

Requirement of Apoptotic Protease-Activating Factor-1 for Bortezomib-Induced Apoptosis but Not for Fas-Mediated Apoptosis in Human Leukemic Cells^S

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ABSTRACT

Bortezomib is a highly selective inhibitor of the 26S proteasome and has been approved for clinical use in the treatment of relapsing and refractory multiple myeloma and mantle cell lymphoma. Clinical trials are also underway to assess the role of bortezomib in several other human malignancies, including leukemia. However, the mechanism(s) by which bortezomib acts remain to be fully understood. Here, we studied the molecular requirements of bortezomib-induced apoptosis using the human T-cell leukemic Jurkat cells stably transfected with or without shRNA against apoptotic protease-activating factor-1 (Apaf-1). The Apaf-1-deficient Jurkat T cells were resistant to bortezomib-induced apoptosis, as assessed by caspase-3 activity, poly(ADP-ribose) polymerase cleavage, phosphatidylserine externalization, and hypodiploid DNA content. In contrast, Apaf-1-deficient cells were sensitive to Fas-induced apoptosis. Bortezomib induced an upregulation of the pro-apoptotic protein Noxa,

loss of mitochondrial transmembrane potential, and release of cytochrome c in cells expressing or not expressing Apaf-1. Transient silencing of Apaf-1 expression in RPMI 8402 T-cell leukemic cells also diminished bortezomib-induced apoptosis. Fas-associated death domain (FADD)-deficient Jurkat cells were resistant to Fas-mediated apoptosis yet remained sensitive to bortezomib. Our results show that bortezomib induces apoptosis by regulating pathways that are mechanistically different from those activated upon death receptor ligation. Furthermore, in silico analyses of public transcriptomics databases indicated elevated Apaf-1 expression in several hematologic malignancies, including acute lymphoblastic and myeloid leukemia. We also noted variable Apaf-1 expression in a panel of samples from patients with acute lymphoblastic leukemia. Our results suggest that the expression of Apaf-1 may be predictive of the response to proteasome inhibition.

Introduction

Bortezomib, a highly selective inhibitor of the 26S proteasome, was approved in 2003 for treatment of relapsed refractory multiple myeloma and, more recently, for treatment of

mantle cell lymphoma (Orlowski and Kuhn, 2008). Clinical trials are underway to assess the efficacy of bortezomib in several other human malignancies, including leukemia (Vink et al., 2006). However, the mechanism(s) by which this drug acts remain to be fully understood. Proteasome inhibitors may act not only on myeloma cells themselves, but also on bone marrow stromal cells (Hideshima et al., 2001). Moreover, bortezomib has a chemosensitizing effect when administered together with other anti-cancer agents; this effect may, in part, be due to the inhibition of the nuclear factor (NF)- κ B pathway (Cusack et al., 2001; Russo et al., 2001). However,

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ABBREVIATIONS: ABT-737, *N*-{4-[4-(4'-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl}-4-(3-dimethylamino-1-phenylsulfanyl)methyl-propylamino-3-nitro-benzenesulfonamide; ALL, acute lymphoblastic leukemia; Apaf-1, apoptotic protease-activating factor 1; B-CLL, B-cell chronic lymphoblastic leukemia; BH3, Bcl-2 homology domain 3; DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MG132, *N*-benzyloxycarbonyl (Z)-Leu-Leu-leucinal; MMP, mitochondrial membrane potential; NC, negative control; NF- κ B, nuclear factor- κ B; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PS, phosphatidylserine; shRNA, short hairpin RNA; TMRE, tetramethylrhodamine-ethyl ester; RNAi, RNA interference; TRADD, tumor necrosis factor receptor-associated death domain; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WT, wild-type; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone.

NF- κ B inhibition alone cannot explain the selective anti-myeloma effect of bortezomib (Hideshima et al., 2001).

Human leukemic cells express abnormally high levels of proteasomes compared with normal peripheral blood cells (Kumatori et al., 1990), and leukemic cells are significantly more sensitive to proteasome inhibition than are normal bone marrow progenitor cells or peripheral blood lymphocytes (Masdehors et al., 1999; Soligo et al., 2001). Furthermore, proteasome inhibitors inhibit leukemic stem cells specifically when compared with normal stem cells (Guzman et al., 2002). Clinical trials conducted to date suggest that bortezomib may only yield minor clinical benefits in leukemia patients when administered as a single drug (Orlowski et al., 2002; Cortes et al., 2004). However, bortezomib enhances the effect of many conventional therapies and may overcome resistance to conventional anticancer drugs, and the effects in combination studies appear promising (Hideshima et al., 2001; Mitsiades et al., 2003; Nikrad et al., 2005; Messinger et al., 2010). However, potential biologic markers of susceptibility to proteasome inhibitors are lacking.

In mammalian cells, the apoptotic machinery is activated mainly via two pathways: the extrinsic pathway, through the engagement of so-called death receptors on the cell surface; and the intrinsic pathway, through activation of mitochondria, with release of proapoptotic factors into the cytosol (Danial and Korsmeyer, 2004). The release of cytochrome *c* from mitochondria serves to trigger the formation of the apoptosome, an oligomeric protein complex consisting of apoptotic protease-activating factor 1 (Apaf-1), procaspase-9, cytochrome *c*, and deoxyadenosine triphosphate (ATP) (Fadeel et al., 2008). This leads, in turn, to the activation of caspase-3, with cleavage of numerous death substrates in the cell, and eventually to the demise of the cell. Studies in recent years have revealed numerous cellular factors that regulate Apaf-1 oligomerization, procaspase-9 recruitment, and apoptosome activation (Fadeel et al., 2008). Binding of death receptors, such as FAS/APO-1/CD95 or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-1 or -2, by a cognate ligand (e.g., Fas ligand or TRAIL) causes the recruitment of adaptor proteins [Fas-associated death domain (FADD) or tumor necrosis factor-associated death domain (TRADD)] and procaspase-8 molecules to the cytosolic portion of the receptor to form the death-inducing signaling complex (DISC) (Danial and Korsmeyer, 2004). In most cells (type I) caspase-8 then activates caspase-3, which is responsible for most of the morphologic and biochemical manifestations of apoptosis, including membrane blebbing and DNA fragmentation. In some cell types (type II), the amount of caspase-8 that is activated in the DISC is low, and mitochondrial amplification of the death signal is required (Scaffidi et al., 1998).

Apaf-1 is transcriptionally and post-translationally regulated during pathologic and physiologic conditions (Fadeel et al., 2008). Indeed, silencing of Apaf-1 protein expression by hypermethylation of the gene promoter has been reported in several human tumors, including malignant melanoma and acute leukemia (Fu et al., 2003). Silencing of Apaf-1 expression in B-cell chronic lymphoblastic leukemia (B-CLL) constitutes a negative prognostic marker in the case of concomitant p53 mutations (Sturm et al., 2006). Jia et al., (2001) reported that Apaf-1 deficiency constitutes a significant mode of resistance to cytochrome *c*-dependent apoptosis in human leukemia cells. In addition, aberrant subcellular localization of the Apaf-1 protein

has been demonstrated in chemoresistant B lymphoma cells, and resistance to etoposide was reversed upon Apaf-1 overexpression (Sun et al., 2005). We previously provided evidence for the importance of Apaf-1 in apoptosis induced by the chemotherapeutic agents etoposide and mitoxantrone in the T-cell leukemic Jurkat cell line (Franklin and Robertson, 2007). Here, we used Jurkat cells with stable silencing of Apaf-1 expression as well as Jurkat cells deficient for FADD expression to determine the molecular requirements for bortezomib-induced apoptosis. For comparison, Jurkat cells with or without Apaf-1 or FADD expression were treated with agonistic anti-Fas antibodies to activate classic, death receptor-mediated apoptosis. Furthermore, we examined Apaf-1 protein expression in leukemic blasts from pediatric patients with acute lymphoblastic leukemia (ALL), and assessed sensitivity toward bortezomib-induced apoptosis *ex vivo*.

