Cell death by bortezomib-induced mitotic catastrophe in natural killer lymphoma cells

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Abstract

The proteasome inhibitor bortezomib (PS-341/Velcade) is used for the treatment of relapsed and refractory multiple myeloma and mantle-cell lymphoma. We recently reported its therapeutic potential against natural killer (NK)-cell neoplasms. Here, we investigated the molecular mechanisms of bortezomib-induced cell death in NK lymphoma cells. NK lymphoma cell lines (SNK-6 and NK-YS) and primary cultures of NK lymphomas treated with bortezomib were examined for alterations in cell viability, apoptosis, cellular senescence, and cell cycle status. Bortezomib primarily induced mitochondrial apoptosis in NK-YS cells and in primary lymphoma cells at the same concentration as reported in myeloma cells. Unexpectedly, SNK-6 cells required a significantly higher median inhibitory concentration of bortezomib (23 nmol/L) than NK-YS and primary lymphoma cells (6-13 nmol/L). Apoptosis was limited in SNK-6 cells due to the extensively delayed turnover of Bcl-2 family members. These cells were killed by bortezomib, albeit at higher pharmacologic concentrations, via mitotic catastrophe—a mitotic cell death associated with M-phase arrest, cyclin B1 accumulation, and increased CDC2/CDK1 activity. Our results suggest that, in addition to cell death by apoptosis at lower bortezomib concentrations, NK lymphoma cells resistant to bortezomib-induced apoptosis can be killed via mitotic catastrophe, an alternative cell death mechanism, at higher pharmacologic concentrations of bortezomib. Hence, activating mitotic catastrophe by bortezomib may provide a novel therapeutic approach for treating apoptosis-resistant NK-cell malignancies and other cancers. [Mol Cancer Ther 2008;7(12):3807–15]

Introduction

According to the WHO classification scheme, natural killer (NK)-cell neoplasms include extranodal NK/T-cell lymphoma (nasal type) and aggressive NK-cell leukemia (1). They are aggressive malignancies with poor treatment outcomes (2). The lymphoma cells are characteristically CD3−CD5−CD56+ and are infected by the EBV. NK-cell lymphomas show a geographic predilection, as they constitute 5% to 10% of all lymphomas in Asia and South America but are extremely uncommon in the West. An optimal treatment for NK-cell malignancies has yet to be found (2). Radiotherapy of localized nasal NK-cell lymphomas may be curative in patients with stage I disease. For patients with stage II or more advanced disease, however, treatment results are unsatisfactory. Despite the availability of prognostic models (3) and accurate lymphoma load assessment by quantification of EBV DNA (4), improvement in treatment results has not been obtained by conventional chemotherapy. Although allogeneic transplantation may rescue some patients, the rapid progression of chemorefractory disease or the toxic side effects of high-dose chemotherapy often preclude such an option. Thus, new therapeutic agents, preferably without dose-limiting effects, are urgently wanted. To achieve meaningful clinical results, a more effective and less toxic agent will be critical to attain disease stabilization before transplantation. Research on new drug development for this disease has been hampered due to the very limited number of bona fide NK lymphoma cell lines that are available (5). Establishment of NK lymphoma cell lines has been very difficult over the years. Similarly, successful development of NK lymphoma primary cultures for the evaluation of therapeutics has been equally challenging, as tumor biopsies are usually small and contain significant necrosis.

Tumorigenesis is generally prevented by the watchdog activity of tumor suppressors that trigger apoptosis. Similarly, anticancer agents often work by inducing cancer cells to undergo apoptosis (6). Cancer cells, however, may still escape apoptosis by enhancing the activity of oncoproteins or inactivating the tumor suppressors. These mechanisms contribute to poor drug responses. Accumulating evidence indicates that, if apoptosis is abnormally suppressed, cancer cells may be eliminated by other mechanisms (7). Among these mechanisms is mitotic catastrophe, a type of cell death that involves abnormal mitosis. When assaulted by drugs, cancer cells tend to bypass the mitotic checkpoints and prematurely reenter the
cell cycle, that is, they divide asymmetrically, leading to cytogenetic chaos and mitotic catastrophe (8–10). New routes to successful cancer treatment may therefore be discovered by investigating the factors that interrupt competent cell division.

