Bortezomib, a proteasome inhibitor, shows substantial anti-tumor activity in a variety of tumor cell lines, is in phase I, II, and III clinical trials and has recently been approved for the treatment of patients with multiple myeloma. The sequence of events leading to apoptosis following proteasome inhibition by bortezomib is unclear. Bortezomib effects on components of the mitochondrial apoptotic pathway were examined: generation of reactive oxygen species (ROS), alteration in the mitochondrial membrane potential ($\Delta \psi_m$), and release of cytochrome $c$ from mitochondria. With human H460 lung cancer cells, bortezomib exposure at 0.1 $\mu$m showed induction of apoptotic cell death starting at 24 h, with increasing effects after 48–72 h of treatment. After 3–6 h, an elevation in ROS generation, an increase in $\Delta \psi_m$, and the release of cytochrome $c$ into the cytosol, were observed in a time-dependent manner. Co-incubation with rotenone and antimycin A, inhibitors of mitochondrial electron transport chain complexes I and III, or with cyclosporine A, an inhibitor of mitochondrial permeability transition pore, resulted in inhibition of bortezomib-induced ROS generation, increase in $\Delta \psi_m$, and cytochrome $c$ release. Tiron, an antioxidant agent, blocked the bortezomib-induced ROS production, $\Delta \psi_m$ increase, and cytochrome $c$ release. Tiron treatment also protected against the bortezomib-induced PARP protein cleavage and cell death. Benzoyloxy carbonyl-VAD-fluoromethyl ketone, an inhibitor of pan-caspase, did not alter the bortezomib-induced ROS generation and increase in $\Delta \psi_m$, although it prevented bortezomib-induced poly(ADP-ribose) polymerase cleavage and apoptotic death. In PC-3 prostate carcinoma cells (with overexpression of Bcl-2, a reduction of bortezomib-induced ROS generation, $\Delta \psi_m$ increase was correlated with cellular resistance to bortezomib and the attenuation of drug-induced apoptosis. The transient transfection of wild type p53 in p53 null H358 cells caused stimulation of the bortezomib-induced apoptosis but failed to enhance ROS generation and $\Delta \psi_m$ increase. Thus ROS generation plays a critical role in the initiation of the bortezomib-induced apoptotic cascade by mediation of the disruption of $\Delta \psi_m$ and the release of cytochrome $c$ from mitochondria.

Recent investigations have demonstrated that apoptosis is regulated by two major pathways (1–3). The first pathway is via the death receptors on the cell surface, such as TNFR1 and Fas/CD95/APO-1, that can directly activate caspase-8 by a signaling complex that includes the cytosolic tail of the receptors and caspase-8 (4). The second pathway regulates the apoptotic cascades by the convergence of the signaling at the mitochondrion, such as those mediated by the Bcl-2 family of proteins. This pathway is involved in the alteration of mitochondrial membrane potentials, the release of cytochrome $c$ into the cytosol, and the activation of caspase-9 (5). It also appears that the mitochondrial control mechanisms underlying apoptosis are involved in the disruption of mitochondrial membrane permeabilization, and the alteration in mitochondrial membrane transition pores, leading to the release of protein effectors (6, 7). Reactive oxygen species (ROS),1 which are the byproducts of normal cellular oxidative processes, have been suggested as regulating the process involved in the initiation of apoptotic signaling. Tan and coworkers (8) showed that an increase in generation of ROS induces cytochrome $c$ release from mitochondria. The mimicking of the ROS elevation in nerve growth factor-deprived sympathetic neurons with exposure to H$_2$O$_2$ resulted in the release of cytochrome $c$ (9). In addition, the initial investigations of the Bcl-2 family of proteins have shown that these proteins play an important role in regulation of mitochondrial-mediated apoptosis (10). For example, the overexpression of Bcl-2 can prevent the release of cytochrome $c$ from mitochondria to the cytosol in apoptotic cells (11). In contrast, the overexpression of the proapoptotic Bcl-2 family, bax results in the facilitation of cytochrome $c$ release and the enhancement of ROS generation (12). Recently, Li et al. (13) reported that p53-induced apoptosis occurs via an increase in the generation of ROS and the disruption of mitochondrial membrane potential without affecting the release of cytochrome $c$.