Materials and Methods

Patient Samples. The study included leukemic cells from seven children with ALL (six patients with T-precursor ALL and one with B-precursor ALL). Informed consent was obtained from each parent in accordance with the conditions of the approval of the study by the local ethics committee (Stockholm, Sweden). Mononuclear cells were isolated from bone marrow samples by centrifugation on a Ficoll/Hypaque gradient (Lymphoprep, Oslo, Norway), and cells were cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide. Upon thawing, lymphoblasts were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY) as described previously (Laane et al., 2007) and subjected to treatment as indicated with bortezomib or cultured without treatment ("spontaneous apoptosis").

Cell Lines. The parental Jurkat human leukemic T-cell line (clone E6.1 for the Apaf-1 experiments and clone A3 for the FADD experiments; described later in this section), Jurkat cells with mutations in FADD (Jurkat FADD mt) cells (a generous gift from Professor John Blenis, Harvard Medical School, Boston, MA) (Juo et al., 1999), the human Burkitt lymphoma cell line Raji, and the human leukemic T-cell line RPMI 8402 (both from European Collection of Cell Cultures, Salisbury, UK) were all cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2% L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY) at 37°C in a humidified 5% CO₂ incubator. For control-transfected (pSUPER) and Apaf-1-deficient (pSUPER-Apaf-1) Jurkat clones (for detailed procedures, see Franklin and Robertson, 2007), 1 mg/ml Geneticin (G418; Invitrogen, Carlsbad, CA) was substituted for penicillin and streptomycin. Silencing of Apaf-1 expression was confirmed by Western blot.

Reagents. The monoclonal agonistic anti-Fas antibody (clone CH-11) was purchased from Nordic Biosite AB (Täby, Sweden) and bortezomib (Velcade) was from Millennium Pharmaceuticals (Cambridge, MA). All other reagents were from Sigma-Aldrich (Stockholm, Sweden) unless stated otherwise.

RNA Interference. The human leukemic T-cell line RPMI 8402 cells were transfected with 200 nM siRNA against APAF1 (Ambion, Austin, TX) or 200 nM non-targeting siRNA [negative control (NC)] (Dharmacon, Lafayette, CO) using the Transit-TKO transfection reagent (Mirus Bio, Madison, WI). The cells were transfected for 36 hours in complete RPMI 1640 medium. Efficiency of RNAi was assessed using Western blot.

Caspase-3-Like Activity. Cell lysates were combined with the fluorogenic, caspase-3-specific substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) in a standard reaction buffer (Jitkaew et al., 2009), and real-time measurements of enzyme-catalyzed release

of AMC were obtained using a Tecan Infinite F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland) operating with Magellan version 6.5 software (Tecan Group Ltd, Männedorf, Switzerland). Fluorescence values were converted to picomoles, and the maximum rate of AMC release (picomoles per minute) was estimated for each sample.

Exposure of Phosphatidylserine. Phosphatidylserine (PS) exposure was determined by flow cytometric detection of the PS-binding protein annexin V using the annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA), as described previously (Uthaisang et al., 2003). Cells were costained with propidium iodide (PI) prior to analysis on a FACScan (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser. Data were analyzed using the CellQuest software (Becton Dickinson) and are reported as “percentage PS exposure” (i.e., percentage of cells positive for annexin V binding).

Mitochondrial Membrane Potential. Loss of mitochondrial membrane potential was detected using tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Eugene, OR) (Jitkaew et al., 2009). In brief, 20 minutes prior to harvesting the cells, 1 ml (TMRE 25 nM) was added to the cell cultures. After washing the cells with PBS, HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂ • 6H₂O, 1.8 mM CaCl₂ • 6H₂O) containing TMRE was added, and the samples were analyzed on a FACScan (Becton Dickinson).

DNA Content. For flow cytometric assessment of DNA content and cell cycle distribution (Karpova et al., 2006), cells were harvested and resuspended in a solution containing PI (50 µg/ml), 0.1% Triton X-100, and 0.1% sodium citrate in PBS. Cells were then analyzed on a FACScan (Becton Dickinson) operating with CellQuest software (Becton Dickinson). Data are depicted as histograms and the percentage of cells displaying hypodiploid DNA content is indicated.

Western Blot Analysis. To obtain total cell lysates, cells were lysed on ice for 30 minutes in radioimmunoassay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate). Cell lysates were then centrifuged, and supernatants were collected. Protein concentration was measured using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL). To obtain cytosolic fractions of Jurkat wild-type (WT), Raji, and RPMI 8402 cells, the ProteoExtract S-PEK mini Kit from Calbiochem was used (Merck4Biosciences, Darmstadt, Germany). First, 30-µg protein of each sample was loaded onto a SDS-gel, transferred onto polyvinylidene difluoride membranes, blocked in 5% milk and probed with antibodies against cytochrome *c* (BD Biosciences, Franklin Lakes, NJ), Apaf-1 (Chemicon, Boronia, Australia), poly(ADP-ribose) polymerase (PARP) (Biomol International, Plymouth Meeting, PA), Noxa (Merck KGaA, Darmstadt, Germany), ubiquitin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), β-actin (DakoCytomation, Glostrup, Denmark) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Nordic Biosite AB), and relevant secondary antibodies (DakoCytomation). Proteins were visualized by enhanced chemiluminescence (Bio-Rad Laboratories). The films were scanned using the Epson Perfection 4490 Scanner (EPSON Europe B.V., Sollentuna, Sweden), and densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

DNA Damage. The comet assay was used to monitor DNA damage. In brief, cells were harvested, washed with PBS, applied in 0.75% low-melting-point agarose to pre-coated agarose glass slides, and lysed in lysis buffer (10 mM Tris, 2.5 M NaCl, 100 mM EDTA). Glass slides were equilibrated in electrophoresis buffer (10 N NaCl, 200 mM EDTA), run at 20 V and 150 mA for 20 minutes, neutralized with neutralization buffer (0.4 M Tris-HCl), stained with ethidium bromide, and mounted on glass slides. At least 100 cells were counted for each sample at 10× or 40× magnification using an inverted Nikon ECLIPSE TE2000-S fluorescence microscope (Nikon Corporation, Kanagawa, Japan) equipped with a Nikon Digital Sight DS-U2 camera and operating with NIS-Elements F software (Nikon Corporation), and the percentage of cells with comet tails were determined. The topoisomerase I inhibitor etoposide was used as positive control.

