The Proteasome Inhibitor Bortezomib Acts Independently of p53 and Induces Cell Death via Apoptosis and Mitotic Catastrophe in B-Cell Lymphoma Cell Lines

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Abstract
Bortezomib is a proteasome inhibitor with proven efficacy in multiple myeloma and non–Hodgkin’s lymphoma. This study reports the effects of bortezomib in B-cell lymphoma cell lines with differing sensitivity to bortezomib to investigate factors that influence sensitivity. Bortezomib induced a time- and concentration-dependent reduction in cell viability in five lymphoma cell lines, with EC_{50} values ranging from 6 nmol/L (DHL-7 cells) to 25 nmol/L (DHL-4 cells) after 72 h. Bortezomib cytotoxicity was independent of p53 function, as all cell lines exhibited mutations by sequence analysis. The difference in sensitivity was not explained by proteasome or nuclear factor-κB (NF-κB) inhibition as these were similar in the most and least sensitive cells. NF-κB inhibition was less marked than that of a specific NF-κB inhibitor, Bay 11-7082. Cell cycle analysis showed a marked G2-arrested population in the least sensitive DHL-4 line only, an effect that was not present with Bay 11-7082 treatment. Conversely, in DHL-7 cells, bortezomib treatment resulted in cells moving into an aberrant mitosis, indicative of mitotic catastrophe that may contribute to increased sensitivity to bortezomib. These studies show that although bortezomib treatment had similar effects on apoptotic and NF-κB signaling pathways in these cell lines, different cell cycle effects were observed and induction of a further mechanism of cell death, mitotic catastrophe, was observed in the more sensitive cell line, which may provide some pointers to the difference in sensitivity between cell lines. An improved understanding of how DHL-7 cells abrogate the G2-M cell cycle checkpoint may help identify targets to increase the efficacy of bortezomib.

Introduction
The ubiquitin-proteasome pathway is essential for the regulated degradation of intracellular proteins in eukaryotic cells (1, 2). The proteasome degrades damaged, oxidized, or misfolded proteins but also plays a vital role in degrading proteins that regulate the cell cycle, activate transcription factors, and control cell growth. The proteasome is an ATP-dependent multicatalytic enzyme expressed in the nucleus and cytoplasm. The complete 26S proteasome complex comprises the 19S regulatory subunits that recognize and remove the polyubiquitinated chain from proteins and a 20S proteolytic core (3). This core particle contains catalytic sites with chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydro-lase-like activity (4). The proteasome plays a key role in cellular metabolism and has important functions in the control of malignancy by its direct or indirect action on transcription factors, such as nuclear factor-κB (NF-κB), cell cycle proteins, signaling molecules, and tumor suppressor genes. Many of these proteins are involved in maintaining the malignant phenotype (5).

Bortezomib (Velcade) is a specific inhibitor of the chymotryptic activity of the proteasome that has cytotoxic activity in several malignant cell lines. It has shown proven efficacy in phase II/III trials in patients with multiple myeloma and malignant lymphoma (6–8). The key component of its mechanism of action was initially thought to be inhibition of NF-κB, which acts as a transcription factor for antiapoptotic proteins, such as Bel-2, c-IAP2, and survivin, but is also known to stabilize p53 and cell cycle proteins, such as p21 and p27 (9–11). We have recently reported large differences in sensitivity to bortezomib in primary lymphoma samples, which correlated with sensitivity to the drug in patients treated in a phase II clinical trial (12). The aim of this study was to investigate the effects of bortezomib in B-cell lymphoma cell lines with differing sensitivity to bortezomib to investigate possible mechanisms of cell death and factors that may influence sensitivity to the drug.

Materials and Methods

Cell Lines
A panel of five B-cell lymphoma cell lines, all derived from patients with transformed follicular lymphoma, was used. DHL-4 and DHL-7 cell lines were kind gifts from Dr. Margaret Shipp (Dana-Farber Cancer Institute, Boston, MA). DoHH2, CRL, and SUD4 cell lines were kind gifts from Prof. Finharr Cotter (The Centre for Haematology, Bart’s and the London School of Medicine, London, United Kingdom). All cells were grown in suspension in RPMI 1640 supplemented with 10% FCS, 1% glutamine, and 1% penicillin (10,000 units/mL) and streptomycin (10,000 μg/mL) at 37°C in a humidified atmosphere with 5% CO₂.