The ubiquitin-proteasome pathway is well known to be responsible for the degradation of the majority of intracellular proteins and relies on the concerted action of two ATP-depend-ent protein complexes, the ubiquitin system, and the proteasome (14, 15). The target proteins that serve as the substrates for this system are generally ubiquitinated by a stepwise process, which involves the sequential reactions catalyzed by the ubiquitin-activating enzyme (E1), ubiquitin-conjugating en-

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1 The abbreviations used are: ROS, reactive oxygen species; $\Delta \psi_m$, mitochondrial membrane potential; DIOC6(3), 3,3'-dihexyloxacarbocya-nine; FACS, fluorescence-activated cell sorter; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; tiron, 4,5-dihydroxy-1,3-benzensulfonyl acid disodium salt; VAD-Fmk, benzoylcarbonyl-VAD-fluoromethyl ketone; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.
zymes (E2s), and ubiquitin ligases (E3s). Then the polyubiquitinated proteins are further recognized and degraded by the 26 S proteasomal multicatalytic protein complex. A number of proteins that have been identified as substrates for the ubiquitin-proteasome system include some short lived functional proteins (16). In addition, other evidence suggests that the ubiquitin-proteasome system is involved in the regulation of cell proliferation, differentiation, survival, and apoptosis (17). Based on these unique properties of the ubiquitin-proteasome system, the targeting of its pathway has recently emerged as a promising approach for the development of anti-cancer agents. Bortezomib (Fig. 1) is part of a new generation of reversible proteasome inhibitors developed by Millennium Pharmaceuticals Inc. (18). This compound displayed highly potent activity against the growth of several cancer cell lines in studies in vitro and in vivo and is currently undergoing evaluations in phase I, II, and III clinical trials (19–25). Recently the Food and Drug Administration (FDA) has approved bortezomib for the treatment of multiple myeloma patients who have received at least two prior therapies and have demonstrated disease progression on the last therapy.2,3

In previous work from our laboratories, we had found that bortezomib has significant potency against cellular proliferation in human non-small cell lung cancer cell lines and that this compound could block cell cycle progression at G2/M phase and caused the induction of apoptosis (28). In this investigation, we have also used the human H460 non-small cell lung cancer cell line as a model to examine the molecular mechanisms of the effect of bortezomib on the induction of apoptosis. We describe a sequence of events related to the effects on the mitochondria by bortezomib exposure of H460 cells that starts within 3–6 h of exposure and increases with longer times. These include increases in mitochondrial derived reactive oxygen species (ROS) and mitochondrial membrane potential (Δψm), as well as the release of cytochrome c from mitochondria into the cytosol. The studies reported below employed the co-incubation of bortezomib with rotenone and antimycin A, two inhibitors of mitochondrial electron transport chain complexes I and II (29, 30). We also made use of a series of co-incubation studies with cyclosporine A (CsA), an inhibitor of the mitochondrial permeability transition pore (31). The opening of the permeability transition pore has been implicated as an important mitochondrial event that occurs during apoptosis, and the degree of mitochondrial membrane permeabilization can be a rate-limiting step of drug-induced apoptosis, a process that is mediated by the BCL-2 family of proteins (31, 32). Studies with the use of these selective mitochondrial inhibitors should help in the elucidation of key mitochondria events related to the activity of bortezomib. Furthermore, we made use of the antioxidant agent tiron, which serves as an ROS scavenger (33, 34) to follow whether bortezomib-induced ROS generation could be effected by the co-incubation of these two agents. The new data resulting from these studies indicate that ROS generation and an increase in Δψm are the early and necessary events for the initiation of bortezomib-induced apoptotic signaling. These findings should aid in the understanding of the pleiotropic mechanisms of action of the proteasome inhibitor bortezomib and provide a basis for the therapeutic use of this compound alone or in combination with other chemotherapeutic agents.

MATERIALS AND METHODS

Chemicals—Bortezomib (VELCADE®), formerly known as PS-341, was supplied by Millennium Pharmaceuticals Inc. (Cambridge, MA) and was dissolved in Me2SO (10 mM) as a stock solution and diluted to the required concentration with PBS. Monoclonal anti-PARP, Bcl-2, and p53 antibodies were purchased from Calbiochem (Cambridge, MA). Monoclonal anti-cytochrome c antibody was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Cell Culture—Human H460 non-small cell lung cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 and 95% air. PC-3/vector and PC-3/Bcl-2 prostate carcinoma cells were a generous gift from Dr. McConkey (Department of Cancer Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX) and maintained in RPMI 1640 medium with 10% fetal bovine serum containing 1 mg/ml G418 sulfate (Geneticin, Invitrogen, Carlsbad, CA) (35).

p53 Transfection—The liposomal vector and the liposomal vector-harbored human wild type p53 cDNA under the control of the human CMV promoter were prepared as described previously (36). The human H358 non-small cell lung cancer cell line with the p53 null gene was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. For p53 transfection, H358 cells (0.7 × 10⁶ cells/well) were plated in a 6-well plate and incubated at 37 °C overnight. The liposomal wild type p53 cDNA or vector was added into the Cell culture.