Normal cellular activities are dependent on the orderly degradation of obsolete cellular proteins by the ubiquitin-proteasome system. The targeted proteins participate in a wide variety of essential cellular activities, such as signal transduction, transcriptional regulation, stress response, and cell cycle control (11). The process is tightly controlled by a network of coordinated enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. The ubiquitinated proteins are targeted for proteolysis by the proteasome. Bortezomib (PS-341/Velcade) is a synthetic small molecule that specifically and reversibly inactivates the proteasome (12). It has been used to treat relapsed and refractory multiple myeloma and mantle-cell lymphoma (11). We have described previously constitutive activation of nuclear factor-κB in NK-cell lymphoma (13). Given the involvement of nuclear factor-κB with tumorigenesis and the action of bortezomib against nuclear factor-κB (11), we have recently investigated and reported its therapeutic potential for NK-cell neoplasms (14). In this study, we have examined the molecular mechanisms of bortezomib-induced cell death in NK lymphoma cell lines and primary lymphoma cells. Our results suggest that, in addition to cell death by apoptosis at lower bortezomib concentrations, NK lymphoma cells that are resistant to apoptosis via bortezomib can be killed via mitotic catastrophe, an alternative cell death mechanism, at higher pharmacologic concentrations of bortezomib. As the apoptosis pathway is often impaired in relapsed and chemorefractory NK-cell malignancy and other cancers, activation of mitotic catastrophe by bortezomib may provide a novel therapeutic approach for the treatment of apoptosis-resistant diseases. Thus, bortezomib has the potential to serve as a more generic antineoplastic agent than thought previously.

Materials and Methods
Approval of this study was obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority, Hong Kong West Cluster (institutional review board reference no. UW 05-070 T/733). Informed consent was obtained in accordance with the Declaration of Helsinki.

NK lymphoma Cell Lines and Primary Cultures
NK lymphoma cell lines SNK-6 (15) and NK-YS (16) were maintained in RPMI 1640 (Hyclone) supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (Hyclone), and 100 units/mL recombinant human interleukin-2 (PeproTech). Primary cultures were set up from fresh tumor specimens obtained from two nasal NK-cell lymphoma patients. In the first patient, the biopsy was obtained from an involved testis during relapse. The tumor tissues were minced and strained through a cell strainer (BD Biosciences). The NK cells were purified from the harvested cells with the NK Cell Negative Isolation Kit (Dynal Biotech ASA) and maintained in the same medium as described above. In the second patient with bone marrow involvement, mononuclear cells were isolated by the Ficoll-Hypaque method from the patient’s bone marrow sample before any treatment, and NK cells were purified and maintained as described above.

Antibodies and Reagents
Primary antibodies against the following proteins were used in this study: caspase-9, caspase-3, poly(ADP-ribose) polymerase, Mcl-1, Bcl-xL, Bcl-2, Bak, Bax, ubiquitin, cyclin B1, phospho-CDC2 (Tyr15), and CDC2 from Cell Signaling Technology; β-actin from Santa Cruz Biotechnology; Bax (clone 6A7) and Bak (clone TC-100) from Calbiochem; and MPM-2 from Upstate. Bortezomib was kindly provided by Millennium Pharmaceuticals. Propidium iodide was obtained from Sigma, 4,6-diamidino-2-phenylindole (DAPI) was from Roche Applied Science, and chloromethylX-rosamine (MitoTracker Red) was from Molecular Probes.

Viable Cell Number Assay
The viable cell number was measured with the AQueous One Solution MTS assay (Promega). Briefly, cells were incubated in a 96-well plate at a density of 2 × 10⁴ per well in 100 μL culture medium. They were treated with various concentrations of bortezomib for 1 day. For each well, 20 μL MTS reagent was added and the plate was incubated at 37°C for 3 h. Absorbance was measured at 492 nm with the Vmax 96-well plate reader (Molecular Devices).