Drugs
Bortezomib [Velcade; [(1R)-3-methyl-1-[(2S)-1-oxo-3-phenyl-2-[(pyrazinyl carbonyl) amino]propyl] amino]butyl] boronic acid] was kindly provided by Millennium Pharmaceuticals (Cambridge, MA), and Bay 11-7082 [(E)-3-[(4-methylphenyl)sulfonyl]-2-propenenitrile] was obtained from Merck Biosciences Ltd. (Nottingham, United Kingdom).

Cell Number and Viability
Cell number and viability were determined by trypan blue exclusion staining. The concentration required to reduce viability by 50% (EC_{50}) at day 3 was calculated using a sigmoidal E_{max} model in GraphPad Prism.

20S Proteasome Activity
Proteasome activity in cytosolic extracts (without protease inhibitors) was examined by measuring the release of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate.
Suc-Leu-Leu-Val-AMC, which is hydrolyzed by chymotryptic activity of the 20S proteasome. The free AMC fluorescence was quantified using a 355/444-nm filter set in a 96-well plate reader. Results were expressed as the percentage of control proteasome activity.

**p53 Mutation Analysis**

**Mutation screening.** DNA from the cell lines was prepared and examined for mutations by single-strand conformational polymorphism (SSCP) and sequence analysis. PCR amplification of the entire coding region of TP53 was carried out using AmpliTaq Gold polymerase (Applied Biosystems, Warrington, United Kingdom). For DNA sequencing, primers were unlabeled; for SSCP, both primers were 5' labeled with Fam or Hex fluorochromes. For SSCP analysis, fluorescent PCR products were diluted 1:40 with water and 1 μL of the diluted product was mixed with 0.5 μL of ROX 500 size standards and 10.5 μL of HiDi formamide (Applied Biosystems). The samples were denatured at 95°C for 2 min and snap cooled on ice. SSCP analysis was carried out on a 3100 Genetic Analyzer (Applied Biosystems) at 18°C and 30°C using 5% GeneScan polymer containing 10% glycerol and 1× Tris-TAPS-EDTA buffer (Applied Biosystems). Data were analyzed using GeneScan 3.1.1 and Genotyper 2.5 software (Applied Biosystems). Mutation detection was by visual inspection of electropherogram traces. Sequencing reactions were carried out using ABI Prism BigDye Terminator Cycle Sequencing kit version 2 (Applied Biosystems) and SeqMan (DNAStar, Madison, WI) software and by visual inspection of electropherograms.

**Western Blot Analysis**

Protein extracts were prepared by lysing cells in Triton X-100 lysis buffer [1× PBS, Triton X-100 (1% v/v), sodium deoxycholate (0.5% w/v), SDS (0.1% w/v), EDTA (1 mmol/L)] with the addition of protease and phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A). Samples were stored at -70°C until Western blot analysis. The protein content of each lysate was determined by use of the Bradford reagent (Sigma-Aldrich Co., Poole, United Kingdom). Cellular lysate (20-50 μg) was resolved by SDS-PAGE, and the gels were then electroblotted onto nitrocellulose membranes. The following primary antibodies were used: rabbit anti-human Bax, mouse anti-human caspase-8, and rabbit anti-human caspase-9 (Oncogene Research Products, Boston, MA), mouse anti-human Bcl-xl, mouse anti-human poly(ADP-ribose) polymerase (PARP), and mouse anti-human β-actin (DAKO Ltd., Cambridge, United Kingdom), mouse anti-human actin (Santa Cruz Biotechnology, Santa Cruz, CA). A horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 was used as secondary antibody for mouse primary antibodies, and anti-rabbit IgG antibody was used for the rabbit (DAKO). The protein bands were visualized using an enhanced chemiluminescence visualization system (ECL Plus, Amersham Life Sciences, Little Chalfont, United Kingdom).

