A coordinated action of Bax, PUMA, and p53 promotes MG132-induced mitochondria activation and apoptosis in colon cancer cells

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Abstract
Targeting the ubiquitin-proteasome degradation pathway has become a promising approach for cancer therapy. Previous studies have shown that proteasome inhibition leads to apoptosis in various cancer cells. The mechanism by which apoptosis occurs are not fully understood and can be cell type and/or inhibitor specific. In this study, we investigated the mechanism of mitochondrial activation by proteasome inhibitors in colon cancer cells. We found that Bax activation and mitochondria translocation were required for apoptosis induced by multiple proteasome inhibitors. In contrast, reactive oxygen species did not seem to be induced by MG132 or bortezomib and antioxidants had no effects on MG132-induced apoptosis. In contrast, treatment with MG132 or bortezomib induced a significant accumulation of p53 and PUMA. Genetic deletion of either p53 or PUMA led to a marked suppression of apoptosis induced by these inhibitors, accompanied with reduced Bax activation and cytochrome c release. Consistently, inhibition of translation by cycloheximide could also effectively abolish the accumulation of p53 and PUMA and suppress MG132-induced Bax activation and apoptosis. These findings thus strongly indicate the critical involvement of p53, PUMA-, and Bax-mediated mitochondrial activation in proteasome inhibitor–induced apoptosis in colon cancer cells. [Mol Cancer Ther 2007;6(3):1062–9]

Introduction
The 26S proteasome, a multicatalytic enzyme complex, is the main intracellular proteolytic system for clearing ubiquitinated short-lived proteins (1). The ubiquitin proteasome system plays an important role in regulating apoptosis and cell cycle by controlling the degradation of important molecules, such as p53, I-κB, and cyclins. Disruption of proteasome function can thus lead to activation of apoptosis, which has recently become a novel strategy for cancer therapy (2). A peptide boronate inhibitor, bortezomib, has been approved recently by Food and Drug Administration for treatment of refractory and relapsed multiple myeloma (3) and is undergoing clinical trials for other types of cancer (2).

Multiple mechanisms can be responsible for the proteasome inhibitor–induced apoptosis (2, 3), which include (a) inhibition of the nuclear factor-κB survival pathway through the prevention of I-κB degradation (2); (b) induction of endoplasmic reticulum stress (4, 5); (c) generation of reactive oxygen species (ROS; refs. 4, 6, 7); (d) activation of c-Jun NH2-terminal kinase (8); (e) accumulation of the pro-death proteins Bik and Bim (9, 10); and (f) induction of the mitochondria pathway (9–14). In addition, proteasome inhibition can activate the death receptor pathway (9, 14) and sensitize cells to killing by tumor necrosis factor–related apoptosis-inducing ligand via the up-regulation of death receptor 5 (15). These mechanisms are not necessarily mutually exclusive and in fact can be closely linked. On the other hand, a particular proteasome inhibitor may preferentially activate certain mechanisms in certain types of cancer cells, which if altered could constitute the basis of tumor-specific resistance to the inhibitors. It is thus important to decipher the particular death pathways in a specific type of tumors to broaden the clinical application spectrum of the proteasome inhibitors.

The Bcl-2 family proteins play important roles in the regulation of apoptosis by targeting to the mitochondria to exert their proapoptotic or antiapoptotic effects (16). The antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, and Mcl-1) promote cell survival. The pro-death molecules Bak and Bax are essential to mitochondrial release of apoptogenic factors, such as cytochrome c and Smac (16). Lack of Bak markedly decreases the cytotoxicity of proteasome inhibitors (12). However, the molecular mechanisms of Bax activation by proteasome inhibition are not clear.

In this study, we defined a previously undisclosed mechanism activated by proteasome inhibitors in the colon cancer cells. We found that inhibition of proteasome led to the accumulation of PUMA and p53, which in turn promote the conformational change and activation of Bax.
Cycloheximide, a general translation inhibitor, suppressed the accumulation of PUMA and p53 and contributed to the reduced Bax activation and apoptosis. These findings could be of therapeutic implication for the use of proteasome inhibitors in colon cancer.