DNA Fragmentation Assay
The DNA fragmentation assay was done as described previously (17). Briefly, cells were harvested after 1 day of exposure to bortezomib, washed once, and resuspended in 200 μL cold PBS. They were lysed by adding 20 μL lysis buffer [0.2 mol/L EDTA, 0.05 mol/L Tris-HCl (pH 8.0), and 5% Triton X-100] and 2 μL of 20 mg/mL proteinase K. Cellular proteins were digested at 35°C overnight. Cell debris was removed by centrifugation at 12,000 × g for 5 min and 200 μL supernatant was gently mixed with 40 μL of 5 mol/L NaCl. Samples were vortexed gently for 20 s to separate histones from DNA and kept at room temperature to achieve complete histone disassociation. Cold absolute ethanol (500 μL) was added 10 min later and the tubes were incubated at -20°C overnight. DNA was recovered by centrifugation at 12,000 × g for 10 min. Pellets were rinsed briefly with 70% ethanol, air dried, and then dissolved in 30 μL Tris-EDTA (pH 7.6) with 100 μg/mL RNase A. Samples were then loaded in a 1.5% agarose gel for electrophoresis. Gel images were obtained with the GelDoc-It Imaging System (UVP).

Flow Cytometry
Flow cytometry was done on the FACSCalibur cytometer (BD Biosciences). Data were collected with the CellQuest software (BD Biosciences) and analyzed with the WinMDI version 2.8 software. For cell cycle analysis, cells were harvested, washed, and fixed with 70% cold ethanol for 2 h. Ethanol was removed by centrifugation at 400 × g for 5 min and the cell pellet was resuspended in PBS with 100 μg/mL...
RNase A. An equal volume of 10 µg/mL propidium iodide was added and the sample was mixed thoroughly. Samples were examined on the FL2 channel and 20,000 events were collected. The distribution of cells in G1, G2, S, and G2-M phases was analyzed with the ModFit LT software (Verity Software House). To detect alteration in the mitochondrial membrane potential (ΔΨm), cells were harvested, washed, and freshly stained with 100 nmol/L chloromethyl-X-rosamine at 37°C for 30 min. Samples were examined on the FL3 channel and 30,000 events were collected.

**Western Blot Analysis**

Protein extracts were prepared by lysing 2 × 10^7 cells in 1 mL cold radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology) and immediately heated at 100°C for 10 min. Samples were kept at room temperature for 30 min before centrifugation at 12,000 × g for 10 min. Supernatant was transferred into a new tube and the protein concentration was measured with the Bradford assay (Bio-Rad). Relative expression levels of the various proteins were determined using anti-CDC2 antibody (Cell Signaling Technology) and protein G-Plus agarose (Santa Cruz Biotechnology). The purified protein was incubated in the reaction buffer with γ-[32P]ATP (Amersham) and histone H1 for 10 min at 30°C. Reaction products were mounted on phosphocellulose paper and radioactivity was measured with a liquid scintillation counter (LS5801; Beckman Instruments). A reaction with recombinant CDK1/cyclin B (Cell Signaling Technology) was done as the positive control. An equal amount of CDC2 served as the reaction input standardization control.

**Mitotic Index**

Mitotic index, which is the ratio of mitotic cells relative to the total number of cells, was calculated from 1,000 cells stained with DAPI (Roche) for each sample.

**Statistical Analysis**

A Student’s t test was used for statistical analysis and *P < 0.05* was considered significant.

**Results**

**SNK-6 Cells Were Less Sensitive to Bortezomib Than NK-YS and Primary NK Lymphoma Cells**

The cytotoxic effect of bortezomib was determined in the NK lymphoma cell lines (SNK-6 and NK-YS) and primary lymphoma cells by measuring cell viability with the MTS assay (Fig. 1A). Similar data from the NK-YS cell line and primary lymphoma cells have been reported by our group (14). The MTS assay was repeated in this study to compare with the data from the SNK-6 cell line. Treated with various concentrations of bortezomib, the lymphoma cells were killed in a dose-dependent manner. The median inhibitory concentration (IC50) of SNK-6 cells was 9 ng/mL (23 nmol/L), whereas the IC50 of NK-YS cells was 3 ng/mL (8 nmol/L), showing that the SNK-6 cells could tolerate higher concentrations of bortezomib than NK-YS cells. The sensitivity of primary NK lymphoma cells was similar to that of NK-YS cell line. The IC50 of primary cultures was 2.4 ng/mL (6 nmol/L) for the first patient and 5 ng/mL (13 nmol/L) for the second.