**NF-κB-Binding Activity**

DHL-4 and DHL-7 cells were cultured at the EC_{50} and 2 × EC_{50} concentrations (cell viability) of bortezomib and Bay 11-7082 for 4, 8, and 24 h, and cell lysates were prepared (Bay 11-7082 EC_{50} = 8 μmol/L for DHL-4 cells and 3.5 μmol/L for DHL-7 cells). The p65 DNA-binding activity of NF-κB was quantified by ELISA by means of the Trans-AM NF-κB p65 Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA) according to the manufacturer's instructions. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p65, visualized by anti-IgG-HRP conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 620 nm. Specificity was established by incubation with a wild-type consensus oligonucleotide (5'-AGTTGAGGAGCTTTCACACGC-3'), which competes with the substrate for binding. This was subtracted from the value obtained for the consensus DNA sequence in the absence of competitor. All samples were analyzed in duplicate.

**Cell Cycle Distribution**

Distinct phases of the cell cycle were distinguished by DNA staining with the fluorescent dye propidium iodide and measured by flow cytometry. Cells were washed in ice-cold PBS, fixed in 70% ethanol, and stained with 500 μg/mL propidium iodide (50 μg/mL and 50 μg/mL RNase A in PBS). Cells were washed again in PBS. Five thousand cells were analyzed for each data point, and percentage of cells in sub-G_1 (apoptotic), G_1, S, and G_2–M phases were determined using the cell cycle analysis program WinMDI.

![Figure 1](cancerresearch.aacrjournals.org)
Mitochondrial Membrane Potential
Cells (5x10^5/mL) were plated in 24-well plates and treated with bortezomib for 24 h. After centrifugation (400 g for 5 min) and washing, cells were resuspended in 500 μL of I × JC-1 reagent (Stratagene, La Jolla, CA) in assay buffer and incubated for 20 min at 37°C and 5% CO2 in a humidified incubator. Cells were then washed twice in cell culture medium, resuspended in 500 μL assay buffer, and immediately analyzed by flow cytometry (FACSCalibur System, BD Biosciences, Oxford, United Kingdom). JC-1 staining was detected simultaneously at 515 to 545 nmol/L (FL-1, JC-1 aggregates, red staining, healthy cells) and 546 to 606 nmol/L (FL-2, JC-1 monomers, green staining, apoptotic cells). Results are presented as cells with decreased mitochondrial membrane potential (MMP; in %).

Immunofluorescent Staining
The monoclonal antibody MPM2 (Biomol, Hamburg, Germany) was used to determine whether cells had entered mitosis by recognizing mitosis-specific phosphoepitopes. DHL-4 and DHL-7 cells were treated with EC50 concentrations (cell viability) of bortezomib for 28 h, as this was the time point where the greatest difference in mitotic entry proteins was observed in preliminary experiments. Cells (25,000) were cytocentrifuged onto a microscope slide using a Shandon Southern Cytospin (Shandon, Pittsburgh, PA), air dried, and fixed in 2% paraformaldehyde for 10 min. Cells were then permeabilized and incubated in a blocking solution (3% normal goat serum in 0.1% Tween 20 in PBS) for 30 min before overnight incubation at 4°C with anti-phospho-Ser/Thr-MPM2 (Upstate, Dundee, Scotland) antibody at a dilution of 1:2,000. After washing, slides were incubated for 2 h in the dark with phycoerythrin (PE)-conjugated anti-mouse secondary antibody (DAKO) at a 1:100 dilution, then incubated with 50 ng/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS for 2 min, washed, and mounted in fluorescent mounting medium (DAKO). Slides were then viewed under a Zeiss Axioskop fluorescence microscope (Zeiss, Welwyn Garden City, United Kingdom) attached to a CCD camera (Photometric Ltd., Tucson, AZ) driven by IPL Labs Spectrum and SmartCapture (Digital Scientific, Cambridge, United Kingdom) software. The filter wheel was set at Texas red (excitation, 540–580 nm/emission, 600–660 nm) and DAPI (excitation, 310–380 nm/emission, 435–485 nm).