Fig. 1. Chemical structure of bortezomib.

Fig. 2. Bortezomib induces apoptosis in H460 cells. Cells were exposed to 0.1 μM bortezomib for the indicated time. A, morphological features of cells exposed to 0.1 μM bortezomib or to the same volume of PBS solution as a control. The photographs were taken 48 h after continuous exposure to bortezomib. The apoptotic cells are indicated by arrows. Magnification, ×400. B, quantitative analysis of apoptotic cell death. After exposure, cells were taken from culture and stained with propidium iodide. The apoptotic (sub-G0/G1) cells were assessed by FACS flow cytometry as described under “Materials and Methods.” C, immunoblotting detection of PARP protein cleavage in cells exposed to 0.1 μM bortezomib for the indicated times.

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2 FDA, available at www.fda.gov/cder/approval/index.htm.
3 FDA, available at www.fda.gov/cder/cancer/whatsnew.htm.
After 3 h of incubation, cells were washed with PBS solution, and reincubated in fresh medium in the presence of 0.05 μM bortezomib or with the same volume of PBS solution as a control. After 24 h of incubation, the cells were taken from culture and prepared for the analysis of ROS production, alteration of Δψm, p53 expression, and apoptosis.

Apoptosis Assay—Cells were treated with 0.1 μM bortezomib for the indicated times. After treatment, cells were harvested and the apoptotic cells were detected by phase-contrast microscopy, or the cells were stained with 1 μg/ml propidium iodide and the apoptotic cells (sub-G0/G1 DNA content) were assessed by FACS analysis (FACScan, BD Biosciences, Franklin Lakes, NJ).

Cell Viability and Cytotoxicity—Exponential growing cells were exposed to bortezomib for the indicated time. Each condition was performed in triplicate, and each experiment was repeated at least three times.

Immunoblotting Analysis—Cells were scraped from the culture, washed twice with PBS, and then suspended in 30 μl of immunoblotting lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1% Triton X-100, and 1% SDS at 0–4 °C for 15 min. After centrifugation at 15,000 × g for 10 min at 0 °C, the supernatants were collected, and the protein amount in each sample was measured by a Bio-Rad DC kit (Bio-Rad, Hercules, CA). The equal amount of sample (50 μg of protein) was subjected to electrophoresis on either 12% or 15% SDS-polyacrylamide gel. Following electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution, and incubated overnight with the corresponding primary antibodies in the blocking solution at 4 °C. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 h, with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:1000). The detected protein signals were visualized by the enhanced chemiluminescence reaction system according to manufacturer’s recommendation (Amersham Biosciences, Indianapolis, IN).

Measurement of Δψm and ROS Generation—The measurement of mitochondrial membrane potential (Δψm) and reactive oxygen species (ROS) generation were performed as described by Marchetti et al. (37). Briefly, cells (5 × 10^4/ml) were exposed to 0.1 μM bortezomib for the indicated times. After exposure, cells were incubated in 40 nM DiOC6(3) and 5 μM dihydroethidine (Molecular Probes, Eugene, OR) at 37 °C for 15 min, and harvested by trypsinization and washed with cold PBS solution for three times. ROS and Δψm were determined by FACS analysis.

Determination of Cytochrome c Release from Mitochondria—H460 cells were treated with 0.1 μM bortezomib for the indicated times and harvested from culture. After washing with cold PBS, cells were incubated in the buffer containing 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin. Following incubation for 10 min on an ice bath, the cells were homogenized with a Dounce homogenizer for 20 strokes, and a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM EDTA, and 0.1% SDS at 0–4 °C. After centrifugation at 1,000 × g for 10 min at 4 °C, the supernatants were centrifuged at 15,000 × g for 30 min. The supernatants were collected and used as the cytosol fraction. The cytochrome c level in the cytosol fraction was detected by immunoblotting analysis. The quantitative analysis of cytochrome c protein in each sample was performed on a scanning densitometer (Bio-Rad GS-670 Imaging Densitometer).

Statistical Analysis—Data are presented as mean ± S.D. of the number of experiments indicated. The comparisons were made with a t test and the difference was considered to be statistically significant if the p value was <0.05.

