. Among the apoptotic-related proteins that we have examined (Bcl-2, Bcl-xL, Mcl-1, cFLIP, Smac, cIAP1, p21), we found that the levels of antiapoptotic members of the B cell leukemia/lymphoma-2 (Bcl-2) family, Bcl-xL and Mcl-1, were decreased, while the p21 level was increased (fig. 5) (data not shown). Thus, bortezomib induced upregulation of proteins that reduce cell growth and survival, and downregulation of the po-tent antiapoptotic factors Bcl-xL and Mcl-1. These data provide a possible explanation for bortezomib-induced cell cycle arrest and cell death.

Combined Bortezomib and TRAIL Treatment: Bortezomib Enhances the Activity of TRAIL

The TRAIL is currently under evaluation as a possible (co)therapy in cancer treatment, but AML samples appear to be significantly resistant to TRAIL-induced apoptosis [32]. However, bortezomib has been reported to sensitize several tumors to TRAIL-mediated death [21, 22, 24, 33]. To examine whether a similar effect could be achieved in AML cells, cells from 7 different patients (6 newly diagnosed, 1 refractory) were treated with bortezomib (0.1 or 0.01 μM) for 3 h and then different doses of TRAIL (10, 50 and 200 ng/ml) were added for a further 24 h. Cell viability was evaluated by trypan blue exclusion (data not
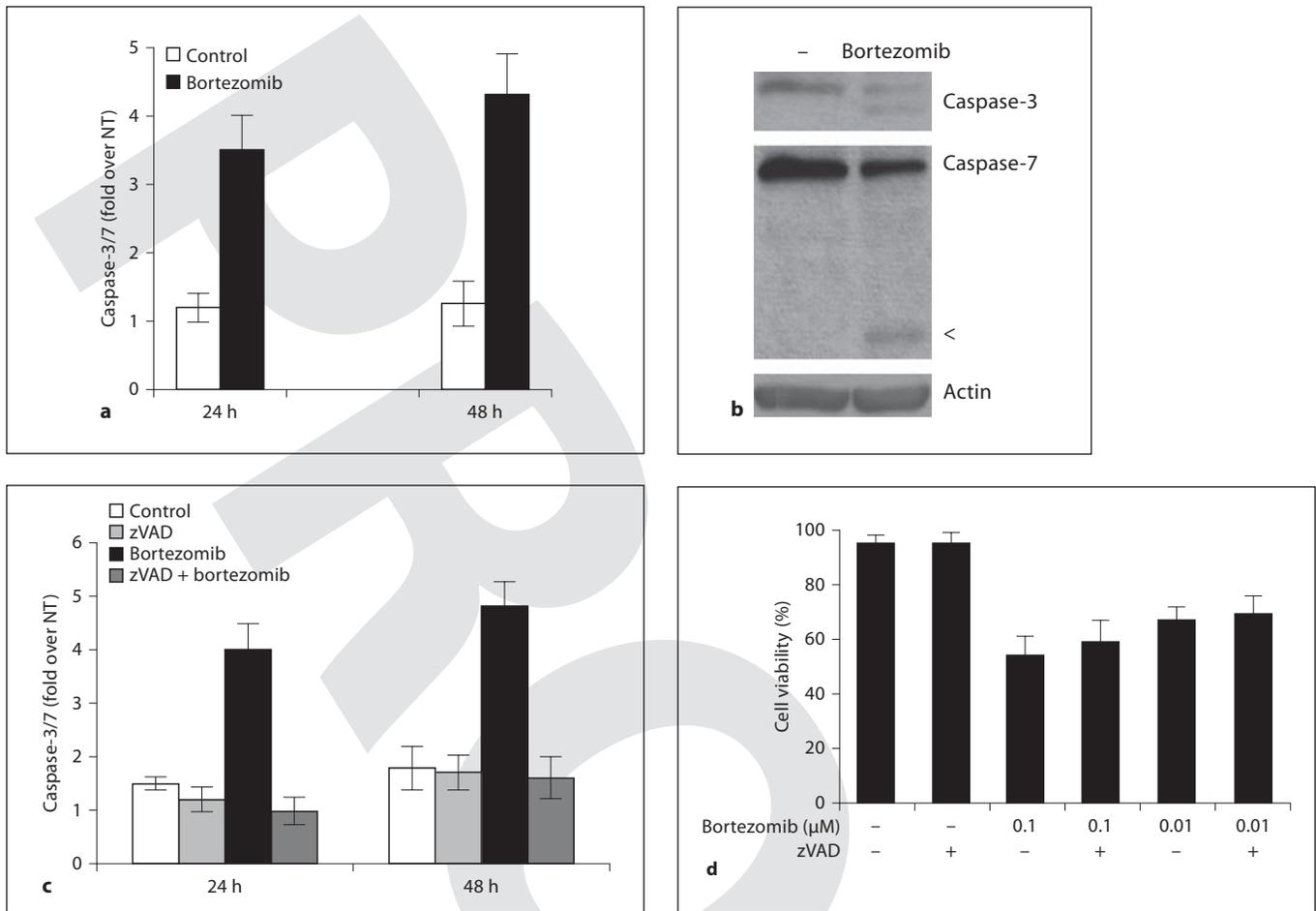
Fig. 3. Activation of caspases in bortezomib-treated AML cells. Cells derived from 10 different patients were incubated in the presence of 0.1 μM bortezomib for 24 and 48 h. Caspase activity was evaluated by cleavage of the fluorogenic substrate Ac-DEVD-AMC using a fluorometric caspase assay (**a**) and by Western blotting (**b**). Data show mean ± SD of ten independent experiments. **c** AML cells were exposed to 0.1 μM bortezomib for 24 and 48 h and caspase activity was evaluated in the absence or presence of 20 μM zVAD-FMK by cleavage of the fluorogenic substrate Ac-DEVD-AMC. NT = ■■■. **d** AML cells were exposed to different doses of bortezomib for 24 h in the presence or absence of 20 μM zVAD-FMK. Percentage of cell viability was determined by the MTS assay. Data show mean ± SD of six independent experiments.