Results
Bortezomib induces a time- and concentration-dependent reduction in cell viability that is independent of p53 function. The effect of bortezomib treatment was assessed in a panel of five cell lines, which showed a variation in sensitivity to bortezomib (Fig. 1A).

Figure 2. A and B, bortezomib activates intrinsic and extrinsic pathways for apoptosis. DHL-4 and DHL-7 cell lines were treated with the EC50 concentration of bortezomib for 18 to 72 h, and protein extracts were analyzed by Western blotting with the indicated antibodies. PARP, caspase-8, and caspase-9 were activated by bortezomib by the appearance of cleavage fragments (CF) from 18 h in DHL-4 and DHL-7 cells. β-Actin was used as a loading control. Bortezomib has varied effects on Bcl-2 family proteins in sensitive and resistant cell lines. Increasing duration of exposure to bortezomib resulted in an increase in Bcl-2 in DHL-4 and DHL-7 cell lines. Bax was not expressed in DHL-4 cells but increased in DHL-7 cells with bortezomib treatment. Bak protein expression increased in DHL-4 cells but was unaffected in DHL-7 cells after exposure to bortezomib. β-Actin was used as a loading control. C, these changes did not result in a change in MMP in either cell line at 6 nmol/L measured by fluorescence-activated cell sorting analysis at 24 h. At 25 nmol/L bortezomib, the percentage of cells with decreased MMP was increased in the more sensitive DHL-7 cells. Columns, mean of three separate experiments; bars, SD.

Figure 3. The specific NF-κB inhibitor Bay 11-7082 causes greater NF-κB inhibition than bortezomib in DHL-4 (A and B) and DHL-7 (C and D) cell lines. Cells were treated with equipotent concentrations (EC50 and 2 × EC50) of bortezomib and Bay 11-7082 for 4, 8, and 24 h, and p65 DNA-binding activity of NF-κB was quantified by ELISA (Bay 11-7082 EC50: 6 μmol/L in DHL-4 cells and 3.5 μmol/L in DHL-7 cells). Columns, mean of three separate experiments; bars, SD.
50%) after exposure to bortezomib for 72 h is shown in Fig. 1B. Shorter durations of exposure (4–12 h) followed by incubation in drug-free medium out to 72 h resulted in similar differences in sensitivity between sensitive (DHL-7) and more resistant (DHL-4) cell lines (data not shown). All cell lines had nonfunctional p53 mutations (Fig. 1B) apart from DoHH2, which had a heterozygous mutation with evidence of function, although reduced, when examined using a functional p53 assay (FASAY; data not shown; ref. 13).

Bortezomib inhibits proteasome activity that is sustained for 24 h in sensitive and more resistant cell lines. In view of differences in sensitivity between DHL-4 and DHL-7 cells, the effect of bortezomib on proteasome activity was examined to elucidate whether less marked inhibition of the proteasome may explain reduced sensitivity in the DHL-4 cell line. There was no difference in total proteasome activity in untreated cells (data not shown). Bortezomib treatment resulted in concentration-dependent inhibition of proteasome activity in both cell lines, observed by 4 h and sustained until 24 h (Fig. 1C and D). There was no significant difference between DHL-4 and DHL-7 proteasome inhibition at 6 nmol/L (59.5 ± 10.5% versus 64.3 ± 9.6%; P = 0.43). At 24 h, there was a trend toward greater proteasome inhibition in DHL-7 cells than DHL-4 cells, a difference that reached statistical significance at 12 nmol/L (P = 0.03; Fig. 1D).