RESULTS

Bortezomib Induces Apoptosis—In our recent studies, we had demonstrated that bortezomib exhibits highly significant activity against the proliferation of human H460 non-small cell lung cancer cells (28). In this work, we used this cell line as a model to determine whether the inhibition of cell growth evoked by this compound is due to an apoptotic death process. Initially, we exposed H460 cells to bortezomib at 0.1 μM, which is a concentration that correlates with the IC50 for a 72-h exposure...
of this cell line as assessed by a MTT assay in our previous report (28). The apoptotic nature of cell death was verified morphologically by phase-contrast microscopy showing that 0.1 μM bortezomib exposed cells for 48 h exhibited a shrinkage in size, membrane blebbing, and the loss of their attachment to the substratum compared with that in untreated control cells (Fig. 2A). For quantitative assessment of drug-induced apoptosis, H460 cells were exposed to 0.1 μM bortezomib for the indicated times, and the extent of apoptotic death was assessed by the determination of sub-G0/G1 cell population following Annexin V staining with propidium iodide as described under “Materials and Methods.” Fig. 2B shows that bortezomib exposure resulted in apoptotic cell death in a time-dependent manner, with apoptosis starting to occur at 24 h of exposure, and after 36–48 h of bortezomib exposure, a significant numbers of cells (38–48%) had progressed to sub-G0/G1 DNA content. By 60–72 h after bortezomib exposure, the sub-G0/G1 cell population reached a peak value (about 60%). To further confirm that the cell death by bortezomib was due to apoptosis, we assessed for the bortezomib-induced cleavage of PARP protein that is mediated by caspases, an event that is commonly used as an apoptotic hallmark. Fig. 2C shows that the PARP protein cleavage was also detected after 24 h of bortezomib exposure, and the extent of the PARP cleavage increased proportional with the duration of the exposure time.

**Effect of Bortezomib on Mitochondrial ROS Generation, ΔΨm Increase, and Cytochrome c Release**—Recent investigations have demonstrated that many apoptotic cascades utilize mitochondria as the nodal point where diverse apoptotic stimuli translate from initiation into execution (38). Mitochondria undergo critical changes, including the collapse of the inner transmembrane potential (ΔΨm), the generation of ROS, and the release of cytochrome c. We proceeded to examine for the possibility that the bortezomib-induced apoptosis could be related to contributions from the mitochondrial pathway. H460 cells exposed to 0.1 μM bortezomib for the indicated time periods were examined for changes in ROS generation and ΔΨm using the specific fluorescence probes, hydroethidine and DiOC6(3). Fig. 3 (A and B, top panels) contains typical fluorescence histograms showing that the exposure to 0.1 μM bortezomib induces a right shift of the hydroethidine and DiOC6(3) fluorescence curves. This is indicative that the bortezomib exposure induces an increase in the ROS generation and ΔΨm. Fig. 3 (A and B, bottom panels) shows that the relative levels of ROS and ΔΨm were elevated as early as 3–6 h after bortezomib exposure and gradually increased with exposure time. The levels of ROS and ΔΨm in untreated control cells were unchanged over all of the incubation time periods. We next explored the effect of bortezomib on the cytochrome c release from mitochondria into the cytosol. Starting at 3–6 h after 0.1 μM bortezomib exposure
Fig. 5. Tiron prevents the bortezomib-induced ROS elevation, and Δψ₁ increase. H460 cells were treated with 0.1 μM bortezomib, or with 0.1 μM bortezomib plus 1 mM tiron, or with the same volume of PBS solution as a control. After 24 h of incubation, the cells were taken and incubated in the presence of 5 μM hydroethidine for determination of ROS generation (A) or in the presence of 40 nM DiOC6(3) for the determination of Δψ₁ (B). C, the protective effect of tiron on bortezomib-induced cytochrome c release from mitochondria into the cytosol. H460 cells were treated with 0.1 μM bortezomib, or with bortezomib plus 1 mM tiron, or with the same volume of PBS solution as a control. After treatment for the indicated time, the cells were taken from culture and washed three times with cold PBS solution. The cytosol fraction was prepared as described under “Materials and Methods.” Cytochrome c (13 kDa) was detected by immunoblotting analysis. A nonspecific binding band (70 kDa) served as the sample loading control. A representative result of two independent experiments is shown.

(Fig. 3C), a small amount of cytochrome c in the cytosol fraction was observed. The cytochrome c levels were gradually elevated with increasing exposure time, reaching a peak value at 24 h, and then decreased after 36–48 h. It is important to note that the level of cytochrome c in the cytosol fraction was barely detected at time 0.