In Silico Analysis of Apaf-1 Expression. Expression of Apaf-1 mRNA was assessed using the Human Gene Expression Map, an

online resource constructed from the integration of 5372 gene expression analyses of 369 different cell and tissue types, disease states and cell lines (Lukk et al., 2010). Statistical analyses of the three probes targeting the APAF1 gene on the Affymetrix HG-U133A (Santa Clara, CA) were performed using one-way analysis of variance with multiple group-wise comparisons to the global mean, and post-hoc tests were applied to identify selected comparisons and globally adjusted *P* values. Comparisons were generated across 15 meta groups (blood neoplasm cell line, blood non-neoplastic disease, breast cancer, germ cell neoplasm, leukemia, nervous system neoplasm, non-breast carcinoma, nonleukemic blood neoplasm, non-neoplastic cell line, normal blood, normal solid tissue, other neoplasm, sarcoma, solid tissue neoplasm cell line, and solid tissue non-neoplastic disease) and 96 biologic groups (cell or tissue types with more than 10 biologic replicates among the 369 states).

Statistics. To estimate differences between treated cells and untreated cells, a one-way analysis of variance with Tukey-Kramer as post hoc test was performed. *P* values < 0.05 were considered significant. Tests were performed using Prism 5.03 software (Graph-Pad Software, Inc., La Jolla, CA). Each experiment was repeated at least 3 to 4 times unless indicated otherwise. Error bars in the graphs depict the standard error of the mean (S.E.M.).

Results

Bortezomib Triggers Apaf-1-Dependent Apoptosis in Leukemic Cells. Previously, we reported that Apaf-1-deficient Jurkat T-cell leukemic cells were resistant to DNA damage- and heat-induced apoptosis (Franklin and Robertson, 2007; Shelton et al., 2010). Here, we wanted to evaluate the importance of Apaf-1 for bortezomib-induced apoptosis. To this end, Jurkat cells were stably transfected with (pSUPER-Apaf-1) or without (pSUPER) shRNA against Apaf-1 (Fig. 1A). We first determined that bortezomib inhibited proteasomal degradation of ubiquitinated proteins to a similar degree in all three cell lines (Supplemental Fig. 1). Next, the parental Jurkat cell line and the two Jurkat clones were treated with bortezomib or agonistic anti-Fas antibody for 6 or 14 hours, and apoptosis was determined by PI staining to detect hypodiploid DNA content and annexin V-labeling to monitor PS exposure. As seen in Fig. 1, B and C, bortezomib induced a modest degree of apoptosis at 6 hours, and this was blocked by the pan-caspase inhibitor zVAD-fmk, demonstrating that bortezomib-induced apoptosis is caspase dependent in this model. After 14 hours of treatment, the degree of bortezomib-induced apoptosis was increased, and Apaf-1-deficient Jurkat cells displayed significantly lower levels of apoptosis when compared with WT or control-transfected cells (Fig. 1, D–E). In contrast, anti-Fas antibody treatment induced similar levels of apoptosis in Jurkat cells with or without Apaf-1 expression (Fig. 1, D and E); a minor decrease in Fas-induced PS exposure was seen at 6 hours in cells lacking Apaf-1, but this difference was not statistically significant (Fig. 1, B and C). The latter data are in accordance with our previous work which indicated that Jurkat cells can undergo Fas-mediated apoptosis without apoptosome-dependent activation of caspase-9 (Shawgo et al., 2009).

To further assess whether Apaf-1 plays a role in bortezomib versus Fas-induced apoptosis, cells were incubated for longer periods of time (24 and 48 hours), and apoptosis was determined using annexin V-FITC labeling. These experiments clearly demonstrated that Fas-mediated apoptosis is Apaf-1-independent (Fig. 1, F and G). Bortezomib triggered some