Materials and Methods

Cell Lines

Parental HCT116 cells and the isogenic Bax−/−, p53−/−, and PUMA−/− cell lines have been described previously (12, 17). HCT116 cells were maintained in McCoy’s 5A, DU145 and HeLa cells were maintained in DMEM, and PC3 cells were maintained in F12K, all with standard supplements. All cell lines were maintained in a 37°C incubator with 5% CO2.

Antibodies and Chemicals

Anti–cytochrome c, Smac, Mcl-1, Bcl-2, Bax (clone 6A7), and caspase-9 antibodies were from BD Biosciences (San Jose, CA). Anti-Bax (N-20) and p53 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–Bcl-x were from Cell Signaling (Beverly, MA). Anti-Bak was from Upstate (Charlottesville, VA). MG132, lactacystin, and N-acetyl-Leu-Leu-norleucinal were from Sigma (St. Louis, MO). Bortezomib was obtained from Millennium Pharmaceuticals as described previously (12).

Quantification of Bax Translocation

Bax-deficient HCT116 cells (2 × 10⁶) were transfected with green fluorescent protein (GFP)–Bax (a generous gift from Dr. Richard Youle; NIH, Bethesda, MD) using Effectene according to the supplier’s protocol (Qiagen, Valencia, CA). After designated treatments, GFP-Bax HCT116 cells cultured on coverslips were first stained with MitoTracker Red (50 nmol/L; Molecular Probes, Eugene, OR) and then fixed with paraformaldehyde (4%, w/v). Cells with punctated GFP-Bax, indicative of Bax translocation to the mitochondria, were quantified using a Nikon fluorescence microscope (Melville, NY) equipped with a SPOT digital camera.

Figure 1. MG132-induced apoptosis in HCT116 cells requires Bax. A, Bax-positive and Bax-negative HCT116 cells were treated with vehicle control or MG132 (1 μmol/L) in the presence or absence of zVAD-fmk (50 μmol/L) for the designated time. Apoptotic cells were quantified by Hoechst 33342 staining. B, Bax-negative HCT116 cells that were stably reconstituted with GFP or GFP-Bax were treated with vehicle control or MG132 (1 μmol/L) and apoptosis was determined as in (A). Points, mean; bars, SD. C and D, MG132 induced cytochrome c (Cyto c) release, caspase-9 (C-9), and caspase-3 (C-3) cleavage in Bax-positive and Bax-negative HCT116 cells (C) or Bax-negative HCT116 cells that were stably reconstituted with GFP or GFP-Bax (D). Cells were treated with MG132 (1 μmol/L) for 24 h. The cytosol fraction was subjected to immunoblot assay and caspase activity measurement.

Measurement of Cell Viability and Caspase Activity

Cells (2 × 10⁶ per well) were seeded into 12-well plates. After indicated treatment, cells were then stained with Hoechst 33342 (10 μg/mL) for 5 min and apoptotic cells with fragmented nuclei were quantified by digital imaging. Caspase assay was conducted as described before (18). Briefly, 15 μg whole-cell lysates were incubated with 2 μmol/L Ac-DEVDD-FMK or Ac-LEHD-FMK (Calbiochem, La Jolla, CA) for caspase-3 or caspase-9 activity, respectively. The change in fluorescence emission (excitation at 405 nm and emission at 500 nm) was monitored and the background signals were corrected. Results were expressed as the fold changes over the control samples.

Subcellular Fractionation and Immunoblot Assay

The preparation of cytosolic fraction and immunoblot analysis for cytochrome c and Smac release, caspase cleavage, and Bax translocation were done as described previously (18). Cytosol fraction was obtained from the supernatant after cells were lysed with 0.05% digitonin in isotonic buffer [10 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA (pH 7.4)] and centrifuged at 12,000 rpm for 10 min. The supernatants were collected for the detection of released mitochondrial proteins, caspase-3 and caspase-9 by immunoblot analysis. The pellet was dissolved in 2% CHAPS in isotonic buffer and used for the detection of Bax translocation to the mitochondria.
Detection of Bax Conformational Changes

Cells were lysed in CHAPS lysis buffer [150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), 1% CHAPS] containing protease inhibitors and subjected to immunoprecipitation with a conformation-sensitive anti-Bax monoclonal antibody (clone 6A7; BD Biosciences). The immunoprecipitates containing the conformationally changed Bax protein were separated by SDS-PAGE followed by immunoblot with a general anti-Bax antibody (N-20).