Effects of the Inhibitors of the Mitochondrial Electron Transport Chain on Bortezomib-induced ROS Generation and Δψ₁ Increase—It has been suggested that the ROS generation is regulated by a mitochondrial electron transport chain (39). To examine the role of the mitochondrial electron transport chain system in regulating bortezomib-induced ROS generation, we evaluated the effects of rotenone, antimycin A, and cyclosporine A (CsA) on ROS generation and the increase in Δψ₁ after co-treatment for 24 h with 0.1 μM bortezomib. Fig. 4 (A and B) shows that the co-treatment with these inhibitors caused a blocking of the bortezomib-induced ROS generation and Δψ₁ increase, as compared with that observed with bortezomib treatment alone. The treatment with these inhibitors alone did not significantly change the levels of ROS and Δψ₁ as compared with that in the untreated control. To further explore whether the blockage of ROS generation and Δψ₁ by rotenone, antimycin A, and CsA could protect against bortezomib-induced cytochrome c release from mitochondria into the cytosol, a series of combination inhibitor plus bortezomib were completed. Fig. 4C shows that co-treatment with 1 μM rotenone, 10 μM antimycin A, or 1 μM CsA caused prevention of the bortezomib-induced cytochrome c release from mitochondria into the cytosol.

Effect of the Antioxidant Agent Tiron on Bortezomib-induced ROS Generation and Δψ₁ Increase—Because bortezomib treatment led to the enhancement of ROS generation, it is possible that alterations in the cellular superoxidant state could play a role in bortezomib-induced apoptosis. To examine this hypothesis, the antioxidant agent tiron was used to perturb the bortezomib-induced ROS generation and then to examine the consequent events. The data in Fig. 5 (A and B) show that co-treatment with 1 mM tiron for 24 h resulted in the complete restoration of bortezomib-induced right shift in the fluorescence curves of ROS and Δψ₁. We also found that the co-treatment with tiron could prevent the bortezomib-induced cytochrome c release from mitochondria. The results in Fig. 5C show that bortezomib-induced cytochrome c release was inhibited starting at 9 h of co-incubation. The release of cytochrome c was almost completely abrogated after 12–24 h of co-incubation. Consequently, we sought to examine whether the abolishment of intracellular superoxide by tiron could rescue the bortezomib-induced apoptotic death. Similar to the morphological changes presented in Fig. 2A, the bortezomib exposure led to a number of cells progressing to apoptotic death; however, the co-treatment with 1 mM tiron resulted in most cells displaying morphological features comparable to the untreated control cells (Fig. 6A). We then investigated the effect of co-treatment with 0.1–1 mM tiron on the bortezomib-induced PARP protein cleavage and cell death. Similar to the arrest of the apoptosis, co-treatment with tiron resulted in a concentration-dependent protection against bortezomib-induced PARP cleavage and cell death. The co-treatment with 1 mM tiron showed a complete inhibition of PARP protein cleavage (Fig. 6B) and caused a significant reduction the H460 cellular death as compared with that in bortezomib treatment.
alone (~8% cell death in 1 mM tiron co-treatment versus ~48% cell death in bortezomib alone, p < 0.001) (Fig. 6C).

Effect of Caspase Inhibitor on Bortezomib-induced ROS Generation and Δψ\textsubscript{m} Increase.—In previous studies, we had shown that bortezomib exposure led to the activation of caspases at the initiation and the execution phase (28), thus we tested whether the ROS generation and change in Δψ\textsubscript{m} were tied to processes in the caspase-dependent pathway. Fig. 7 (A and B) contains profiles that show that 50 μM Z-VAD-fmk did not affect the bortezomib-induced elevation of ROS generation and Δψ\textsubscript{m}. Under the same experimental conditions, we found that bortezomib-induced PARP protein cleavage and cell death were significantly inhibited by 50 μM Z-VAD-fmk (Fig. 7, C and D). These two lines of data suggest that the generation of ROS and disruption of mitochondrial functions were not through the caspase-dependent pathway, and the ROS generation and change in Δψ\textsubscript{m} comprised an upstream event resulting from caspase activity in the bortezomib-induced apoptosis.

Effect of Overexpression of Bcl-2 on Bortezomib-induced ROS Generation and Δψ\textsubscript{m} Increase.—It is known that the Bcl-2 family of proteins play critical roles in the regulation of mitochondria-dependent apoptosis (11, 40). As shown in Fig. 8A, the PC-3/Bcl-2 cells were found to display more resistance to bortezomib than that of PC-3/Vector cells. The exposure to bortezomib at 0.1 μM for 72 h caused a 90% cell death in PC-3/Vector cells but resulted in just 60% cell death in the PC-3/Bcl-2 cells. The numbers of apoptotic cells in PC-3/Bcl-2 cells were markedly lower than that in the PC-3/Vector cells in a bortezomib concentration-dependent manner, indicating that the overexpression of Bcl-2 protein was able to prevent the bortezomib-induced apoptosis (Fig. 8B). We found that the relative levels of ROS and Δψ\textsubscript{m} in PC-3/Bcl-2 cells exposed to different concentration of bortezomib were markedly lower than those in the PC-3/Vector cells (Fig. 8, C and D). The data suggest that both bortezomib-induced accumulation of ROS and increase in Δψ\textsubscript{m} may be regulated by Bcl-2 and its related pathway.