Bortezomib treatment causes cell death via activation of caspases and apoptosis and affects Bcl-2 family of proteins. The most sensitive cell line (DHL-7) and the most resistant cell line (DHL-4) were used to examine possible mechanisms of cell death and factors that may determine sensitivity. Treatment with the EC50 concentration (% viability) of bortezomib resulted in activation of caspase-8 and caspase-9 with PARP cleavage observed in both the DHL-4 and DHL-7 cell lines (Fig. 2A). Bortezomib treatment resulted in an increase in the antiapoptotic protein Bcl-2 in both cell lines and differing effects on proapoptotic proteins. Bortezomib resulted in an increase in Bax in DHL-7 cells. This protein was not expressed in DHL-4 cells, but here, Bak was increased after exposure to bortezomib (Fig. 2B). These changes only resulted in a decrease in MMP in the more sensitive DHL-7 cells at the higher concentration (25 nmol/L) at 24 h (Fig. 2C). After 72 h of exposure to 25 nmol/L bortezomib, changes were similar in DHL-4 and DHL-7 cells (data not shown).

NF-κB–binding activity after treatment with bortezomib or the specific NF-κB inhibitor Bay 11-7082. Treatment of DHL-4 and DHL-7 cells with EC50 concentrations (% viability) of bortezomib resulted in concentration-dependent inhibition of p65 NF-κB–binding activity at 4, 8, and 24 h. An equipotent concentration (% viability) of Bay 11-7082, however, resulted in greater inhibition of activity than bortezomib at all times, which

Figure 4. Bortezomib treatment results in different cell cycle effects in DHL-4 and DHL-7 cell lines, whereas Bay 11-7082 treatment has equivalent effects in the two cell lines. A and B, cells were exposed to the EC50 concentrations of bortezomib and Bay 11-7082 for 24 to 72 h, and cell cycle distribution was analyzed by flow cytometry. Bortezomib treatment results in a time-dependent G2-M cell cycle arrest in DHL-4 from 24 to 72 h. A, bortezomib treatment results in apoptosis without a G2-M arrest observed in the DHL-4 cells. B, increasing duration of exposure to Bay 11-7082 had equivalent effects on cell cycle distribution in DHL-4 and DHL-7 cells with no G2-M arrest observed in the DHL-4 cells. C, increasing concentrations of bortezomib after 72 h of exposure result in a G2-M cell cycle arrest in the DHL-4 cell line only. D, increasing concentrations of Bay 11-7082 have comparable effects on cell cycle distribution in DHL-4 and DHL-7 cell lines at 72 h. Columns, mean of three separate experiments; bars, SD. *, P < 0.05; **, P < 0.001 versus respective untreated controls.
was more apparent at the higher concentration in both DHL-4 (Fig. 3A and B) and DHL-7 (Fig. 3C and D) cells. In the bortezomib-treated cells, NF-κB activity returned toward control levels by 24 h.

**Effects of bortezomib on cell cycle distribution.** Flow cytometric analysis revealed significant changes in the distribution profile of DHL-4 and DHL-7 cells treated with bortezomib, which occurred in a concentration- and duration-dependent manner. The cell cycle effects were not consistent across the cell lines. In the DHL-4 cell line, a time- and concentration-dependent marked G2-M arrest occurred as indicated by an increase in the percentage of cells at this phase [0 nmol/L: 20.9 ± 1.6% versus EC50 (25 nmol/L): 47.0 ± 3.7%; P < 0.001, t test]. This was mirrored by a decreasing number of cells within the S and G1 phases. Increasing concentrations of bortezomib and increased duration of exposure resulted in an emptying of cells from this phase with a consequent increase in the sub-G1 (apoptotic) cell population (Fig. 4A and C). In the more sensitive DHL-7 cell lines, a G2-M block did not occur [DHL-7 (0 nmol/L): 18.6 ± 4.8% versus EC50 (6 nmol/L): 18.3 ± 4.8%]. Instead, a concentration- and duration-dependent increase in apoptosis was observed with a consequent decrease in the percentage of cells in the G1 phase. There was no significant change in the S or G2-M phases of the cell cycle in the DHL-7 cell lines (Fig. 4A and C). Treatment with NF-κB inhibitor Bay 11-7082 did not show a G2-M block in the DHL-4 cell line (Fig. 4B and D).