**Apoptosis Was Limited in SNK-6 Cells Treated with Bortezomib**

To identify the mechanism of death, we examined the apoptotic activity in both cell lines and compared the

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expression of both proapoptotic and antiapoptotic molecules associated with the mitochondrial pathway. In the DNA fragmentation assay that distinguishes apoptosis from other types of cell death, exposure to 15 ng/mL bortezomib (a concentration used throughout the study unless otherwise specified) led to apoptosis in 24 h in the NK-YS and the primary lymphoma cells. There was, however, no evidence of a DNA degradation ladder in the SNK-6 cells (Fig. 1B). Consistent with the above findings, morphologic examination on NK-YS and primary lymphoma cells showed the typical features of apoptosis, evident with significant DNA hyperchromicity (chromatin condensation) and karyorrhexis (nuclear fragmentation). In the SNK-6 cells, however, the morphologic sign of apoptosis was not evident (Fig. 1C). These findings were also supported by the measurement of the ΔΨₘ and cleavage of caspases. With exposure to bortezomib, the ΔΨₘ depolarized in NK-YS cells in 6 h, whereas it took 9 h in SNK-6 cells (Fig. 1D). Consistent with the ΔΨₘ data, cleavages in caspase-9, caspase-3, and poly(ADP-ribose) polymerase were also limited in SNK-6 cells (Fig. 2A).

SNK-6 Cells Treated with Bortezomib Showed Antiapoptotic Bias

To better understand the diminished apoptotic response to bortezomib in SNK-6 cells, we examined the expression profiles of representative Bcl-2 family members that have antiapoptotic and proapoptotic activities. As expected, Bcl-xL levels decreased in the apoptosis-sensitive NK-YS cells. The expression levels, however, remained unchanged in SNK-6 cells (Fig. 2A). Additionally, antiapoptotic Bcl-2 expression levels increased progressively in SNK-6 cells, whereas they slightly decreased in the NK-YS cells in which the band corresponding to the proapoptotic cleaved Bcl-2 protein was clearly present at 24 h. Moreover, an increase in Mcl-1 expression levels was detected early after bortezomib treatment in both cell lines, although its turnover differed significantly between them. Mcl-1 expression increased steadily in SNK-6 cells and remained high at 24 h, whereas Mcl-1 expression in NK-YS cells increased in the first 6 h but dropped later at 24 h (Fig. 2A). In addition to these antiapoptotic molecules, we also examined the expression and activation of proapoptotic molecules from the Bcl-2 family. With bortezomib, Bak expression levels decreased slightly in both cell lines (Fig. 2A) and its conformational change, a hallmark of activation (18), coincided between them (Fig. 2B). In contrast, Bax expression levels remained roughly the same in both cell lines (Fig. 2A). The Bax conformational change differed temporally between them, with the active form (19) present at 6 h in NK-YS cells while appearing at 24 h in SNK-6 cells.
To compare the apoptosis potentials of these two cell lines, we calculated the Bcl-2/Bax ratio. In SNK-6 cells, the Bcl-2/Bax value increased by 30%, from 1.83 at 0 h to 2.35 at 6 h, whereas it slightly decreased by 10%, from 1.85 to 1.66, in NK-YS cells in the same time frame. Thus, there appeared to be an overall bias toward antiapoptotic conditions in SNK-6 cells in response to bortezomib.

**Bortezomib Induced Mitotic Catastrophe in SNK-6 Cells**

Mitotic catastrophe is a form of cell death associated with abnormal mitosis and often accompanied by cellular senescence (20). Based on the above findings, we investigated if bortezomib at higher concentrations might induce cellular senescence and mitotic catastrophe in the SNK-6 cell line. We examined both cell lines for senescence-associated β-galactosidase activity, a hallmark of cellular senescence (21). Unexpectedly, senescent cells remained scarce in SNK-6 cells, whereas the number of senescent cell increased at 6 h but dropped dramatically at 24 h due to apoptosis in NK-YS cells (Fig. 2C). The morphology of SNK-6 cells after adding bortezomib was consistent with a drug effect on mitosis. The dying cells appeared in the cell culture from 12 h, with a significant accumulation of enlarged, distorted, flattened, spindle-shaped cells.