Measurement of ROS Production

Superoxide anion was detected as described previously (18). Briefly, following a designated treatment, cells were incubated with 2.5 μmol/L dihydroethidium for 30 min at 37°C. The cells were washed, resuspended in PBS, and measured by flow cytometry for the level of ethidium, a red fluorescence product derived from dihydroethidium in the presence of superoxide anion.

Results

Proteasome Inhibition Induces Bax-Dependent Apoptosis in HCT116 Cells

A proteasome inhibitor, bortezomib, had been found to induce apoptosis in HCT116 cells in a Bax-dependent way (see below; ref. 12). But how Bax is activated is not known. To address this issue, we would like to first confirm the finding using different proteasome inhibitors. The Bax-positive HCT116 colon cancer cells contain one copy of functional Bax gene, and the Bax-negative HCT116 cells were derived by genetic deletion of the Bax gene (17). Indeed, Bax-positive HCT116 cells were much more sensitive than Bax-negative cells to apoptosis induced by MG132, lactacystin, or N-acetyl-Leu-Leu-norleucinal in a dose- and time-dependent manner (Fig. 1A; Supplementary Fig. S1A). In addition, Bax-deficient cells that were reconstituted with GFP-Bax recovered the sensitivity to MG132 induced-apoptosis (Fig. 1B). Consistently, we found that MG132 induced the release of cytochrome c only in Bax-positive parental cells or in Bax-deficient cells stably reconstituted with GFP-Bax (Fig. 1C and D). Neither Bax-deficient parental cells nor the GFP-only reconstituted subline would release cytochrome c following MG132 treatment. As the result, the activation of caspase-9 and caspase-3 was also dependent on Bax (Fig. 1C and D) and the apoptosis could be very well inhibited by zVAD-fmk, a general caspase inhibitor (Fig. 1A).

It seems that the importance of Bax for MG132-induced apoptosis was not restricted in HCT116 cells. MG132 could induce cytochrome c release, caspase activation, and apoptosis in Bax-positive PC3 prostate cancer cells but not in Bax-negative DU145 prostate cancer cells (Supplementary Fig. S2).

Proteasome Inhibition Induces Bax Translocation in Cancer Cells

The dependence of proteasome inhibitors on Bax to induce apoptosis indicated that Bax was activated by these chemicals. Bax normally resides in the cytosol and translocates to the mitochondria on apoptotic stimulation. MG132 treatment resulted in an increased level of Bax in the mitochondrial fraction in HCT116 cells (Fig. 2A). Consistently, we found that MG132 induced the release of cytochrome c only in Bax-positive parental cells or in Bax-deficient cells stably reconstituted with GFP-Bax (Fig. 1C and D). Neither Bax-deficient parental cells nor the GFP-only reconstituted subline would release cytochrome c following MG132 treatment. As the result, the activation of caspase-9 and caspase-3 was also dependent on Bax (Fig. 1C and D) and the apoptosis could be very well inhibited by zVAD-fmk, a general caspase inhibitor (Fig. 1A).

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Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
indicating the mitochondrial location (Fig. 2C). Notably, only those cells with punctated GFP-Bax went into apoptosis based on the morphology of the nuclei (Fig. 2B), further confirming the critical role of Bax in MG132-induced apoptosis.