shown) and the MTS assay (fig. 6a). Whereas treatment with TRAIL as a single agent was ineffective, cells treated with bortezomib alone or in association with TRAIL exhibited many of the morphological characteristics of apoptosis (cells shrinking and fragmenting in multiple membrane-bound apoptotic bodies) as confirmed by the COMET assay (fig. 6b). The effects of bortezomib and TRAIL were additive and occurred within 24 h in all the samples of primary AML cells examined. The use of zVAD did not significantly modify the toxicity induced by TRAIL plus bortezomib (data not shown).

The effect found was statistically significant and the combination of the two compounds made it possible to use low doses of bortezomib and TRAIL to produce a considerable cytotoxic effect. This indicates that bortezomib treatment makes AML cells permissive to TRAIL-mediated cytotoxicity.

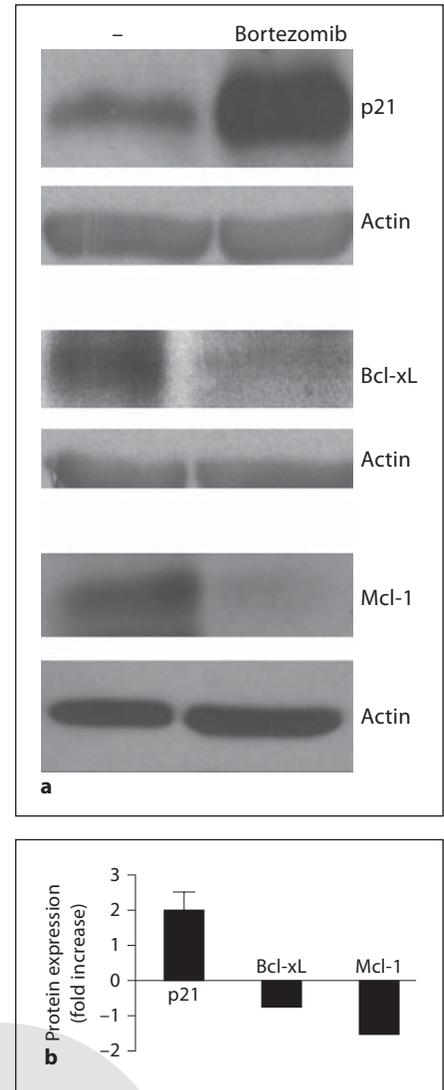*Expression of TRAIL Receptors in AML Primary Cells*