**Figure 2.** High pharmacologic concentrations of bortezomib-induced mitotic catastrophe in the SNK-6 cell line that was insensitive to apoptosis and cellular senescence. **A,** Western blots of the apoptosis-related proteins in the bortezomib-treated NK lymphoma cell lines. Relative expression levels of the indicated proteins were quantified by densitometric analysis of the Western blots. β-Actin was used as the protein loading control and the data are presented at the bottom of the Western blots. For Western blots showing multiple bands, densitometric analysis was done only for the full-length protein bands. **B,** active forms of Bak and Bax were examined with immunofluorescence in bortezomib-treated NK lymphoma cell lines using the conformation-specific monoclonal antibody TC-100 and 6A7 (both in green). Nuclei were counterstained with DAPI (blue). Fluorescent microscopy; original magnification, ×400. **C,** senescent cells (blue) were detected in both lymphoma cell lines after treatment with bortezomib. Original magnification, 200 ×. **D,** morphologic analysis of bortezomib-induced mitotic catastrophe in SNK-6 cells. Various morphologic alterations were consistent with those of mitotic catastrophe (i-iii): the enlarged, flattened, spindle-shaped cells (long-tailed arrow); multinucleate cells (arrowhead); and plasma membrane blebbing in the dying cells (short-tailed arrow). Inverted phase-contrast microscopy; original magnification, ×800. Additionally, these enlarged, spindle-shaped dead cells (open arrow) were detected with MPM-2 antibody (green; iv-ix). Nuclei were counterstained with DAPI (blue). Fluorescent microscopy; original magnification, ×800.
nuclear membranes were absent, the chromosomes spread erratically, and their plasma membrane was occasionally undulated and generated blebs around the cells (Fig. 2D, i-iii). To identify if they were associated with mitosis, these bortezomib-treated SNK-6 cells were examined by immunofluorescence with the MPM-2 antibody, which recognizes the mitosis-specific phosphorylated proteins, thus serving as a marker of mitotic cells (22). As expected, bortezomib induced an accumulation of MPM-2-positive cells among the SNK-6 cells with the chromosomes in total disarray. This was in sharp contrast to the normal mitotic cells from the control (Fig. 2D, iv-ix).

**Bortezomib-Induced Mitotic Catastrophe Was Associated with G2-M Arrest in SNK-6 Cells**

To quantify the effect of bortezomib on mitosis, we compared the mitotic indices of SNK-6 and NK-YS cells after drug treatment. Bortezomib caused a significant increase in the mitotic index in SNK-6 cells but not in NK-YS cells (Fig. 3A). From the mitotic index data, we estimate that ~25% of SNK-6 cells were killed through mitotic catastrophe after drug treatment. To further explore the effect of bortezomib on mitotic arrest, SNK-6 and NK-YS cells were treated with two concentrations (5 and 15 ng/mL) and subjected to cell cycle analysis (Fig. 3B). In NK-YS cells, apoptosis was induced in both concentrations of bortezomib. The sub-G1 population from apoptotic cells increased progressively in a time-dependent manner, with a stable distribution of the cells in the G1 and G2-M phases. In contrast, SNK-6 cells survived in 5 ng/mL bortezomib, and this concentration did not have any effect on the distribution of the cell cycle phases. When exposed to 15 ng/mL bortezomib, however, SNK-6 cells displayed G2-M arrest after 9 h, with the tetraploid G2-M cells increasing and outnumbering the diploid G1 cells. Once again, the evidence for apoptosis was still lacking, as the sub-G1 population from apoptotic cells remained consistently low in SNK-6 cells. Together, these findings indicated a mitotic cell death associated with the G2-M arrest.

**Bortezomib-Induced Mitotic Catastrophe Caused Cyclin B1 Accumulation and Increased CDC2/CDK1 Activity in SNK-6 Cells**

Because we have identified that bortezomib induced the G2-M arrest in the progress of mitotic catastrophe, we next investigated whether the cyclin B1/CDC2 complex played a role in these events. As cyclin B1 is regulated by the ubiquitin-proteasome system (23), we examined by...
Western blot analysis the amount of ubiquitin, cyclin B1, and CDC2 (CDK1) in the bortezomib-treated SNK-6 cells (Fig. 4A). With a steady increase in the ubiquitin levels, the cyclin B1 levels increased moderately and reached a plateau after 9 h. In the same time frame, however, the total CDC2 levels declined slightly, with the phospho-CDC2 (Tyr15) levels remaining stable. To achieve a better understanding of the cyclin B1/CDC2 complex activity in the progress of mitotic catastrophe, we measured the CDC2 kinase activity in the bortezomib-treated SNK-6 cells. A marginal increase in CDC2 kinase activity was detected at the early stage of mitotic catastrophe (~9 h after adding bortezomib), and ultimately, a 2-fold increase in the kinase activity was observed after 24 h (Fig. 4B).