**Bortezomib has variable effects on G2-M cell cycle proteins in DHL-4 and DHL-7 cells.** The effect of bortezomib exposure duration on G2-M cell cycle proteins was investigated after 20, 24, and 28 h (determined from preliminary experiments; data not shown). In the DHL-4 cell line, bortezomib treatment resulted in an increase in cyclin B from 20 h, which was sustained until 28 h. Cyclin-dependent kinase 1 (Cdk1) was not affected by bortezomib. Although a decrease in Wee-1 was observed in DHL-4 cells, it remained present throughout these time points of exposure consistent with cell cycle arrest at G2 (Fig. 4). In the DHL-7 cell line, bortezomib resulted in a decrease in cyclin B from 20 h and, to a greater extent, at the 28-h time point. At 20 and 24 h, expression of Wee-1 reduced and it disappeared by 28 h. Cdk1 levels were also reduced (Fig. 5).

**MPM2 staining shows mitotic catastrophe in more sensitive DHL-7 cells.** MPM2 staining of untreated DHL-4 and DHL-7 cells revealed cells in all stages of mitosis to be present, although the number of mitotic cells was reduced in the DHL-4 line (Fig. 6). After exposure to the EC50 concentration (% viability) of bortezomib for 28 h, DHL-7 cells continued to enter into mitosis; however, normal mitosis was not observed. Instead, aberrant mitosis occurred in a significant proportion of cells, with anaphase being attempted in cells with incomplete nuclear condensation or unequal distribution and separation of DNA (Fig. 6B). Similar changes were observed in treated DHL-4 cells (Fig. 6D), although to a reduced extent and with far fewer cells entering into mitosis, consistent with the flow cytometry results.

**Discussion**

Bortezomib is one of several exciting new agents emerging for the treatment of malignant lymphoma, having shown 20% to 50% response rates as a single agent in patients with recurrent, refractory disease (7, 8, 12). However, the majority of patients do not respond to single agent therapy and increased understanding of the mechanism of action of this drug and possible mechanisms of resistance would be a valuable step toward improving its efficacy. Using cell lines with differing sensitivity to bortezomib, we show that, although bortezomib treatment had similar effects on apoptotic and NF-κB signaling pathways in these cell lines, different cell cycle effects were observed and induction of a further mechanism of cell death, mitotic catastrophe, was observed in the more sensitive cell line.

In all five lymphoma cell lines studied, bortezomib was cytotoxic at low nanomolar concentrations, comparable with data from other studies (9, 11), but differential sensitivity between the lines was observed, with the most sensitive cell line DHL-7 having an EC50 of 6 nmol/L compared with 25 nmol/L in the most resistant DHL-4 cells. Sensitivity was independent of p53 activity as all cell lines had evidence of nonfunctional mutations and is in keeping with findings in mantle cell lymphoma (MCL) prostate cancer and myeloma (9, 14, 15). Differences in proteasome activity and proteasome inhibition were unable to explain differences in sensitivity to bortezomib, as activity in untreated cells did not differ between the two cell lines and concentration-dependent proteasome inhibition was equivalent in DHL-4 and DHL-7 cell lines at 4 h. At 24 h, there was a trend to greater recovery in the DHL-4 cell line; however, concentrations that caused significant sustained inhibition (e.g., 12 nmol/L) were not sufficient to cause cell death, providing evidence that reaching a “threshold” of proteasome inhibition does not inevitably result in cell death. Similar findings have been reported elsewhere (16, 17).

Bortezomib treatment at the EC50 concentration of the DHL-4 and DHL-7 cell lines resulted in apoptosis with cleavage of PARP, a substrate for active caspase-3, and occurred via activation of both caspase-8 and caspase-9. Chauhan et al. examined the effects of bortezomib on DHL-4 cells and a similar lymphoma cell line, DHL-6. They used a low concentration of bortezomib (10 nmol/L) that had no effect on DHL-4 cell viability to show that resistance in the DHL-4 cell line was due to resistance to apoptosis, in turn due to overexpression of the heat shock protein 27 (Hsp27; ref. 17). In similar studies, Hideshima et al. (18) showed that bortezomib treatment of DHL-4 cells did not result in cleavage in caspase-8, caspase-9, caspase-3, or PARP. Here, we show that DHL-4 cells are not resistant to bortezomib-induced apoptosis but that a higher
concentration of bortezomib is required for its induction and cell death to occur.

