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Bortezomib Blocks Bax Degradation in Malignant B-Cells during Treatment with TRAIL

Feng-Ting Liu*, Samir G. Agrawal*, John G. Gribben[‡], Hongtao Ye[¶],
Ming-Qing Du[¶], Adrian C. Newland* and Li Jia*

*Centre for Haematology, Institute of Cell and Molecular Science, [‡]Medical Oncology, Institute of Cancer, Barts and the London Queen Mary, University of London, [¶]Division of Molecular Histopathology, Department of Pathology, University of Cambridge

Address correspondence to:

Dr. Li Jia,

Centre for Haematology,

Institute of Cell and Molecular Sciences,

Queen Mary University of London,

4 Newark Street, London E1 2AT, UK.

Tel: 0044-207-882-2280; Fax: 0044-207-882-3170; email: L.jia@qmul.ac.uk.

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Abstract

Pro-apoptotic Bcl-2 family member Bax is a crucial protein in the induction of apoptosis and its activation is required for this process. Here we report that Bax is a short-lived protein in malignant B-cells and Bax protein levels decreased rapidly when protein synthesis was blocked. Malignant B-cells were relatively resistant to TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis and this correlated with low basal Bax protein levels. Furthermore, during treatment with TRAIL, the resistant cell lines showed prominent Bax degradation activity. This degradation activity was localized to mitochondrial Bax and could be prevented by truncated Bid (tBid), a BH3-only protein; in contrast, cytosolic Bax was relatively stable. The proteasome inhibitor Bortezomib is a potent drug in inducing apoptosis *in vitro* in malignant B-cell lines and primary chronic B-lymphocytic leukemic (CLL) cells. In CLL cells, Bortezomib induced Bax accumulation, translocation to mitochondria, conformational change, and oligomerization. Accumulation and stabilization of Bax protein by Bortezomib sensitized malignant B-cells to TRAIL-induced apoptosis. This study reveals that Bax instability confers resistance to TRAIL, which can be reversed by Bax stabilization with a proteasome inhibitor.

Key Words: Apoptosis, Bax, B-cell malignancy, CLL, protein degradation.

Introduction

Bax is a critical element in the induction of apoptosis and having adequate levels of intracellular Bax protein are crucial for cells to die by apoptosis in response to death signals. During the apoptotic process, Bax translocates from the cytosol to the mitochondria and undergoes conformational changes. Insertion into the mitochondrial membrane is essential for the pro-apoptotic activity of Bax. However, Bax protein has a shortened half-life in cancer cells due to greatly increased proteasome-dependent degradation activity.¹ Low levels of, or absent, Bax protein in malignant cells are associated with significant resistance to cancer therapy. Instability of Bax has been found in several types of malignant cells, including Jurkat T-cell and pre-B acute lymphoblastic leukemia 697 cell lines,² primary chronic lymphocytic leukemic cells (CLL),³ cervical cancer Hela cell line,⁴ and advanced human prostate cancer.¹ Decrease of Bax protein was significantly correlated with a poor prognosis in prostate cancer¹ and primary superficial-spreading melanoma.⁵

Recently, studies have revealed that many pro-apoptotic molecules are substrates and targets for ubiquitin/proteasome degradation, including p53,⁶ tBid,⁷ Bax,^{1,4} ARTS,⁸ NOXA⁹ and Bim.¹⁰ Alteration of the stability of these proteins through the ubiquitin/proteasome regulated pathway generally contributes to apoptosis resistance and poor prognosis in cancer cells. However, it is unclear whether the status of protein degradation by the ubiquitin/proteasome system could be altered by the treatment of cancer.

The levels of all intracellular proteins are determined by the duality of regulation: protein synthesis versus degradation. Basal Bax protein levels or the ratios of Bax/Bcl-2 have been considered to be critical factors for the sensitivity of malignant B-cells to anti-cancer drugs.^{11,12} Killing of malignant cells by induction of Bax expression commonly occurs with

DNA damaging agents via p53-dependent and p53-independent pathways.^{13,16} Proteasome inhibitors also have the ability to increase or maintain Bax protein levels - which are critical for Bax activation - when used alone or in combination with other agents.¹⁷⁻²⁰ TNF-related apoptosis inducing ligand (TRAIL) is a promising anti-cancer agent. However, malignant B-cells are resistant to TRAIL-induced apoptosis.^{21,22} We have previously found that Bax protein levels decline in leukemic cell lines during treatment with TRAIL.²³ It is unclear whether this could be one of the mechanisms of the resistance of malignant B-cells to TRAIL.