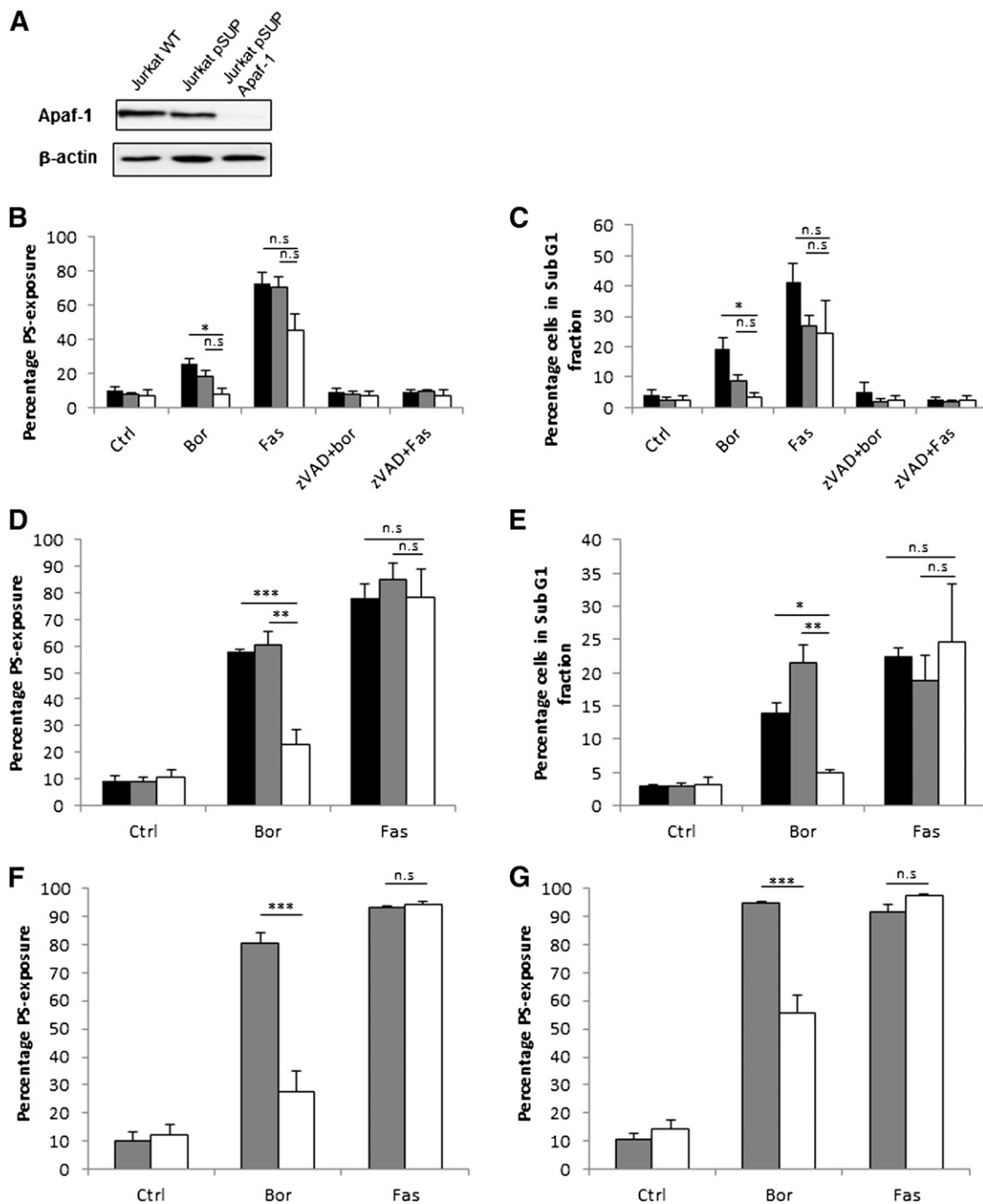


Fig. 1. Bortezomib fails to induce apoptosis in Jurkat cells lacking Apaf-1. (A) Apaf-1 protein levels were determined in total cell lysates from Jurkat WT cells and Jurkat cells transfected with (pSUPER-Apaf-1) or without shRNA against Apaf-1(pSUPER). β -Actin expression was monitored to control for equal loading of protein. (B and C) Jurkat WT (black bars), pSUPER (gray bars), and pSUPER-Apaf-1 (white bars) were treated with 50 nM bortezomib or agonistic anti-Fas antibody (250 ng/ml) for 6 hours, and apoptosis was assessed by annexin V-FITC assay (B) and PI staining to determine the percentage of cells in sub-G1 fraction (C). The pan-caspase inhibitor, zVAD-fmk (20 μ M) was added 30 minutes prior to the apoptosis agonists. (D and E) Jurkat WT (black bars), pSUPER (gray bars), and pSUPER-Apaf-1 (white bars) were treated with 50 nM bortezomib or anti-Fas antibody (250 ng/ml) for 14 hours, and apoptosis was assessed by percentage PS exposure (D) and percentage cells in sub-G1 fraction (E). (F and G) Jurkat pSUPER (gray bars) and pSUPER-Apaf-1 (white bars) were treated with 50 nM bortezomib or anti-Fas antibody (250 ng/ml) for 24 hours (F) and 48 hours (G), and apoptosis was assessed by the annexin V-FITC assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

apoptosis at 24 and 48 hours in Apaf-1-deficient Jurkat cells, but the differences between Apaf-1-expressing cells and Apaf-1-deficient cells remained significant (Fig. 1, F and G), thus demonstrating that bortezomib-induced apoptosis in the Jurkat cell line is largely Apaf-1-dependent.

To obtain further evidence for a role of Apaf-1 in bortezomib-induced apoptosis, additional cell lines were employed. We

previously reported that the human B-lymphoma Raji cells are resistant to etoposide-induced apoptosis due to plasma membrane sequestration of Apaf-1 in lipid rafts (Sun et al., 2005). Indeed, these cells displayed decreased cytosolic Apaf-1 expression in comparison with Jurkat cells (Supplemental Fig. 2A). We also evaluated the human T-ALL cell line RPMI 8402 and found that the Apaf-1 expression was similar to that seen

in the Jurkat cell line. Subsequently Jurkat, RPMI 8402 and Raji cell lines were treated with bortezomib for 14 h and apoptosis was measured using the caspase-3 assay and PS exposure. As seen in Supplemental Fig. 2, B and C, bortezomib induced apoptosis in Jurkat and RPMI 8402 cells but not in Raji cells. Next, we tested whether Apaf-1 expression was required for apoptosis induction in the RPM 8402 cell line. To this end, RPMI 8402 cells were treated for 36 hours with siRNA specific for Apaf-1 or with non-targeting siRNA (NC) followed by 12 hours of treatment with 50 nM bortezomib (Supplemental Fig. 3, A and B). A reduction in PS exposure as well as in caspase-3 like activity was seen in Apaf-1 siRNA-treated RPMI 8402 cells compared with cells transfected with NC siRNA. The decrease in mitochondrial membrane potential was similar in Apaf-1 siRNA-treated RPMI 8402 cells and in the nontransfected and untreated control cells (Supplemental Fig. 3, C and D).

Taken together, our data suggest that bortezomib-induced apoptosis in T-cell leukemic cells is Apaf-1 dependent.

Bortezomib Triggers Caspase-Independent Mitochondrial Activation. Almond et al. (2001) previously reported that the proteasome inhibitors, lactacystin and MG132 [*N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinall], induce mitochondrial cytochrome *c* release and caspase-dependent apoptosis in B-CLL cells. To further study the molecular signaling events in bortezomib-treated leukemia cells, we examined the effects of bortezomib on mitochondrial membrane potential and cytochrome *c* release. Jurkat cells were treated with bortezomib or anti-Fas antibody for 6 or 14 hours, and mitochondrial membrane potential was measured. Jurkat cells with or without Apaf-1 expression both displayed a dissipation of mitochondrial membrane potential (Fig. 2, A and B). The anti-Fas antibody-induced drop of mitochondrial membrane potential was blocked by zVAD-fmk as expected, whereas the bortezomib-induced drop of mitochondrial membrane potential was unaffected by the pan-caspase inhibitor (Fig. 2B), indicating caspase-independent signaling upstream of mitochondria in the case of bortezomib. Furthermore, cytochrome *c* was released from mitochondria upon treatment of cells with bortezomib or anti-Fas antibody irrespective of Apaf-1 expression (Fig. 2C). Caspase-3 is a downstream executioner caspase that can be activated following DISC formation or apoptosome activation (Danial and Korsmeyer, 2004). Cleavage of procaspase-3 to its active form leads to proteolysis of such downstream substrates as PARP, a nuclear protein. Bortezomib induced caspase-3 activation at 6 hours and 14 hours, as evidenced by DEVD-AMC cleavage, in Jurkat cells expressing Apaf-1 but not in cells with stable silencing of Apaf-1 (Fig. 2, D–E). In addition, bortezomib only triggered PARP cleavage in Jurkat cells expressing Apaf-1 (Fig. 2F). By comparison, Fas ligation resulted in PARP cleavage in cells with or without Apaf-1 expression.

The apoptotic machinery in cancer cells can also be activated following DNA damage. Moreover, it has been suggested recently that bortezomib may act, at least in part, by disrupting essential DNA damage repair pathways (Chen et al., 2010). However, bortezomib induced negligible DNA damage in Jurkat cells in comparison with the conventional chemotherapeutic agent, etoposide, as assessed using the comet assay (Supplemental Fig. 4). Proteasome inhibitors were previously shown to trigger Noxa-mediated apoptosis in melanoma and myeloma cells (Qin et al., 2005). Noxa is a so-called BH3-only

protein that is known to act on mitochondria. To assess whether Noxa may play a role in the present model, we determined the level of Noxa expression following bortezomib treatment. As shown in Fig. 2G, bortezomib treatment led to the induction of Noxa in parental Jurkat cells as well as in control-transfected and Apaf-1-deficient clones. Taken together, our results show that Apaf-1 is critically required for bortezomib-induced caspase-3 activation and apoptosis in leukemic cells. However, upstream signaling events, including the induction of Noxa, drop in mitochondrial membrane potential and release of cytochrome *c*, are unimpaired in Apaf-1-deficient cells. Although caspase inhibition was unable to inhibit dissipation of the mitochondrial membrane potential, we wanted to examine the potential role of death receptor-dependent caspase-8 activation in bortezomib-induced apoptosis. Therefore, Jurkat cells with or without Apaf-1 expression were treated with bortezomib for 14 hours, and cleavage of procaspase-8 into its active form was assessed by Western blot (Fig. 3A). As a positive control for caspase-8 activation, Jurkat WT cells were treated with anti-Fas antibody for 6 hours. Bortezomib induced detectable but very low levels of caspase-8 cleavage in all three cell lines with or without Apaf-1 expression when compared with anti-Fas treatment (Fig. 3A). This could potentially be explained by caspase-3-mediated activation of procaspase-8 downstream of the activation of mitochondria. To exclude the involvement of death receptor activation in this model, Jurkat WT (clone A3) and FADD mt were treated with bortezomib or anti-Fas antibody. Jurkat FADD mt cells were completely resistant to Fas-induced apoptosis, whereas bortezomib treatment resulted in the same level of PS exposure in both Jurkat WT (clone A3) and Jurkat FADD mt cells (Fig. 3, B and C).