Examination of the Bcl-2 family of proteins revealed that, in contrast to observations in multiple myeloma cell lines, the antiapoptotic protein Bcl-2 was not reduced by bortezomib and, in fact, an increase was observed in both DHL-4 and DHL-7 cells (11). In MCL cell lines, no alteration of Bcl-2 protein expression is observed after bortezomib treatment, but instead, a phosphorylated proapoptotic cleavage product of Bcl-2 is formed, an effect not observed in these lymphoma cell lines (9). Bortezomib did have an effect on two proapoptotic members of the Bcl-2 family examined. Bax, not expressed in the DHL-4 cell line, was increased by bortezomib in DHL-7 cells, whereas, in DHL-4 cells, Bak was increased by bortezomib treatment. The effect of bortezomib on the Bcl-2 family of proteins did not result in a significant change in mitochondrial membrane permeability in the DHL-4 cells at 24 h, but an increase in permeability (due to a decrease in potential) was observed in DHL-7 cells at the higher concentration (25 nmol/L) of bortezomib. Several authors have reported up-regulation of Bax as a result of proteasome inhibition, and cytochrome c release via elevation of Bax proteins has been observed in Jurkat cells (19, 20). In MCL cell lines, bortezomib treatment does not result in an overall increase in Bax or Bak expression but conformational changes consistent with their activation are observed (21). There, bortezomib also induced a decrease in Bid protein level as a consequence of its cleavage mediated by caspase-3 activation. These data suggest that, in some cell lines, different proapoptotic proteins are up-regulated to increase mitochondrial membrane permeability with the subsequent release of cytochrome c and induction of apoptosis. The absence of a decrease in MMP in DHL-4 cells may partially explain resistance to apoptosis in the DHL-4 cells. However, this cannot fully explain differences in

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Figure 6. Staining with the immunofluorescent antibody MPM2 shows that the more sensitive DHL-7 cells are progressing into mitosis and undergoing mitotic catastrophe. DHL-4 and DHL-7 cells were treated with EC50 concentrations of bortezomib, and cytospins of untreated and treated cells were prepared. After permeabilization, cells were incubated with anti-phospho-Ser/Thr-MPM2 overnight and, after exposure to PE-conjugated anti-mouse secondary antibody and the DNA stain, DAPI, viewed under a fluorescence microscope. MPM2-positive cells stain red and the DNA stain, DAPI, appears blue. Untreated DHL-7 (A) and DHL-4 (C) cells show mitosis proceeding normally, with some cells staining positive for MPM2 with normal chromatid separation and chromosome migration. B, in bortezomib-treated DHL-7 cells, mitosis continues to occur as cells stain positive for MPM2, but mitosis is aberrant and cells show various abnormalities (three examples shown) resulting in asymmetrical distribution of DNA. D, there are fewer cells undergoing mitosis in DHL-4–treated cultures, consistent with the G2-arrested population shown in Fig. 4A.
sensitivity between the cell lines, as the EC_{50} concentration of DHL-7 failed to induce a significant change in the permeability at this time point.

As inhibition of NF-κB is thought to be an important mediator of activity of bortezomib, its effects were compared with that of a specific inhibitor, Bay 11-7082. In MCL cell lines and primary cultures, NF-κB inhibition seems to play a significant role, as similar effects are observed with Bay 11-7082 and bortezomib (9). In the DHL-4 and DHL-7 cell lines, the effect of bortezomib was found not to be solely attributable to its effect on NF-κB activity, as equipotent concentrations (with respect to % viability) of Bay 11-7082 resulted in greater NF-κB inhibition than bortezomib. In addition, no differences were found between the cell lines with either drug.