Bortezomib (PS-341, Velcade®) is a novel, first-in-class proteasome inhibitor with anti-tumor activity against a number of hematological and non-hematological malignancies.²⁴ *In vitro* studies showed that Bortezomib selectively inhibits proteasome-dependent degradation of p53,²⁵ IκB,²⁶ p21,²⁷ Noxa,^{9,28} and TRAIL receptors DR4 and DR5.²⁹ Proteasome inhibitors have been shown to be a promising approach for overcoming the resistance of tumor cells to TRAIL-induced apoptosis.²⁹⁻³⁵

Overexpression of Bcl-2 is one of the characteristics of malignant B-cells. However, the role of Bax on malignant B-cell survival and resistance to therapy has not been widely studied. This study has looked at Bax protein instability in malignant B-cells and the critical role of Bortezomib in the induction of Bax activation.

Materials and Methods

Materials

Bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA). Soluble TRAIL was from Biomol Ltd. (Exeter, U.K.). Annexin V-FITC kit, anti-Bax clone 6A7 and clone 3 monoclonal antibodies were purchased from BD Biosciences (San Diego, CA). M-450 Rat anti-Mouse IgG₁ Dynalbeads was from (DynaL Biotech Ltd, Wirral, U.K.). The anti-Bax 2D2 monoclonal antibody (clone YTH-2D2) and the recombinant caspase-8 cleaved Bid were from the R & D Systems (Oxon, U.K.). The anti-Bcl-2 (100), anti-Bcl-XL and anti-Bax antibodies were from Santa Cruz, CA. MitoTrackerTM red CMXRos was from Molecule Probes (Oregon, USA). RNeasy mini kit was from QIAGEN Ltd. (West Sussex, U.K.). The first-strand synthesis kit was purchased from UK-Invitrogen Ltd. (Paisley U.K.). **The proteasome substrate III, Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) was from Alexis Biochemicals (Nottingham, UK).** Cycloheximide (CHX), propidium iodide, **7-Amino-4-Methylcoumarin (AMC)**, the monoclonal anti- β -actin antibody, ubiquitin, anti-ubiquitin serum, protease inhibitor cocktail and other chemicals were from Sigma (Dorset, U.K.).

Cell culture and clinical samples

EBV transformed human B cell, the HRC57 cell line (is indicated as HRC in this article, provided by CRUK cancer cell services), and the human diffuse large B cell lymphoma cell lines, CRL,³⁶ DoHH2,³⁷ and DHL-4³⁸ and human leukemic cell line K562 were used in this study. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 25 mmol/L HEPES, 2.0 mmol/L L-glutamine at 37°C in a 5% CO₂ humidified incubator. Peripheral blood was obtained after written informed consent from patients with CLL

after clinical approval. The mononuclear cells were isolated by density centrifugation over Ficoll.

Apoptosis assays by flow cytometry

Apoptosis was determined by both Annexin V and DNA content assays. The Annexin V assay was performed according to the protocol of the Annexin V-FITC kit. Whole cells in the binding buffer suspension were stained with 5 μ l of Annexin V-FITC and 10 μ l of propidium iodide (PI) for 15 minutes at the room temperature in the dark. Annexin V-FITC and PI fluorescence were measured on the FL1-H and FL3-H channels, respectively, by flow cytometry (Becton Dickinson FACScan). Annexin V positive cells (both PI negative and positive) were defined as apoptotic. DNA content was measured by flow cytometry as described previously.¹⁵

Preparation of cellular fractions

Cells (5×10^7) were washed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and suspended in 1ml of Buffer A (250 mmol/L sucrose, 10 mmol/L HEPES-KOH, pH 7.4, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, protease inhibitor cocktails, 20 μ mol/L cytochalasin B) and incubated for 20 minutes on ice. Cells were then broken with a glass Dounce homogenizer. Nuclei were separated by spinning at $790 \times g$ for 10 minutes at 4°C . The post-nuclear supernatant was further spun at $10,000 \times g$ for 10 minutes at 4°C to separate cytosol and mitochondria. The crude mitochondrial pellet was purified by passing through a sucrose gradient (100-300 mmol/L) cushion at $9,000 \times g$ for 8 minutes.³⁹

Proteasome activity assay

Cytosolic or mitochondrial proteins (50 μ g) were diluted to 90 μ l with flurogenic assay buffer (20 mmol/L PIPE-KOH, pH 7.4; 10 mmol/L DTT, 10% sucrose, 1.0 mmol/L EDTA, and 0.1% CHAPS). The reaction was initiated by the addition of 10 μ l of 400 μ mol/L (final

concentration was 40 $\mu\text{mol/L}$ fluorescent substrate, Suc-LLVY-AMC for the chymotrypsin-like peptidase activity of the 20S proteasome. After incubation at 37°C for 15 minutes, the reaction was stopped by the addition of 50 μl of 1% sodium acetate trihydrate in 175 mmol/L acetic acid and cooling on ice. The fluorescence at 380/460 nm for AMC release by the proteasome cleavage was measured using a Bio-Tek SynergyTM HT Multi-Detection Microplate Reader (Vermont, USA). Measurements were calibrated against a standard linear regression curve of AMC. Proteasome activity was defined as $\mu\text{mol/L}$ AFC release per mg protein per minute ($\mu\text{mol/L}/\text{min}/\text{mg}$ protein).