Discussion
We have shown that low concentrations of bortezomib induce apoptosis in the NK-YS cell line and in primary NK lymphoma cells. However, in SNK-6 cells, which were resistant to apoptosis at low drug concentrations, bortezomib induced mitotic catastrophe at higher pharmacologic concentrations. Mitotic catastrophe is a type of cell death induced by the abnormal activations of cyclin B and CDC2, resulting in deficient cell cycle checkpoints (the DNA structure checkpoint and the spindle assembly checkpoint; ref. 10). It is usually triggered by the abnormal mitosis that culminates in aberrant chromosome segregations. Although its molecular mechanism needs further investigation, some facts about this unique mitotic cell death have been clearly identified. Unlike apoptosis, which is basically dependent on caspase activation (24), mitotic catastrophe may be mediated in a caspase-dependent or caspase-independent fashion (8). In some cases, mitotic catastrophe shares the same signaling pathway with apoptosis. More often, when apoptosis is deficient, a programmed cell death can still be executed through mitotic catastrophe in response to mitotic failure independent of apoptosis (10).

From our observation of the bortezomib-induced cell death in a lymphoma cell line (SNK-6) that was resistant to apoptosis, we identified mitotic catastrophe with characteristics of M-phase arrest, cyclin B1 accumulation, and elevated CDC2/CDK1 activity. These bortezomib-induced alterations may interfere with mitosis, and as a result, mitotic catastrophe is triggered without the typical apoptosis activation.

We examined SNK-6 cells for expression of antiapoptotic Bcl-2 family members and found a global delay in the degradation of Mcl-1, Bcl-xL, and Bcl-2 proteins and an elevated Bcl-2/Bax ratio. This was accompanied with a delay in the conformational activation of Bax. The alterations in the Bcl-2 family members represent an overall antiapoptotic bias in SNK-6 cells. Recent studies have shown that an accumulation in Mcl-1 protein accounts for the inhibition to apoptosis in bortezomib-treated cancer cells (25–27). Similarly, in our study, both NK lymphoma cell lines showed Mcl-1 protein increases in response to bortezomib treatment. Apoptosis, however, was still mediated in NK-YS cells, indicating that an increase in Mcl-1 protein might merely compromise the bortezomib-induced apoptosis in NK-cell lymphomas.

In the Western blot for caspase-3 of the bortezomib-treated NK lymphoma cell lines, an extra band of ~20 kDa was detected between the full-length and the cleaved caspase-3 (Fig. 2A). This band probably represents the intermediate cleavage product of the sequential two-step process in the caspase-3 activation. It has been reported that the caspase-3 precursor is first cleaved at Asp175/Ser176 (between the large and the small subunits) to produce the p12 subunit and the p20 peptide (28). The p20 peptide is then cleaved at Asp28/Ser29 to generate the mature p17 subunit. With the caspase-3 antibody that recognizes the full length (~35 kDa) and the large subunit of caspase-3 (~17 kDa), we detected this ~20 kDa band on the Western blot. It most likely represents the partial cleavage product that results from cleavage at Asp175/Ser176, which occurs before cleavage at Asp28/Ser29 during processing of the prosegment.

Cleavage of Bcl-2, which is a proteolysis product of active caspase-3 (29–32), generates a 23 kDa Bax-like fragment...
that was present in NK-YS but not in SNK-6 cells following 24 h treatment with bortezomib. Bcl-2 cleavage at Asp\textsuperscript{34} removes the NH\textsubscript{2}-terminal BH4 domain, which is required for apoptosis inhibition as it permits formation of a heterodimer with Bax. The truncated form of Bcl-2 cannot bind to Bax and therefore releases Bax to form the proapoptotic homodimer. In addition, the Bax-like cleavage fragment of Bcl-2 can induce apoptosis by inducing release of cytochrome \( c \) into the cytosol. Such processing therefore promotes apoptosis in NK-YS cells via a positive amplification loop, from caspase-mediated Bcl-2 cleavage to increased mitochondrial cytochrome \( c \) release and further activation of caspases.