Apaf-1 Protein Expression Differs in ALL Patient Cells: Association with Bortezomib-Induced Apoptosis.

Next, we examined Apaf-1 protein expression by Western blot in primary samples from patients with ALL (Table 1). Apaf-1 protein expression was found to vary between the samples, and in one patient (ALL6), Apaf-1 expression was undetectable (Fig. 4). Cells from these ALL patients were also subjected to treatment *ex vivo* with bortezomib for 24 hours, and apoptosis was determined by annexin V-labeling (Table 1). Following 24 hours of *ex vivo* culture in the absence of cytokines, a substantial fraction of the leukemic blasts undergo spontaneous apoptosis (Laane et al., 2007). For this reason, it was difficult to correlate the responsiveness to bortezomib with Apaf-1 expression. Notwithstanding, in the patient sample (ALL6) apparently deficient for expression of Apaf-1, we noted the lowest increase in apoptosis in response to bortezomib treatment *ex vivo* (Fig. 4; Table 1).

Apaf-1 mRNA Expression in Various Malignancies, Including Subtypes of Leukemia: In Silico Studies.

Finally, in light of the fact that Apaf-1 expression appeared to be a key determinant of bortezomib-induced apoptosis in the Jurkat model, we explored the Human Gene Expression Map for the expression of Apaf-1 transcripts in a diverse range of human malignancies (Lukk et al., 2010). First, the three probes targeting the Apaf-1 gene were compared relative to 15 major groups (Table 2). Consistently higher expression of Apaf-1 was identified in the following groups: blood neoplasm cell line, blood non-neoplastic disease, leukemia, and normal blood. Next, the selected hematologic malignancy groups (i.e., leukemia and nonleukemic blood neoplasm groups) were

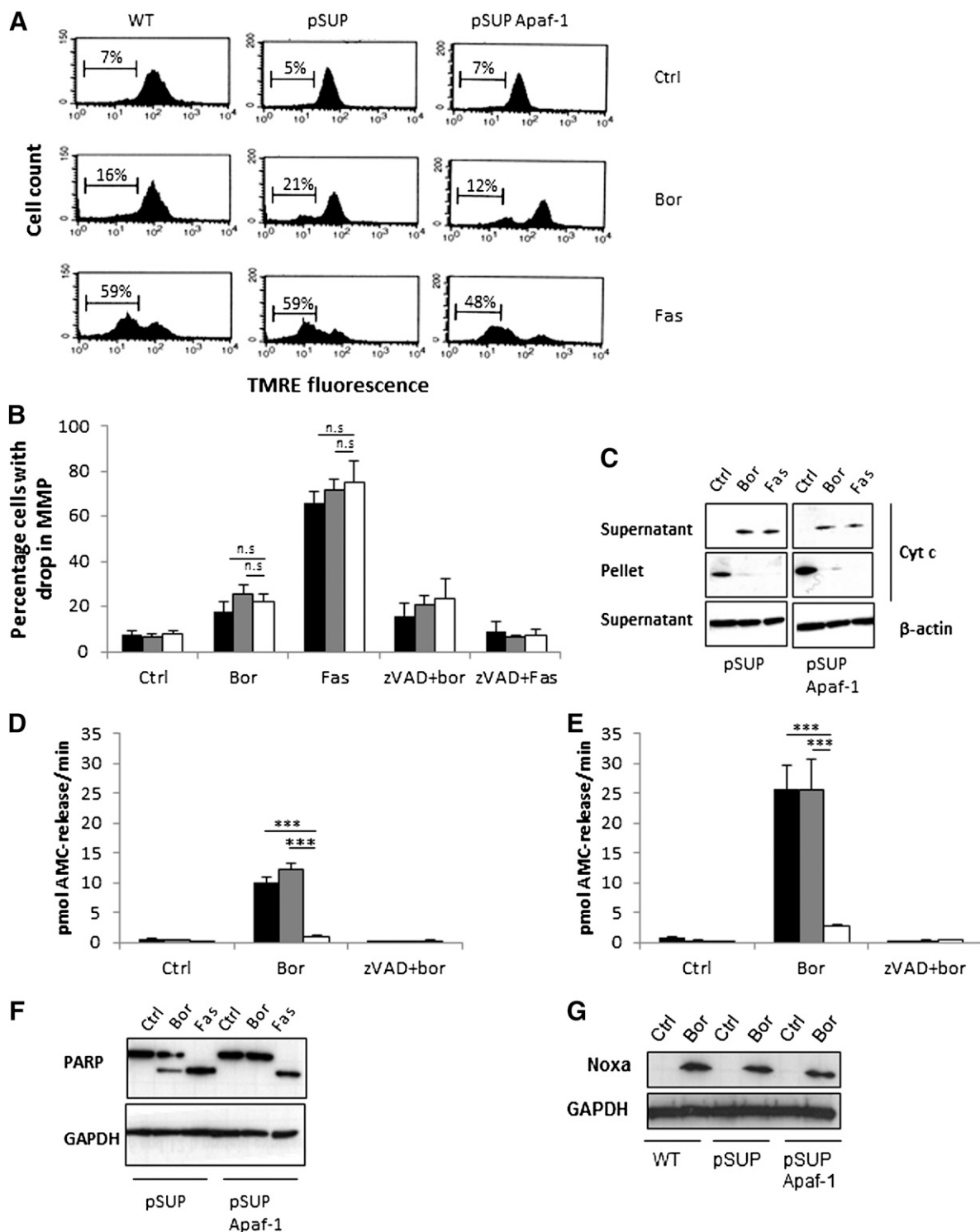


Fig. 2. Bortezomib induces cytochrome *c* release and dissipation of mitochondrial membrane potential irrespective of Apaf-1 expression. (A) Mitochondrial membrane potential in Jurkat WT, pSUPER, and pSUPER-Apaf-1 after treatment with 50 nM bortezomib for 6 hours. The percentages of cells with a drop in potential are shown. (B) Quantification of the drop in mitochondrial membrane potential (see panel A for representative results) in Jurkat WT (black bars), pSUPER (gray bars), and pSUPER-Apaf-1 (white bars) after treatment with 50 nM bortezomib or anti-Fas antibody (250 ng/ml) for 6 hours. The pan-caspase inhibitor, zVAD-fmk (20 μ M) was added 30 minutes prior to the addition of bortezomib and anti-Fas antibody. (C) Western blot analysis of the expression of cytochrome *c* in the supernatant (cytosolic) and pellet (mitochondria) fractions of Jurkat pSUPER and pSUPER-Apaf-1 after treatment with bortezomib or anti-Fas antibody (250 ng/ml) for 6 hours. (D and E) Caspase-3-like activity in Jurkat WT, pSUPER and pSUPER-Apaf-1 after treatment with 50 nM bortezomib for 6 hours (D) or 14 hours (E). The pan-caspase inhibitor zVAD-fmk (20 μ M) was added 30 minutes prior to the addition of bortezomib. (F) Jurkat pSUPER and pSUPER-Apaf-1 were treated with 50 nM bortezomib or anti-Fas antibody (250 ng/ml) for 14 hours, and the level of PARP and cleaved PARP (a marker of caspase-3 activation) was determined. GAPDH was monitored to control for equal loading. (G) Jurkat WT, pSUPER, and pSUPER-Apaf-1 cells were treated with or without 50 nM bortezomib for 6 hours, and the expression of Noxa was determined using specific antibodies. GAPDH was monitored to control for equal loading. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