**MG132 Does Not Increase ROS Generation in HCT116 Cells**

ROS could be important in proteasome inhibition–induced apoptosis in some cancer cells (4, 6, 7). We thus would like to examine whether ROS played a role in MG132-induced Bax activation and apoptosis. We found that neither MG132 nor bortezomib could significantly promote superoxide anion production in the Bax-positive HCT116 despite that these cells were capable of generating superoxide anion when stimulated with UV (Fig. 3A; Supplementary Fig. S3). We also failed to detect any hydrogen peroxide production using 2,7'-dichlorodihydrofluorescein diacetate in MG132-treated HCT116 cells (data not shown). Consistently, antioxidants, such as Mn (III) tetraakis (4-benzoic acid) porphyrin, a mimetic of superoxide dismutase, N-acetylcysteine, a precursor of intracellular glutathione, and Tiron, a free radical scavenger, all failed to inhibit MG132-induced apoptosis (Fig. 3B), Bax translocation (Fig. 3C), mitochondrial release of cytochrome c, and caspase-3 activation (Fig. 3D) in HCT116 cells. Taken together, these data suggest that ROS did not seem to play a significant role in MG132-induced Bax activation and apoptosis in colon cancer cells.

**PUMA and p53 Promote MG132-Induced Apoptosis by Activating Bax**

Inhibition of proteasome function can lead to the accumulation of many short-lived proteins. Interestingly, MG132 treatment led to increased levels of several antiapoptotic proteins, such as Mcl-1, Bcl-2, and Bcl-xL (Supplementary Fig. S4A), which are able to suppress Bax activation. However, the successful induction of apoptosis by MG132 in these cells indicated that these antiapoptotic proteins must have been antagonized to allow the activation of Bax. The BH3-only pro-death Bcl-2 family proteins can activate Bax either directly, such as Bid (19, 20), or indirectly by antagonizing the anti-death Bcl-2 family proteins, such as PUMA (20, 21). The level of Bid was not significantly increased due to the inhibition of its degradation or reduced due to caspase cleavage following MG132 treatment, although tumor necrosis factor-α treatment could induce the cleavage in these cells (Supplementary Fig. S4B and D). These observations suggested that Bid might not be involved in the activation of Bax in this context. In contrast, the level of PUMA was significantly increased in cells treated with MG132 or bortezomib (Supplementary Fig. S4B and C). PUMA expression is mainly controlled by p53 (22, 23), which is normally rapidly degraded via the proteasomes (24). Indeed, the amount of p53 was dramatically increased following MG132 or bortezomib treatment (Supplementary Fig. S4B and C).
Notably, p53 can activate Bax by transcriptionally up-regulating PUMA (22, 23) and by its transcriptionally independent function (25, 26). We then asked whether p53 and PUMA would contribute to proteasome inhibition–induced Bax activation and apoptosis. Genetic deletion of PUMA or p53 in HCT116 cells significantly reduced mitochondrial release of cytochrome c and Smac and the activation of caspase-3 following MG132 treatment despite the presence of Bax (Fig. 4A and B). Consequently, apoptosis was significantly reduced in these cells (Fig. 4C). The importance of PUMA and p53 in proteasome inhibitor–induced apoptosis was also confirmed in the same panel of HCT116 cells treated with bortezomib (Supplementary Fig. S5). Furthermore, in another Bax-positive colon cancer cell line, RKO, we found that MG132 treatment also up-regulated the expression of p53 and PUMA (data not shown) and inhibition of Bax or PUMA expression using the specific small interfering RNAs led to significant resistance to MG132-induced apoptosis and caspase activation (Supplementary Fig. S6).

To examine whether deletion of PUMA or p53 would affect MG132-induced Bax activation, we did immunoprecipitation assay using an antibody that specifically recognized Bax in the activated conformation (clone 6A7; BD Biosciences; ref. 27). As shown in Fig. 4D, p53 was not expressed in the p53-deficient HCT116, and as the result, PUMA accumulation was not seen in this cell line treated with MG132. In PUMA-deficient cell line, despite that there was an accumulation of p53 following MG132 treatment, there was no PUMA expression. Furthermore, deletion of PUMA led to a noticeable reduction of MG132-induced conformationally changed Bax, whereas deletion of p53 completely inhibited such a conformational change. In all these cases, the effect of p53 deletion seemed to be more potent than PUMA deletion, suggesting that PUMA is only one of the mediators of p53 effects. Overall, these data strongly indicate that p53 and PUMA can promote MG132-induced Bax activation and in turn MG132-induced mitochondrial activation and apoptosis.