Previous studies have documented an increase in the tetraploid population in cancer cells treated with bortezomib (33). It was suggested that bortezomib might induce apoptosis through G\textsubscript{2}-M-phase arrest. Using the SNK-6 line that is relatively resistant to apoptosis, we have further characterized bortezomib-induced mitotic catastrophe. As illustrated in Fig. 5, cyclin B1 is degraded through the ubiquitin-proteasome pathway (11, 23). Bortezomib inhibits proteasome activity and stabilizes cyclin B1. As a result, cyclin B1 accumulates in the cells. CDC2 is a cyclin-dependent kinase that controls entry and exit of mitosis. Recent evidence shows that mitotic exit is reversibly regulated by CDC2 kinase activity (34). Briefly, mitotic entry is enforced by an increase in active CDC2, whereas mitotic exit is triggered by a decrease. If cyclin B1 persists, as in bortezomib-treated SNK-6 cells, it may maintain CDC2 in an active form and in turn lead to mitotic arrest. Interfering with mitosis, however, does not completely arrest the cell cycle in M phase. Indeed, mitotic checkpoint slippage occurs in the presence of an active checkpoint (35). This slippage then results in the observed cytogenetic chaos, multinucleate cell formation, and mitotic catastrophe.

Earlier observations on the effects of cyclin B1 accumulation induced by proteasome inhibitors are informative. One study has shown that mitotic arrest induced by MG132 is associated with the presence of relatively high amounts of cyclin B1 but that MG132-arrested cells unexpectedly present a low CDC2 activity (36). There is also evidence that CDC2 inactivation, which drives the cell cycle out of mitosis, is triggered by a dissociation of cyclin B1 from CDC2 rather than its degradation by proteasome (37). To clarify these events, we examined CDC2 expression and activation status in the SNK-6 cells treated with bortezomib. In our study, CDC2 activity increased over 24 h with bortezomib, supporting an association of cyclin B1 accumulation with the maintenance of CDC2 activity during mitotic catastrophe.

Our present results provide an early indicator that bortezomib may be an effective treatment for NK-cell lymphomas. The IC\textsubscript{50} of bortezomib in NK-YS cells, patient-derived lymphoma cells (6-13 nmol/L), and in SNK-6 cells (23 nmol/L) are clinically achievable, as the IC\textsubscript{50} for normal peripheral blood mononuclear cells is \( \geq 100 \) nmol/L (11). In the absence of preclinical studies, the present bortezomib administration schedules in lymphoma patients have been derived from the management of multiple myeloma. The finding of an alternative cell death mechanism at higher pharmacologic concentrations and in different lineages of hematopoietic malignancies supports the utility of exploring more intensive infusion schedules in refractory lymphoma patients.

In this study, we investigated an unusual mechanism of cell death, mitotic catastrophe, in a NK lymphoma cell line that was resistant to apoptosis. In response to chemotherapy, cancer cells may protect themselves from destruction by suppressing apoptosis. Under some circumstances, however, these refractory cancer cells are prone to bypass the cell cycle checkpoints (6), leading to cell death via mitotic catastrophe (38). We present evidence here that bortezomib has the unusual attribute to potentiate both apoptosis and mitotic catastrophe and may therefore be

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**Figure 5.** Schematic diagram of molecular events in the bortezomib-induced M-phase arrest and mitotic catastrophe. Normally, the active CDC2/cyclin- B1 complex plays an essential role in driving the cell cycle through the M phase (39). At the G\textsubscript{2}-M-phase transition, the inhibitory phosphoryl group (Tyr\textsuperscript{15}) is removed from CDC2 by the phosphatase CDC25. This process is counteracted by the kinase Wee1. Active CDC2 is able to promote cyclin B1 phosphorylation, and phosphorylated cyclin B1 allows the whole complex to enter the nucleus. Nuclear translocation of CDC2/cyclin B1 triggers activation in various mitosis-associated molecules that drive cell cycles in progress. The anaphase-promoting complex/cyclosome (APC/C) complex forms during anaphase and acts as the ubiquitin E\textsubscript{3} ligase that labels cyclin B1 with ubiquitins. Once these ubiquitin tags are recognized, cyclin B1 is degraded by proteasome, a critical step in driving the cell cycle out of M phase. Proteasome inhibitors, such as bortezomib, enable cyclin B1 stabilization and thus play a role in M-phase arrest as well as in mitotic catastrophe.
uniquely effective in treating many refractory cancers. Our findings contribute to an emerging novel therapeutic strategy in which induction of mitotic catastrophe is exploited.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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