Effect of Overexpression of p53 on Bortezomib-induced ROS Generation and Δψ\textsubscript{m} Increase.—p53 overexpression may be a factor involved in the ROS generation and this effect may be necessary for the p53-dependent apoptosis (13). In our previous work with H460 cells, we had shown that bortezomib exposure induced the stabilization and accumulation of intracellular p53 protein (28). The data plotted in Fig. 9 (A and B) indicate that p53 transfection led to a significant elevation of ROS and Δψ\textsubscript{m}. The bortezomib exposure caused the elevation of levels of ROS and Δψ\textsubscript{m} in all tested cells to a similar extent, suggesting that ROS production and change in Δψ\textsubscript{m} by bortezomib were not further enhanced in H358 cells transfected with p53. Under the same experimental conditions, the p53 transfection caused cell apoptotic death (~13% of apoptotic cells in untreated H358/p53 cells versus ~6% of apoptotic cells in untreated H358 or H358/Vector cells). In addition, the p53 transfection strongly activated bortezomib-induced apoptosis, i.e. ~29% of cells exhibited apoptotic death in the bortezomib-treated H358/p53 cells, whereas only ~10–12% of cells exhibited apoptotic death in the bortezomib-treated H358 and H358/Vector cells (Fig. 9C). Moreover, the immunoblotting analysis revealed that a transient transfection of p53 cDNA resulted in a higher expression of p53, and with bortezomib exposure, led to a remarkable accumulation of p53 protein in H358/p53 cells. None of the p53 signal was seen in either H358 cells or in the H358/Vector cells treated with or without bortezomib. These data suggest that p53 overexpression is involved in the regulation of bortezomib-induced apoptotic pathway but not in the ROS generation and mitochondrial damage.
DISCUSSION

In our previous work, we have shown that bortezomib is a highly active proteasome inhibitor with strong activity against a variety of tumor cell lines in studies in vitro and in vivo. We also found that bortezomib exposure caused cell cycle arrest at the G2/M phase in H460 cells. The G2/M phase arrest by bortezomib was shown to occur via drug-induced stabilization of p53 protein and induction of p21 and MDM2 proteins, as well as the accumulation and stimulation of G2/M phase-related regulators such as cyclins A and B (28). Furthermore, our previous studies have demonstrated that bortezomib exposure leads to Bcl-2 phosphorylation and cleavage, which are associated with the events of the bortezomib-induced G2/M phase arrest and apoptosis (41). In the present work, we have further examined the molecular mechanisms of action of bortezomib in terms of the sequence of events involved in the inhibition of cell proliferation and cell death. Our results show that the exposure of H460 cells with low concentration of bortezomib resulted in apoptotic cell death as verified from morphological observations and the assessment of sub-G0/G1 cells by FACS analysis (Fig. 2). In addition, we verified that bortezomib induces the disruption of mitochondrial function, via the observed elevation of mitochondrial derived ROS production, and an increase in \( \Delta \psi_m \), along with the cytochrome c release from mitochondria into the cytosol (Fig. 3). The time course studies showed that the elevation of ROS generation, \( \Delta \psi_m \) increase, and the cytochrome c release occurred as early as 3–6 h post exposure to bortezomib, indicating that these events were earlier than that in apoptotic execution such as DNA fragmentation and PARP protein cleavage as described in Fig. 2. Interestingly, the disruption of mitochondrial function and the induction of apoptosis can be completely rescued following the abolishment of the intracellular superoxidant state by the antioxidant agent tiron. These results suggest that the elevation in ROS generation is a required event for the bortezomib-induced apoptosis. In addition, we found that the elevation of ROS generation and \( \Delta \psi_m \) increase were induced in H460 cells by other types of proteasome inhibitors such as MG-132, PSI (Z-IE(OtBu)AL-CHO) and lactacystin, indicating that the induction of ROS production and \( \Delta \psi_m \) increase are not restricted to bortezomib (data not shown). It appears to be significant that the elevation in ROS generation and increase in \( \Delta \psi_m \) are early events in the disruption of mitochondrial function.