Inhibition of Protein Synthesis Suppresses MG132-Induced Bax Activation and Apoptosis

Because the inhibition of proteasome by MG132 led to the accumulation of proapoptotic proteins p53 and PUMA, we wondered whether inhibition of such an accumulation would reverse MG132-induced apoptosis. We thus examined the effects of cycloheximide, a general translational inhibitor. Cycloheximide eliminated MG132-induced accumulation of p53 and PUMA (Fig. 5A). Cycloheximide could effectively inhibit Bax translocation based on both subcellular fractionation study and GFP-Bax assay (Fig. 5B and C). MG132-induced Bax conformational change could also be blocked by cycloheximide (Fig. 5D). Consequently, cycloheximide inhibited MG132-induced mitochondrial cytochrome c and Smac release (Fig. 5E; Supplementary Fig. S7A). It also inhibited MG132-induced activation of caspase-3 and caspase-9 (Fig. 5E; Fig. Supplementary S7A–C). Collectively, these effects of cycloheximide contributed to the suppression of MG132-induced apoptosis (Fig. 5F). It is conceivable that the elimination of PUMA and p53 accumulation could constitute a major mechanism by which cycloheximide inhibited MG132-induced Bax activation and apoptosis in HCT116 cells.

Discussion

The ubiquitin-proteasome degradation pathway plays an essential role in protein homeostasis, which is fundamental to the regulation of cell survival. Therefore, proteasome has become an attractive target for cancer therapy. Induction of apoptosis in various cancer cells by the inhibition of
proteasome has been well documented (2, 3), in which the mitochondria apoptotic pathway can play important roles (9–14). However, how the mitochondria pathway is activated is not entirely clear and could vary in different cells with different inhibitors. In the present study, we confirmed that the proapoptotic protein Bax was required for the proteasome inhibition–induced apoptosis in colon cancer cells. We further revealed that the accumulation of proapoptotic proteins, p53 and PUMA, promoted Bax activation and the subsequent mitochondria activation and apoptosis.

The mitochondria apoptotic pathway is mainly regulated by the Bcl-2 family proteins. This family of proteins consists of both antiapoptotic proteins (Bcl-2, Mcl-1, Bcl-xL, etc.) and proapoptotic proteins (Bid, Bax, etc.; ref. 16). The proapoptotic proteins could be further classified into multidomain proteins, such as Bax and Bak, and BH3-only molecules, such as PUMA and Bid. It has been generally thought that the BH3-only molecules activate the downstream multidomain Bax and Bak, which then initiate the mitochondrial release of apoptogenic factors. It has also been shown that some of the BH3-only molecules, such as tBid and Bim, can directly bind to and activate Bax, whereas most of other BH3-only molecules seem to activate Bax indirectly by binding to and antagonizing the antiapoptotic Bcl-2 family proteins (19–21). One important BH3-only molecule is PUMA (23, 28). PUMA can activate Bax through binding to Bcl-xL and dissociate the interaction of Bax and Bcl-xL (21, 22). Liberated Bax is responsible for cytochrome c release and apoptosis. Indeed, PUMA-deficient cells were significantly resistant to apoptosis induced by proteasome inhibitors (this study) and many other stimuli (29, 30).