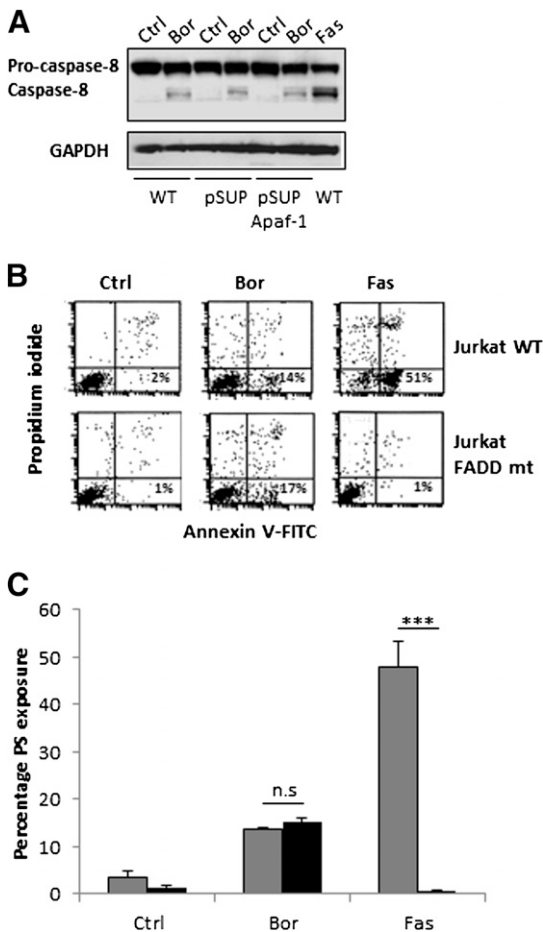


Fig. 3. Bortezomib-induced apoptosis is FADD-independent. (A) Western blot analysis of procaspase-8 cleavage after treatment with bortezomib for 14 hours in Jurkat WT, pSUPER, and pSUPER-Apaf-1. Jurkat WT treated with anti-Fas antibody (250 ng/ml) for 6 hours was used as a positive control for procaspase-8 cleavage. (B) Jurkat WT (clone A3) and Jurkat FADD mt were treated with 50 nM bortezomib or agonistic anti-Fas antibody (250 ng/ml) for 14 hours, and apoptosis was assessed using the annexin V-FITC assay. The percentages of apoptotic (PS-positive cells) are indicated. (C) Quantification of PS exposure in Jurkat WT (clone A3) (gray bars) and Jurkat FADD mt (black bars) was treated as indicated in panel (B). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

assessed relative to 96 biologic groups. Assessment of the six leukemic subtypes relative to 96 biologic groups demonstrated consistently increased expression of Apaf-1 in ALL, acute myeloid leukemia, chronic myeloid leukemia, and precursor T-lymphoblastic leukemia (Table 3). In contrast, the acute promyelocytic leukemia subtype displayed an inconsistent pattern, and two of three Apaf-1 probes for myelogenous leukemias were not significantly differentially expressed. The subtypes within the nonleukemic blood group showed increased expression for B-cell lymphoma, whereas anaplastic large cell lymphoma and CD138+ plasma cell lymphoma displayed variable expression patterns of the three probes targeting Apaf-1 (Supplemental Table 1). Our assessment of the 18 cell lines in the blood neoplasm cell line group indicated similar Apaf-1 expression levels, with the exception of the promyelocytic leukemic cell line HL60 that showed increased levels (Supplemental Table 2). Overall, these bioinformatics data from multiple tumor types show elevated Apaf-1 expression in several hematologic malignancies, thus

implying the potential for proteasome inhibitors including bortezomib in treatment regimens for such patients.

Discussion

Proteasome inhibitors including bortezomib have attracted considerable attention as potential anticancer agents, but the mechanism(s) by which proteasome inhibitors induce apoptosis is poorly understood. In the present study, we provided evidence using the human Jurkat T-cell leukemic cell line with or without stable silencing of the key adaptor protein, Apaf-1, that bortezomib-induced apoptosis but not Fas (death receptor)-mediated apoptosis is dependent on Apaf-1 expression. Furthermore, we noted that expression of Apaf-1 was variable in a panel of pediatric ALL patient samples, and Apaf-1 expression was absent altogether in one patient. The primary cells presented with a high degree of spontaneous apoptosis upon ex vivo culture; however, the Apaf-1-deficient sample presented the lowest sensitivity toward bortezomib-induced apoptosis, thus providing correlative evidence for a role of Apaf-1 in bortezomib-induced cell killing (defects in other apoptosis signaling pathways may also come into play). Moreover, bioinformatics analysis of publically available transcriptomics data from multiple tumor types demonstrated elevated Apaf-1 expression in several hematologic malignancies, including (among the six leukemic subtypes) ALL, acute myeloid leukemia, chronic myeloid leukemia, and precursor T-lymphoblastic leukemia.

We have previously reported that human Raji lymphoma cells that are deficient for cytosolic Apaf-1 expression (Sun et al., 2005) are resistant to the proteasome inhibitor lactacystin (Sun et al., 2007). As shown in the present study, these cells are also insensitive to bortezomib-induced caspase-3 activation, whereas the T-ALL cell line RPMI 8402 with cytosolic Apaf-1 expression is highly sensitive to bortezomib-induced caspase-3 activation. In addition, transient interference of Apaf-1 expression in the RPMI 8402 cell line decreased the responsiveness toward bortezomib-induced apoptosis, further supporting the Apaf-1-dependent cell death-inducing effects of bortezomib in cells of leukemic origin. Fang et al. (2012) reported very recently that the receptor tyrosine kinase C-KIT that is aberrantly expressed in acute myeloid leukemia binds and phosphorylates heat shock protein 90, which sequesters Apaf-1; the authors also reported that bortezomib dephosphorylates Hsp90 and releases Apaf-1, resulting in formation of the apoptosome and activation of caspases. Hence, these recent findings further point toward a role for Apaf-1 in bortezomib-triggered apoptosis, at least in certain types of leukemia.

The results reported here are in accordance with the previous observation that the proteasome inhibitors, lactacystin and MG132, trigger cytochrome *c*-dependent caspase activation in B-CLL cells (Almond et al., 2001). Moreover, our data show that bortezomib induces apoptosis by regulating pathways that are different from those activated upon Fas ligation. The upstream signaling events merit attention: how does inhibition of the 26S proteasome by bortezomib lead to activation of mitochondria? We can apparently exclude DNA damage-induced responses, as we could not detect significant DNA damage in bortezomib-treated cells. We can also exclude p53-dependent signaling, since Jurkat cells lack p53 (Karpnich et al., 2006). Some studies have suggested that