Mechanistically, it is possible that resistance of AML blasts to TRAIL-induced cell death is due to the low expression of the death-inducing receptors TRAIL-R1 and TRAIL-R2. Since proteasome inhibitors have been shown to increase TRAIL-R1 and TRAIL-R2 expression levels in a variety of cell lines, the action of bortezomib could be linked to this upregulation. Immunohistochemistry
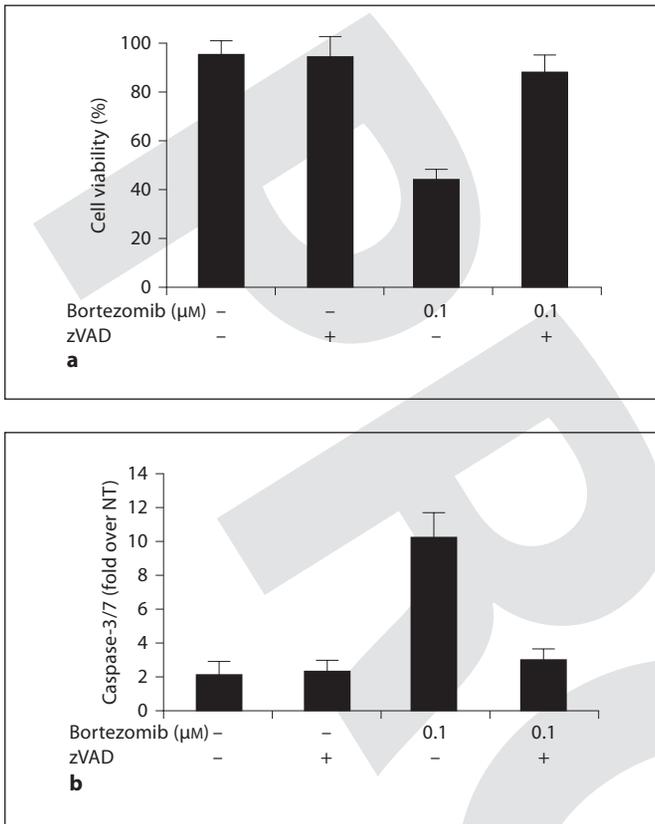
Fig. 4. Jurkat cell line was incubated in the presence of 0.1 μM bortezomib for 24 h in the presence or absence of 20 μM zVAD-FMK. **a** Cell viability was measured by MTS. **b** Caspase activity was evaluated by cleavage of the fluorogenic substrate Ac-DEVD-AMC. Mean ± SD of three independent experiments is shown. NT = ■■■.

Fig. 5. Immunoblot analysis of expression of pro- and antiapoptotic proteins in AML cells untreated or treated with bortezomib. **a** Expression of Bcl-xL, p21 and Mcl-1 in AML cells treated for 24 h with 0.1 μM bortezomib. One representative experiment of eight performed is shown. **b** Densitometric analysis of immunoblot experiments in AML cells treated for 24 h with 0.1 μM bortezomib. Data represent mean ± SD of eight independent experiments.

on formalin-fixed paraffin-embedded sections of four AML BM specimens confirmed the lack of TRAIL receptors, in contrast with the high expression detected in epithelial colon carcinoma cells (fig. 7a).

We therefore evaluated the surface expression of TRAIL receptors after bortezomib treatment in samples from 8 AML patients (5 newly diagnosed, 1 relapsed, 2 refractory) to determine whether the differences in tumor cell sensitivity to TRAIL correlate with TRAIL receptor variations. Most of these samples had already shown high sensitivity to the TRAIL-sensitizing effect of bortezomib. Flow-cytometric analysis revealed that the pattern of cell surface expression of TRAIL receptors in AML cells was significantly ($p < 0.05$) altered when the cells were treated with bortezomib (fig. 7b). Exposure of blast cells to bortezomib significantly increased the surface expression of both TRAIL-R1 and TRAIL-R2 in all

the primary samples. This increased receptor expression, particularly TRAIL-R2, was confirmed by immunofluorescence (data not shown).