**Fig. 7. Effect of Z-VAD-fmk on bortezomib-induced ROS generation, \( \Delta \psi_m \) increase, and apoptosis.**

A. Z-VAD-fmk did not change the bortezomib-induced ROS generation. The ROS generation was assessed by the incubation with hydroethidine for 15 min following 24 h of exposure of H460 cells to 0.1 \( \mu \)M bortezomib, or to 50 \( \mu \)M Z-VAD-fmk, or to the same volume of PBS solution as a control. B. Z-VAD-fmk did not change the bortezomib-induced \( \Delta \psi_m \). The increased \( \Delta \psi_m \) was assessed by the incubation with DiOC6(3) for 15 min following 24 h of treatment of H460 cells with 0.1 \( \mu \)M bortezomib, or with 50 \( \mu \)M Z-VAD-fmk, or with the same volume of PBS solution as a control. C. Z-VAD-fmk prevented the PARP cleavage after 48 h of treatment of H460 cells with 0.1 \( \mu \)M bortezomib, or plus with 50 \( \mu \)M Z-VAD-fmk, or with the same volume of PBS solution as a control. D. Z-VAD-fmk effectively reduced the bortezomib-induced cell death. H460 cells were treated with 0.1 \( \mu \)M bortezomib, or with 50 \( \mu \)M Z-VAD-fmk alone, or with 0.1 \( \mu \)M bortezomib plus 50 \( \mu \)M Z-VAD-fmk, or with the same volume of PBS solution as a control. After 48 h of incubation, the cell death was assessed by trypan blue exclusion as described under “Materials and Methods.” The data represent the mean ± S.D. of three independent experiments (**, \( p < 0.001 \); statistically significant comparison with that in bortezomib treated alone).
bortezomib-induced apoptotic cascade and appear to have a major contribution in the initiation of apoptotic signals by proteasome inhibitors.

In this work, we have demonstrated that bortezomib exposure results in a dramatic elevation of the ROS generation in a time-dependent manner. However, it is not known how the inhibition of ubiquitin-proteasome pathways can directly induce the ROS generation and the disruption of mitochondrial function. One possible explanation for the elevation in ROS generation is that bortezomib could directly or indirectly interact with the ROS generation system and results in an increase in the production of O$_2$. However, we have no direct evidence to verify that the elevation in ROS generation could be due to the bortezomib-induced production of free radical O$_2$. Another possibility is that bortezomib could act on the mitochondrial electron transport system and subsequently cause an elevation in ROS generation. These data with the inhibition of mitochondrial electron transport chain by rotenone, antimycin A, and CsA suggest that bortezomib-induced ROS generation and Δψm increase may be associated with mitochondrial electron transport chain complexes and/or with the modulation of mitochondrial membrane permeabilization (Fig. 4). The other possibility is that bortezomib treatment could alter the oxidation-reduction metabolic pathways and metabolites such as alteration in the intracellular glutathione level, xanthine/xanthine oxidase system, and/or changes in NADPH oxidase in the membrane and the mitochondrial electron transport system (42, 43). Moreover, recent reports have shown that ROS production may be associated with the activation of NFκB family (44). It therefore should be considered that the ROS elevation by bortezomib could be due to a direct or indirect interference with NFκB-related pathways (45). Finally, bortezomib treatment could prevent protein degradation and induce gene transcription (46). Accordingly, we believe that the bortezomib-induced ROS generation could be associated with the activation of the transcription of ROS-related genes and/or accumulation of its related products. Indeed, a recent investigation has shown that a ROS-related gene product such as PIG3, a gene homolog of oxidoreductase, was verified to be involved in p53-induced elevation of ROS production (47).

It has been known that Δψm is a component of the overall moving proton force that drives the ATP generation in mitochondria (48). Recent investigations have shown that an alteration in mitochondrial Δψm was implicated in the induction of apoptosis. For example, the increase in Δψm was observed in L929 cells triggered into apoptosis by growth factor withdrawal (49), and renal proximal tubular cells induced by cisplatin treatment into apoptosis (50). In this work, we have demonstrated the close relationship between the increase in ROS generation and increased Δψm in bortezomib-treated H460 cells. Furthermore, we have found that the prevention of ROS generation with antioxidant agent tiron led to a complete restoration of Δψm (Fig. 5). These results are consistent with another report that described how a pro-oxidant state of cerebellar granule neurons caused by the deprivation of nerve growth factor is able to elevate Δψm and induce apoptosis (42). The increase in Δψm could be due to the disruption of mitochondrial functions on the inner membrane, including the reduction of mitochondrial electron transport flow rate and the instability of F$_{0}$F$_{1}$-ATPase to effectively pump protons to the mitochondrial matrix (51). An alternative mechanism that could cause the increase in Δψm could be the closure of the voltage-dependent anion channel on the mitochondrial outer membrane (52). Moreover, cytochrome c release from mitochondria may be in-