TABLE 1
Characteristics of ALL patients

| Patient | Age (years) | Immune Origin | Karyotype | Leukocytes $\times 10^9$ /liter | Status | Apaf-1/ β -Actin Ratio | % Cell Death in Medium Alone (24 h) | % Cell Death in Bortezomib Treated (24 h) |
|---------|-------------|---------------|---------------------------------------|---------------------------------|-------------------------------|------------------------------|-------------------------------------|---|
| ALL 1 | 3 | T cell | 46XX | 519 | CR | 0.4 | 88 | 96 |
| ALL 2 | 8 | T cell | 46,XX,del(11)(q23-25,del(12)(p13) | 588 | MRD | 0.5 | 40 | 82 |
| ALL 3 | 2.5 | B precursor | 46XYt(10;14) | 28.1 | CR2 | 0.7 | 94 | 98 |
| ALL 4 | 14 | T cell | 47,XY,+del(6)(q?21),t(11;19)(q23;p13) | 144.8 | CR | 0.4 | 77 | 98 |
| ALL 5 | 16 | AML/T cell | 46XY,t(7;12)(p15;p13)[19]/46,XY[7] | 17.9 | MRD | 0.6 | 68 | 84 |
| ALL 6 | 11 | T cell | 46XY[27] | 4.1 | Relapsed disease ^d | 0.1 | 73 | 75 |
| ALL 7 | 6 | T cell | 46,XY,del(6)(q2?1)23] | 316 | CR | 0.5 | 45 | 87 |

CR, complete remission; CR2, complete remission after first relapse; MRD, minimal residual disease.

activation of caspase-8, normally activated following death receptor ligation (Calvaruso et al., 2006) or in combination treatment with other proteasome inhibitors (Chauhan et al., 2008), may play a role in bortezomib-induced apoptosis (Liu et al., 2007; Zhao et al., 2008). We noted that bortezomib was able to induce low levels of procaspase-8 processing in Jurkat cells, whereas Fas-induced processing of procaspase-8 was more prominent. However, Jurkat FADD-deficient and WT cells showed the same sensitivity toward bortezomib-induced apoptosis, whereas FADD-deficient cells were completely resistant to Fas-induced apoptosis; this finding demonstrates that, in this model, death receptor activation is not required for the apoptosis-inducing effects of bortezomib. Other investigators have reported that proteasome inhibitors trigger Noxa-mediated apoptosis (Qin et al., 2005; Gomez-Bougie et al., 2007). Indeed, silencing of Noxa, a BH3-only protein that engages the intrinsic, mitochondria-dependent apoptosis pathway, has been shown to decrease bortezomib-induced apoptosis in CLL cells (Baou et al., 2010). Thus, p53-independent upregulation of Noxa (Pérez-Galán et al., 2006) constitutes a potential link between proteasome inhibition and mitochondrial activation, possibly explaining why bortezomib-induced mitochondrial events are caspase-independent. Our data support this model, as bortezomib triggers the induction of Noxa in the p53-null Jurkat cell line regardless of Apaf-1 expression. In sum, our data indicate that cytochrome *c*/Apaf-1-dependent activation of caspases downstream of mitochondria serves as a critical step leading to apoptosis in response to bortezomib (Fig. 5). In contrast, Fas-induced apoptosis in Jurkat (type II) cells appears to be Apaf-1-independent but may still very well depend on release of mitochondrial factors, including second mitochondrial activator of caspases (smac) or Omi/HtrA2 (Shawgo et al., 2009).

A recent study published since the inception of the present work offers further support for a role for Noxa in the regulation of bortezomib-induced apoptosis in lymphoid cells. Smith et al. (2011) reported that Noxa was shown to interact with Bcl-2. Notably, the authors reported that Noxa siRNA markedly diminished the ability of bortezomib to induce apoptosis in Jurkat cells. Moreover, bortezomib-induced killing of Jurkat cells was enhanced by the Bcl-2/Bcl-xL antagonist ABT-737 [*N*-(4-[4-(4'-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl)-4-(3-dimethylamino-1-phenylsulfanylmethyl-propylamino)-3-nitro-benzenesulfonamide] and by Bcl-2 downregulation but diminished by Bcl-2 overexpression, suggesting that Bcl-2 overexpression is a potential mechanism of bortezomib resistance in lymphoid cells. Overall, it was concluded that Bcl-2 is able to protect cells from bortezomib-induced apoptosis, at least in part by interacting with proapoptotic Noxa (Smith et al., 2011).

Matondo et al. (2010) have suggested that proteasome activation status reflects bortezomib sensitivity of hematologic malignancies, including acute myeloid leukemia. Our results using the Jurkat T-cell line suggest, in addition, that the level of expression of Apaf-1 is a critical determinant of the sensitivity of leukemic cells to bortezomib. We determined that the Apaf-1 protein expression varied in leukemic blasts from patients with ALL, albeit in a small set of patients. Jia et al. (2003) have previously reported that Apaf-1 deficiency is one mechanism underlying primary ALL blast resistance to cytochrome *c*-induced activation of caspase-3. The downregulation of Apaf-1 expression in different subtypes of leukemia was suggested to be due to DNA methylation (Fu et al., 2003). Indeed, loss of Apaf-1 expression due to transcriptional silencing as a result of hypermethylation of DNA was also reported in metastatic melanoma cells (Soengas et al., 2001). To complement our in vitro studies, we determined the expression of Apaf-1 in normal and cancer samples using the public Human Gene Expression Map (Lukk et al., 2010). The in silico analysis indicated that the level of expression of Apaf-1 differs significantly between different subtypes of leukemia. It will be of interest to determine whether the expression of Apaf-1 correlates with response to treatment with proteasome inhibitors in a larger number of samples. Moreover, the bioinformatics analysis demonstrated that Apaf-1 mRNA expression is increased in the Jurkat cell line, derived from

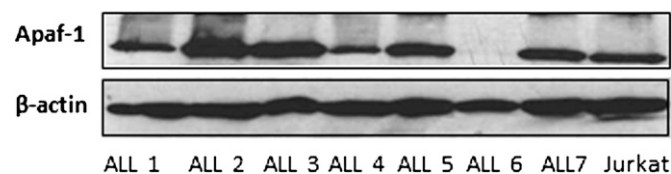


Fig. 4. Apaf-1 expression in ALL patient cells. The protein expression of Apaf-1 was determined in total cell lysates from leukemic blasts from pediatric ALL patients (refer to Table 1 for details). β -Actin expression was monitored to control for equal loading of protein.

TABLE 2

Analysis of APAF1 expression in the Human Gene Expression map for 15 meta groups

| Meta-group | APAF1 | | | | | |
|---|-------------------|-----------------------|-------------------|------------------------|-------------------|------------------------|
| | 204859_s_at | | 211553_s_at | | 211554_s_at | |
| | Expression Change | P Value | Expression Change | P Value | Expression Change | P Value |
| Blood neoplasm cell line (n = 166) | +/- | 0.282 | + | 5.37×10^{-7} | + | 1.09×10^{-8} |
| Blood non neoplastic disease (n = 388) | + | $<1 \times 10^{-10}$ | + | 8.87×10^{-5} | + | $<1 \times 10^{-10}$ |
| Breast cancer (n = 672) | - | $<1 \times 10^{-10}$ | - | $<1 \times 10^{-10}$ | - | $<1 \times 10^{-10}$ |
| Germ cell neoplasm (n = 71) | +/- | 0.293 | - | 5.18×10^{-10} | +/- | 0.88 |
| Leukemia (n = 567) | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ |
| Nervous system neoplasm (n = 112) | +/- | 0.059 | - | $<1 \times 10^{-10}$ | - | $<1 \times 10^{-10}$ |
| Non breast carcinoma (n = 258) | - | 4.45×10^{-9} | - | 9.65×10^{-8} | - | 1.39×10^{-10} |
| Non leukemic blood neoplasm (n = 334) | - | $<1 \times 10^{-10}$ | + | 2.55×10^{-8} | + | $<1 \times 10^{-10}$ |
| Non neoplastic cell line (n = 262) | +/- | 0.954 | + | 0.012 | + | 0.025 |
| Normal blood (n = 467) | + | $<1 \times 10^{-10}$ | + | 1.61×10^{-10} | + | $<1 \times 10^{-10}$ |
| Normal solid tissue (n = 566) | - | $<1 \times 10^{-10}$ | +/- | 0.542 | - | 6.35×10^{-4} |
| Other neoplasm (n = 167) | - | 1.71×10^{-5} | - | 1.62×10^{-5} | - | 7.2×10^{-6} |
| Sarcoma (n = 104) | +/- | 0.233 | - | 2.16×10^{-7} | - | 0.017 |
| Solid tissue neoplasm cell line (n = 831) | - | $<1 \times 10^{-10}$ | + | 1.52×10^{-6} | + | $<1 \times 10^{-10}$ |
| Solid tissue non-neoplastic disease (n = 377) | - | $<1 \times 10^{-10}$ | +/- | 0.112 | +/- | 0.849 |