Furthermore, samples where TRAIL receptors were upregulated by bortezomib treatment were more sensitive to low-dose bortezomib plus TRAIL exposure than the other samples.
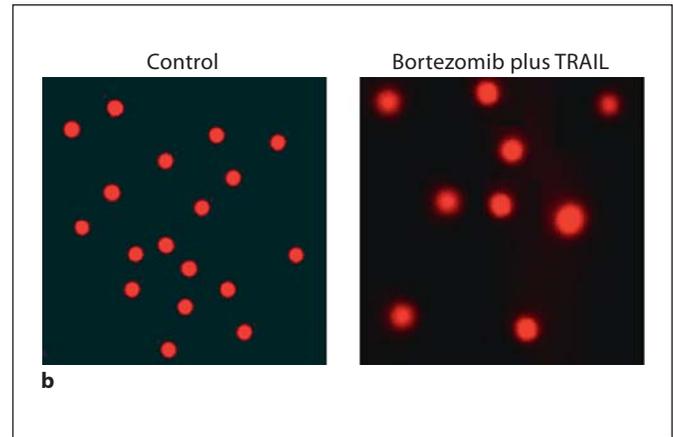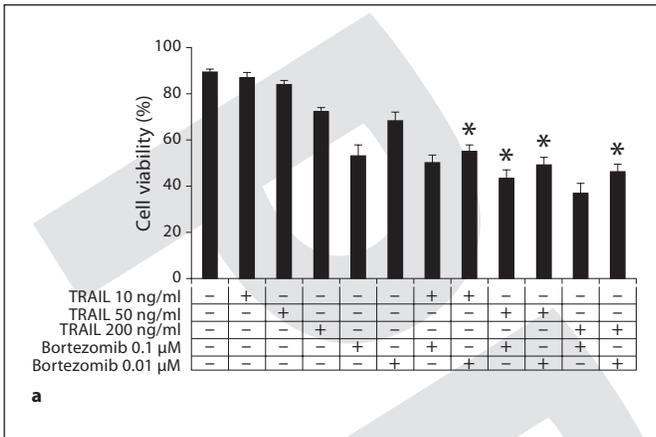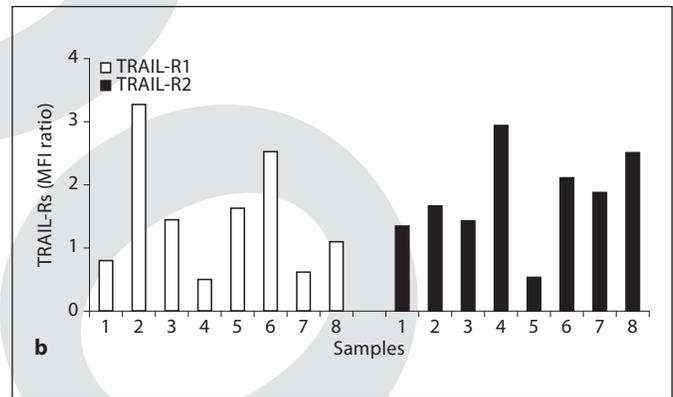

Fig. 6. Bortezomib-mediated sensitization to TRAIL in AML primary cells. **a** AML cells were incubated with 0.01–0.1 μM bortezomib for 3 h prior to the addition of various concentrations (10, 20 and 50 ng/ml) of TRAIL. Bortezomib was present the whole 24 h of incubation with TRAIL. After 24 h, cell viability was assessed by MTS assay. The values are averages of seven independent experiments. **b** AML blasts from 2 patients at diagnosis and 1 resistant patient were stained by COMET assay after exposure to 0.1 μM bortezomib plus 200 ng/ml TRAIL for 24 h. One representative experiment of three is shown. * p < 0.05.
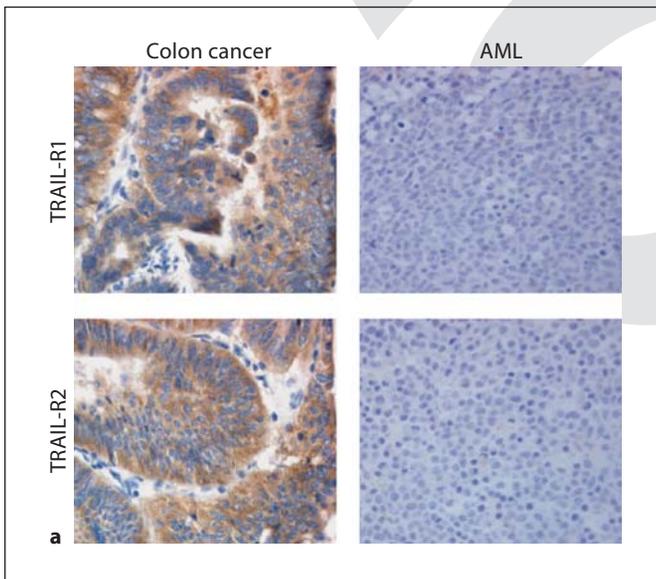
Fig. 7. Proteasome inhibition modulates surface expression of TRAIL receptors. **a** Immunohistochemical analysis of TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2) on formalin-fixed paraffin-embedded sections of AML BM and colon cancer specimens. The results are representative of four independent experiments with samples from different AML patients. Original magnification ×40. **b** AML cells were treated with 0.1 μM bortezomib for 24 h and subsequently analyzed for surface expression of TRAIL-R1 and TRAIL-R2 by flow cytometry as described in Materials and Methods. Data are expressed as mean fluorescence intensity (MFI) ratio between the specific staining of treated cells and control staining of untreated cells where ratio <1 means a decrease in expression and ratio >1 means an increase in expression of TRAIL-Rs. These data represent results from 8 independent experiments. Samples 1, 2, 3, 5, 6 are from newly diagnosed patients, samples 4 and 8 are from resistant patients, sample 7 is from a relapsed patient.