The accumulation of PUMA following MG132 treatment was p53 dependent, as such the accumulation was not observed in p53-deficient cells (Fig. 4). PUMA is transcriptionally up-regulated by p53 (22, 23). Inhibition of proteasome function resulted in the blockage of normal degradation of p53 and in turn up-regulated PUMA. p53 and PUMA could work together not only via the transcriptional coupling but also via collaborative protein interactions at the mitochondria. Thus, it has been shown that PUMA can dissociate Bax–Bcl-xL interaction (21), as well as p53–Bcl-xL interaction (26). Together, this could

![Figure 5](image-url)

**Figure 5.** Inhibition of protein translation reverses MG132-induced accumulation of p53 and PUMA and suppresses Bax activation. **A,** Bax-positive HCT116 cells were treated with MG132 plus cycloheximide (CHX; 10 μg/mL) for 24 h. Total cell lysates were prepared for the immunoblot assay. **B,** the membrane fraction was prepared from cells treated as in (A), with 1 μmol/L MG132, to detect Bax. **C,** Bax-negative HCT116 cells that were stably reconstituted with GFP-Bax were treated with MG132 in the presence or absence of cycloheximide for 24 h. Cells showing punctuate GFP-Bax were quantified. **D,** total cell lysates from cells treated as in (B) were subjected to immunoprecipitation using the 6A7 anti-Bax antibody or a control antibody as in Fig. 4. **E,** Bax-positive cells were treated with MG132 (1 μmol/L) in the presence or absence of cycloheximide (10 μg/mL) for 24 h. The cytosol fraction was prepared for immunoblot assay (caspase-3). **F,** Bax-positive or Bax-negative cells that were stably reconstituted with GFP or GFP-Bax were treated with MG132 (1 μmol/L) in the presence or absence of cycloheximide (10 μg/mL) for 24 h and apoptosis was determined by Hoechst 33342 staining. Columns, mean; bars, SD.
allow a direct and potent interaction of p53 with Bax for the activation of the latter (25, 31). Deletion of p53 led to a complete inhibition of Bax conformational change and subsequent mitochondria activation and apoptosis (Fig. 5). This study thus indicates that the accumulation of p53 and PUMA could be the critical factor for promoting Bax-dependent mitochondria activation in the colon cancer cells following treatment with MG132 as well as other proteasome inhibitors. Furthermore, the conclusion is also supported by the finding with cycloheximide, which effectively reduced the level of p53 and PUMA. Although cycloheximide could also suppress the accumulation of prosurvival molecules, such as Mcl-1 or Bcl-xL, the protective effect of cycloheximide against MG132 at the proper dose and time frame probably indicates that the pro-death mechanisms are mostly affected. On the other hand, suppression of the anti-death molecules, such as Mcl-1, could enhance the proteasome inhibitor–induced apoptosis via new mechanisms, such as promoting Bak activation (32).

Proteasome inhibitors could activate different apoptotic mechanisms in different cells. Although we do not consider the p53-PUMA-Bax cascade as the sole mechanism for proteasome inhibitor–induced mitochondria activation and apoptosis in this and other cases (6, 9, 10, 12), we did find that ROS that could be related to mitochondria activation did not seem to be relevant in our study. Several studies reported that ROS was generated by proteasome inhibitors and participated in the induction of apoptosis (4, 6, 7). In our study, we found that MG132 and bortezomib failed to increase ROS production in HCT116 cells (Fig. 3; Supplementary Fig. S3). In addition, MG132-induced apoptosis could not be inhibited by several antioxidants (Fig. 3). The same findings could be also shown in the HeLa cells (data not shown). It has to be pointed out that some of the earlier studies used a specific antioxidant, Tiron, to show the role of ROS in bortezomib-induced apoptosis (4, 7). A recent study (33), however, showed that Tiron could specifically bind to bortezomib and directly inhibited bortezomib, but this inhibitory effect was not observed for other antioxidants, such as Mn (III) tetrakis (4-benzoic acid) porphyrin, or for other nonboronated proteasome inhibitors, such as MG132. Thus, one has to be cautious in interpreting the role of ROS in proteasome inhibitor–induced cell death. Our study suggests that the ROS participation in proteasome inhibitor–induced apoptosis could be cell type specific.

In summary, this study shows that inhibition of proteasome function induces accumulation of proapoptotic proteins p53 and PUMA, which promotes Bax activation, mitochondria release of proapoptotic factors, and apoptosis. The identification of this mechanism in colon cancer cells may have therapeutic implications for this type of cancer.

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References
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