Assessment of APAF1 expression in the Human Gene Expression Map (Lukk et al., 2010). The database encompasses 5372 samples hybridized to the Affymetrix HG-U133A platform for comparison of the expression levels relative to 15 meta-groups. The number of samples is listed for the respective meta groups. Direction of change and P values, respectively, are indicated. Significant increased expression is indicated by plus (+), significant decreased expression indicated by minus (-) and non-differentially expressed is indicated by +/- . A P value of 0.05 was considered significant.

a 14-year old patient with ALL more than three decades ago (Schneider et al., 1977).

Cell lines as model systems of tumors are essential in cancer research. However, most cell lines have been selected under in vitro conditions for a long period of time, which may affect many cellular pathways and processes (Sandberg and Emberg, 2005). Our results show that the Jurkat cell line is a good model, as the mRNA expression matches the expression in primary patient samples.

The proteasome is a promising emerging target in the treatment of leukemia, and both preclinical studies and clinical trials indicate that proteasome inhibition acts synergistically with conventional chemotherapeutic agents and steroids to improve antileukemic responses (Vink et al., 2006). Moreover, the utility of expression profiling of components of the apoptotic machinery, including Apaf-1, was recently demonstrated by Ragusa et al. (2010). The authors noted that downregulation of Apaf-1 and upregulation of cIAP1 and cIAP2 was associated with a predisposition to minimal residual disease and relapse in AML patients. Apaf-1 is a critical component of the apoptosome, a protein complex that acts as a cytosolic platform for the activation of executioner caspases downstream of mitochondria (Fadeel et al., 2008). The current in vitro studies

clearly show that bortezomib-induced apoptosis in human leukemia cells is dependent upon the expression of Apaf-1. More studies are needed on primary patient samples. Nevertheless, the present results suggest that individual tailoring of anticancer treatment based on bortezomib or similar drugs should take into consideration not only proteasome status (Matondo et al., 2010) but also apoptosome status.

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Authorship Contributions

Participated in research design: Ottosson-Wadlund, Söderhäll, Grandér, Robertson, Fadeel.

Conducted experiments: Ottosson-Wadlund, Ceder, Preta.

Contributed analytical tools: Pokrovskaja, Heyman.

Performed data analysis: Ottosson-Wadlund, Ceder, Preta, Grandér, Hedenfalk, Fadeel.

Wrote or contributed to the writing of the manuscript: Ottosson-Wadlund, Ceder, Grafström, Hedenfalk, Fadeel.

TABLE 3

Assessment of APAF1 in different human leukemia relative to the 96 biologic groups Human Expression Map

| Group | APAF1 | | | | | |
|---|-------------------|----------------------|-------------------|-----------------------|-------------------|-----------------------|
| | 204859_s_at | | 211553_s_at | | 211554_s_at | |
| | Expression Change | P Value | Expression Change | P Value | Expression Change | P Value |
| Acute lymphoblastic leukemia (n = 95) | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ | + | 5.49×10^{-9} |
| Acute myeloid leukemia (n = 295) | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ |
| Acute promyelocytic leukemia (n = 18) | - | 0.018 | + | 1.27×10^{-6} | +/- | 0.19 |
| Chronic myeloid leukemia (n = 44) | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ | + | $<1 \times 10^{-10}$ |
| Myelogenous leukemia (n = 37) | +/- | 0.269 | + | 6.69×10^{-4} | +/- | 0.685 |
| Precursor T-lymphoblastic leukemia (n = 59) | + | 0.003 | - | $<1 \times 10^{-10}$ | + | 0.003 |

Assessment of APAF1 expression in the Human Gene Expression Map (Lukk et al., 2010). The database encompasses 5372 samples hybridized to the Affymetrix HG-U133A platform and enables the comparison of the expression levels relative to 96 biologic groups. Here, the expression of APAF1 in subtypes of leukemia was determined relative to the 96 groups (Kwiecinska et al., 2011). Direction of change and P values, respectively, are indicated. Significant increased expression is indicated by plus (+), significant decreased expression indicated by minus (-) and non-differentially expressed is indicated by +/- . A P value of 0.05 was considered significant.

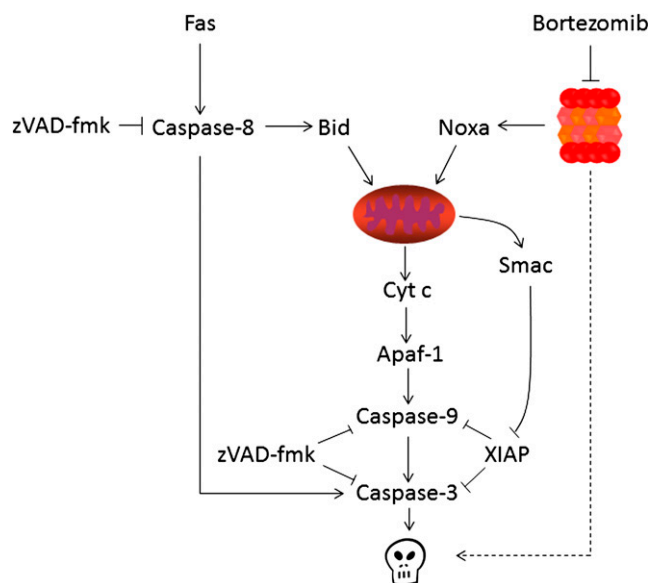


Fig. 5. Schematic view of bortezomib-induced apoptosis. Bortezomib inhibits the proteasome, leading to upregulation of Noxa and activation of Bid and cytochrome *c*. Smith et al. (2011) recently demonstrated that Noxa interacts with Bcl-2 (not shown) and that Bcl-2 overexpression is a potential mechanism of bortezomib resistance. In cells expressing Apaf-1, cytochrome *c* serves as a coactivator of the apoptosome, with ensuing caspase activation and dismantling of the cell by apoptosis. In Apaf-1-deficient cells, bortezomib-induced apoptosis is blocked, as demonstrated in the present study. Ligation of the death receptor, Fas (also known as APO-1 or CD95) leads to caspase activation and apoptosis independently of cytochrome *c*-dependent apoptosome activation (see also Shawgo et al., 2009). Following prolonged exposure to bortezomib, some Apaf-1-independent apoptosis is observed (dotted line). This could theoretically occur through caspase-8 and/or caspase-9 activation independently of the DISC and the apoptosome, respectively, but further studies are needed to clarify whether this is the case.

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