In vitro Bax degradation assay

Mitochondria or cytosol in 5 mg/ml protein concentration were incubated in the Buffer B (250 mmol/L sucrose, 10 mmol/L HEPES-KOH, pH 7.4, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, protease inhibitor cocktail 1:100, 50 $\mu\text{g}/\text{ml}$ creatine phosphokinase, 10 mmol/L phosphocreatine, 2 mmol/L ATP). After the addition of 2 $\mu\text{g}/\text{ml}$ ubiquitin, the reaction mixture was incubated at 37°C for up to 3 hours in the Buffer B (without any detergent). Equal amount of reaction mixtures were taken out at each indicated time point to determine the Bax protein level by Western blotting.

Detection of Bax conformational change and ubiquitination by immuno-precipitation

After treatment with Bortezomib, cells were washed with PBS and lysed with the Chaps buffer (10 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1% CHAPS, 1 mmol/L DTT, 0.1 mmol/L PMSF, 3 $\mu\text{g}/\text{ml}$ aprotinin, 25 $\mu\text{g}/\text{ml}$ leupeptin, and 25 $\mu\text{g}/\text{ml}$ pepstain). M-450 Rat anti-Mouse IgG₁ Dynalbeads (20 μl) were pre-incubated with 1 μg of anti-Bax (6A7) antibody at 4°C on the rotor for 3 hours. The cell lysates were normalized for protein content. 500 μg of total protein in 300 μl Chaps lysis buffer were then mixed with Bax (6A7) antibody-loaded Dynalbeads.

Immuno-precipitation was performed at 4°C on a rotator overnight. After rinsing 4 times with lysis buffer, beads were collected with a Dynal Magnetic Particle Concentrator. Conformationally changed Bax protein was eluted with 25 µl of loading buffer for Western blotting. The anti-Bax 2D2 antibody was used to detect the conformationally changed Bax¹⁶ and the polyclonal anti-ubiquitin serum was used to determine Bax-ubiquitin conjugation.

Immuno-fluorescence analysis of Bax translocation

To detect Bax translocation to mitochondria, intact cells were first labeled with the mitochondrion-specific dye, MitoTrackerTM red CMXRos. Cells in culture medium were incubated with 100 nmol/L MitoTrackerTM at 37°C for 15 minutes. After washing, cells were fixed/permeabilized on slides. Cells were incubated with the anti-Bax clone 3 antibody, which only detects active Bax, at a 1:50 dilution for 1 hour and then incubated with FITC-conjugated anti-mouse secondary antibody (Sigma) at a 1:20 dilution. Slides were washed with PBS, air dried at 4°C in the dark and stained with DAPI before viewed under a Zeiss Axioskop fluorescence microscope (Zeiss, Germany).^{15,17,39}

RT-PCR

Total RNA was extracted with the RNeasy mini kit. 1 to 5 µg RNA was used for reverse transcription. cDNA was synthesized with the first-strand synthesis kit and amplified with Bax primers (5'CCCTTTTGCTTCAGGGTTTC, and 3' primer TGTTACTGTCCAGTTCGTCC size 151bp) or actin primers (5' GGAACGGTGAAGGTGACAG and 3' primer GGGACAA AAAGGGGGAAG size 338bp). PCR program was set up as below: cDNA templates denatured at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 56°C for 20 seconds, and an extension at 72°C for 20 seconds. Additional extension was at 72°C for 10 minutes. The PCR product was detected in 2% agarose gel.

Results

Bax is a short-lived protein in malignant B-cell lines

The expression of the Bcl-2 family of proteins was determined in the malignant B-cell lines, CRL, DoHH2 and DHL-4 and compared with the EBV- transformed B-cell line, HRC. Malignant B-cells overexpressed Bcl-2 protein and some of them also had higher levels of Bcl-XL. Bax protein expression was significantly lower in the malignant B-cell lines, CRL and DoHH2, compared with HRC cells. **In agreement with a previous study,³⁸ neither** Bax nor Bak protein was detected in the DHL-4 cell line. Expression of Bak was not significantly altered in the CRL and DoHH2 cell lines in comparison with HRC (Figure 1 A). Importantly, Bax mRNA levels were similar in these cell lines and were not consistent with their protein levels (Figure 1. B). The DHL-4 cell line, which lacked Bax protein expression, had plentiful Bax mRNA. These results suggested that the regulation of Bax expression in these cell lines may occur at the post-translational level.