One of the most striking findings of the study was that bortezomib treatment resulted in a G2-M cell cycle arrest in DHL-4 cell line, an effect not observed in the DHL-7 cell line, or either cell line with the specific NF-κB inhibitor. The proteasome is intimately involved in regulation of progression of the cell cycle from G2 into mitosis through the temporal degradation of both positive and negative regulators of this process (22). Inhibition of the proteasome may therefore result in several effects, and cell cycle proteins involved in this transition were investigated to see whether it could explain any differences between the two cell lines. In the DHL-4 cell line where a G2-M arrest was observed, cyclin B increased and Wee-1 (a mitotic inhibitory protein) degradation was prevented (22). Cyclin B is normally broken down by proteasome-mediated degradation during metaphase, and increased expression is observed during G2 due to increased transcription. These results provide confirmatory evidence, in addition to the cell cycle distribution data, that bortezomib treatment results in the arrest of DHL-4 cells at the G2-M checkpoint. Chauhan et al. (17) showed that the DHL-4 cell line overexpresses Hsp27 and it was this that mediates resistance to bortezomib, as inhibition of Hsp27 using small interfering RNA led to increased sensitivity to bortezomib and transfection of Hsp27 into sensitive DHL-6 cells conferred resistance. Hsp27 is known to prevent cells from undergoing apoptosis by preventing release of cytochrome c from the mitochondria; thus, it may contribute to resistance to bortezomib, but as little change in MMP was observed in the DHL-7 cell line, this effect cannot offer the only explanation. It is, however, possible that induction of Hsp27 (or other Hsps) may contribute to the differences in cell cycle effects observed in the cell lines, as Hsp27 overexpression has been shown in ovarian cells treated with etoposide or vincristine, which was shown to correlate with growth arrest and G2-M accumulation (23). In a preliminary analysis of gene expression profiling of DHL-4 and DHL-7 cells done at this institution, DHL-4 cells were again found to overexpress Hsp27 with increased expression after treatment (data not shown). It is thus possible that a checkpoint protein, such as chk1, may be a Hsp27 client and an increase in Hsp27 acts to produce the G2 arrest observed in the DHL-4 cells.

In DHL-7 cells, bortezomib treatment resulted in degradation of cyclin B and Wee-1 and staining with MPM2 provided evidence that DHL-7 cells had entered into mitosis. Aberrant mitosis, however, seemed to have occurred as haphazard asymmetrical segregation of chromatid clusters in abnormal-appearing anaphase was observed. These findings are consistent with a form of cell death called “mitotic catastrophe,” a term used to describe cell death that occurs from aberrant mitosis (24, 25). Several key regulatory proteins, such as the aurora kinases and other chromosomal passenger proteins, are involved in regulating chromatid segregation and chromosome migration. Proteasome inhibition may have a marked effect on the degradation of these regulatory proteins, or their substrates, resulting in the abnormal mitotic cells observed.

Some reports indicate that mitotic catastrophe shares a common pathway with apoptosis-induced cell death, whereas others report that it is an independent pathway (26, 27). It is likely that mitotic catastrophe in apoptosis-competent cells is followed by apoptosis as was observed in these cell lines (28). A similar phenomenon has been described in bortezomib-treated non–small cell lung cancer cell lines (29).

Malignant cells often have cell cycle checkpoint abnormalities, and this may be why they are particularly susceptible to the induction of mitotic catastrophe (30). The studies reported here show the cell-specific effects of bortezomib and provide some pointers to the difference in sensitivity between cell lines. It seems that DHL-7 cells abrogate the G2-M checkpoint with consequent mitotic catastrophe and cell death, whereas, in the DHL-4 cells, activation of the G2-M checkpoint may allow repair of damaged DNA, thereby reducing sensitivity to the drug. An improved understanding of control of the checkpoint may help identify targets to increase the efficacy of bortezomib in cells less sensitive to the drug. The use of RNA interference may be helpful to determine which of these changes are crucial to the cytotoxic effects of bortezomib.

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