Discussion

Our data on AML cells from de novo and relapsed/refractory patients indicate that bortezomib, at doses fully compatible with those reached in vivo, displays a considerably higher cytotoxic activity than the conventional chemotherapeutic agents currently used for treating AML. Moreover, we have demonstrated that low-dose bortezomib sensitizes AML cells to TRAIL-mediated apoptosis, indicating that the two molecules may act together in eradicating AML.

The antitumor effect against leukemic cells of a combination of proteasome inhibitors with other drugs such as anthracyclin [34], arsenic trioxide [35] and more recently TRAIL itself has already been described by different authors. Particularly, Riccioni et al. [27] showed that M4 and M5 AML display a high sensitivity to bortezomib-mediated apoptosis and this effect is increased when TRAIL is added.

Proteasome inhibitors are generally held to block NF-κB activation by inhibiting IκB degradation and consequently preventing the translocation of NF-κB to the nucleus. However, this mechanism does not seem to be important in killing AML blasts. In fact, we found that NF-κB remains in the nucleus in AML samples after bortezomib treatment. We observed that AML cells exposed to bortezomib show marked reductions in Bcl-xL and Mcl-1 together with activation of executioner caspases such as caspase-3 and 7. Pretreatment of cells with zVAD inhibits caspase activation but failed to block cell death suggesting that caspase activation may not be required for the cytotoxic effect exerted by bortezomib. Since we have examined MMP by JC-1 assay and we have observed loss of MMP after bortezomib treatment, the intrinsic mitochondrial pathway could be critical in executing cell death in AML samples.

We also observed a sustained upregulation of the cyclin-dependent kinase inhibitor p21 in all the AML cells after bortezomib treatment. This probably causes the cells to accumulate in the G2-M phase of the cell cycle, as in other tumor cells exposed to bortezomib [36]. Thus, bortezomib targets AML cells by affecting both proliferation and survival.

The discovery that TRAIL potently and selectively targets neoplastic cells while sparing normal cells sparked widespread interest in its use as a novel anticancer molecule. A synergistic apoptotic response has been observed when TRAIL is combined with chemotherapeutic agents or with proteasome inhibitors such as bortezomib. Specifically, treatment with bortezomib sensitizes resistant prostate, colon and bladder cancer cell lines to TRAIL-induced apoptosis by upregulating TRAIL death receptors. Leukemic blasts have been shown to be invariably resistant to TRAIL-mediated apoptosis even if the TRAIL death pathway is present and can function. Lack of sensitivity to the TRAIL apoptotic pathway can be explained by different mechanisms involving reduced expression of the death receptors (only a minority of the AML cells express TRAIL-R1 and TRAIL-R2; or there is overexpression of internal regulators of the apoptotic machinery such as cFLIP, which inhibits caspase-8 activation, or Bcl-2/Bcl-xL, which inhibits Bax/Bak-mediated release of cytochrome c). Thus, the ability of bortezomib to upregulate TRAIL-R1 and TRAIL-R2 while reducing the expression of Bcl-2 and Bcl-xL may well explain its capacity to sensitize AML cells to TRAIL-induced apoptosis.

This is consistent with findings in other cell lines treated with bortezomib in combination with TRAIL. The increase of TRAIL receptor expression could be promoted by the lack of protein degradation that follows proteasome inhibition or may be due to the stabilization of p53, which is known to regulate DR expression in many cell types [21–23, 37]. Therefore, although bortezomib alone is sufficient to induce death in AML blasts, the combination of the two compounds may allow lower doses of bortezomib to be used while maintaining a high cytotoxic effect.

Conclusion

Our data demonstrate that bortezomib exerts a considerable cytotoxic activity on AML cells, which is increased by combination with TRAIL. It is noteworthy that both proteasome inhibitors and TRAIL have previously been reported as relatively nontoxic toward normal hematopoietic cells. Although further preclinical studies are required to assess the possible use of bortezomib and TRAIL in the treatment of AML, such a high cytotoxic activity, together with the good in vivo tolerability of the two molecules, may provide the basis for future studies aimed at improving the clinical outcome of AML patients.

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