![Figure 8](image_url)

**Fig. 8.** Effect of overexpression of Bcl-2 on bortezomib-induced apoptosis, ROS generation, and Δψm increase in human PC-3 prostate carcinoma cells. The Bcl-2 stable transfectant of PC-3/Bcl-2 and PC-3/Vector cells were exposed to varying concentrations of bortezomib for 72 h. After exposure, the cell survivals were assessed by a MTT assay (A), or the cells were exposed to different concentrations of bortezomib for 48 h. After exposure, cells were harvested and stained with propidium iodide for determination of apoptotic death by FACS analysis (B). The data represent the mean ± S.D. of three independent experiments. PC-3/Vector and PC-3/Bcl-2 cells were exposed to varying concentrations of bortezomib for 24 h. After exposure, the cells were taken and incubated in the presence of 5 μM hydroethidine for the determination of ROS generation (C), or in the presence of 40 nm DiOC6(3) for the determination of Δψm (D). The data are expressed the relative levels of ROS and Δψm compared with that in the untreated control cells as a 100%. Each bar represents the mean ± S.D. of three independent experiments.
volved in the opening of the permeability transition pore of the inner mitochondrial membrane. This opening should result in swelling of the mitochondria and the disruption of the outer mitochondrial membrane. In this work, we have examined the effect of bortezomib on mitochondria structures observed by electron microscopy and found that bortezomib treatment caused the mitochondria structural changes as early as 3–6 h after exposure (data not shown), suggesting that the increase of ∆ψm and cytochrome c release may be due to the alteration in the mitochondrial structure. Furthermore, the data with the antioxidant agent, tiron, suggest that the elimination of the intracellular superoxidant state by tiron can block the bortezomib-induced apoptosis. 

Besides the alteration of mitochondrial functions, the ROS generation may be regulated by caspase activation caused by apoptotic stimuli (56). In this work, we demonstrated that the ROS generation was an upstream event of caspase activation in the bortezomib-induced apoptosis. This is consistent with the reports by Polyak et al. (48).

There is accumulating evidence that the cytochrome c release from mitochondria is controlled by Bcl-2 families (55). Bcl-2 protein functions as an anti-apoptotic molecular rheostat that prevents cytochrome c release into the cytosol by a variety of apoptotic stimuli (56). In this work, we demonstrated that the enforced Bcl-2 overexpression in human PC-3 prostate carcinoma cells results in a protection from the bortezomib-induced ROS generation and ∆ψm increase. Furthermore, the overexpression of Bcl-2 effectively reduces the bortezomib-induced apoptosis (Fig. 8). In our previous studies, the phosphorylation and cleavage of Bcl-2 protein were induced by bortezomib exposure (41). This series of findings suggests that the inactivation of Bcl-2 protein may be implicated in the regulation of bortezomib-induced apoptotic cascades, including the disruption of mitochondrial function and ROS generation.

p53 is a transcription factor that not only induces growth arrest through transcription of the genes such as p21 and p27 but also triggers apoptotic signals via the induction of apoptosis-related genes (26, 57). There is an accumulation of evidence showing that p53-induced apoptosis may be involved in the activation mitochondrial pathway through the elevation of ROS generation and the alteration of ∆ψm in some types of cells (13, 27). In the work present here, we have used the p53 null genotype of H358 cells to examine the effect of p53 overexpression on the bortezomib-induced ROS generation and apoptosis.
ROS Generation and Mitochondrial Dysfunction by Proteasome Inhibitor

Our data showed that the transient transfection of p53 cDNA caused a significant induction of ROS generation and ΔΨm increase. This result is consistent with the data reported by Li and coworkers (13). It is worth noting that the p53 transfection strongly increased bortezomib-induced apoptosis but did not further enhance the bortezomib-induced ROS generation and ΔΨm increase (Fig. 9). All of these data suggest that the elevation in ROS generation and mitochondrial injury are not likely to depend on the p53 pathway, although the bortezomib-induced apoptosis was significantly activated by p53 overexpression.

In summary, our results demonstrate that the ROS generation is a key factor in bortezomib-induced apoptosis, because the abrogation of ROS generation can prevent the increase in ΔΨm and cytochrome c release from mitochondria, as well as apoptosis. In addition, the inactivation of bcl-2 cleavage may be implicated in the regulation of mitochondrial dysfunction in bortezomib-induced apoptosis. Although transfection of wild-type p53 does not directly enhance the bortezomib-induced apoptosis, transient transfection of p53 cDNA strongly increased bortezomib-induced apoptosis but did not increase. This result is consistent with the data reported by Li et al. (13).

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