The stability of Bax protein was tested in HRC, CRL, and DoHH2 cell lines after they were treated with cycloheximide (CHX), a protein synthesis inhibitor, and the lifespan of Bax protein was examined by Western blotting. Bax protein was found to be stable in the transformed HRC cell line but not in the malignant B-cell lines (Figure 1 C). **However, CHX-mediated Bax degradation in both CRL and DoHH2 cell lines was completely inhibited by pre-treatment with Bortezomib (Figure 1 D).** The expression of Bak remained unchanged in all cell lines tested suggesting that Bak protein is relatively stable (results not shown). These results indicate that Bax, but not Bak, is a short-lived protein in malignant B-cells and its instability varies among these cells. **Importantly, Bax degradation can be inhibited by Bortezomib.**

TRAIL induces Bax degradation in malignant B-cell lines

The malignant B-cells with low Bax protein expression, CRL and DoHH2, were found to be more resistant to TRAIL-induced apoptosis compared with the HRC cell line. DHL-4 cells, which had no detectable Bax protein, were highly resistant to TRAIL (Figure 2 A). The inverse correlation of Bax protein levels and sensitivity to TRAIL led us to examine the stability of Bax during treatment with TRAIL. A reduction in Bax protein was detected in the TRAIL-resistant CRL and DoHH2 cell lines, but not in the sensitive HRC cell line (Figure 2 B), indicating that Bax protein degradation occurs in malignant B-cells during TRAIL treatment in resistant cell lines. The expression of Bcl-2 and Bak was not significantly altered (results not shown). These results demonstrate that treatment with TRAIL is associated with enhanced Bax instability in resistant B-cell lines.

Proteasome inhibition promotes apoptosis in the TRAIL-resistant DHL-4 cell line

As shown above, the DHL-4 cell line lacks Bax protein expression but not Bax mRNA (Figure 1). Bortezomib at 20 nmol/L induced neither Bax accumulation nor apoptosis in DHL-4 cells. We were interested in whether proteasome inhibition could lead to an accumulation of Bax protein and TRAIL sensitization in the DHL-4 cell line. After DHL-4 cells were incubated with a high dose of Bortezomib (100 nmol/L) for 24 hours, Bax protein accumulation was observed by Western blotting, whereas, the Bax mRNA levels remained unchanged (Figure 3 A and B). DHL-4 cells were highly resistant to TRAIL-induced apoptosis, showing no apoptosis when cells were treated with TRAIL alone. High dose Bortezomib caused an accumulation of Bax protein in DHL-4 cells and sensitized these cells to TRAIL-induced apoptosis (Figure 3 C). These results further confirmed that the down-regulation of Bax in malignant B-cells is regulated at the post-translational level. Bax degradation contributes to resistance of malignant

B-cells to TRAIL-induced apoptosis, which can be reversed by proteasome inhibition leading to stabilization of Bax protein levels.

Bax degradation occurs in the mitochondrion and can be prevented by tBid or Bortezomib

To understand why TRAIL triggers Bax degradation and whether it is associated with Bax translocation to mitochondria, the location of Bax degradation was tested. Mitochondria isolated from the human leukemic K562 cell line were used as a model because they possess an inactive form of the Bax protein.^{16,40} Cytosol was extracted from the DHL-4 cell line, which does not contain detectable Bax protein (Figure 1 A). **The resting proteasome activity was tested in both cytosolic and mitochondrial fractions. Proteasome activity was found to be mainly located in the cytosol. Interestingly, the DHL-4 cytosol had greater proteasome activity than K562 cytosol (Figure 4 A1). Bortezomib significantly inhibited proteasome activity in both DHL-4 and K562 cytosol (Figure 4 A2). Mitochondrial Bax degradation occurred** when K562 mitochondria were incubated with K562 cytosol **in the presence of ubiquitin and it** was prevented by tBid. Interestingly, Bax protein degradation was not observed in the K562 cytosol (Figure 4 **B**). The interaction of tBid with Bax at the mitochondrial level is a critical intermediate step in TRAIL-induced apoptosis in type II cells.⁴¹ The effect of tBid in Bax degradation was tested at the mitochondrial level. **Mitochondrial Bax protein was stable when the K562 mitochondria were incubated in the degradation buffer containing ubiquitin for 3 hours, indicating that cytosol is required for the degradation. Similarly, Bax was also relatively stable when the K562 mitochondria were incubated with K562 cytosol without ubiquitin (Figure 4 C).** When the K562 mitochondria were incubated in the DHL-4 cytosol **in the absence of ubiquitin,** the mitochondrial Bax protein disappeared within 30 minutes, indicating that the mitochondrial Bax is susceptible to the degradation system present in the

DHL-4 cytosol, **probably due to the higher proteasome activity in the DHL-4 cytosol.** Bax degradation was prevented in the presence of tBid (Figure 4 **D**). **To test whether the mitochondrial Bax degradation is proteasome-dependent, the DHL-4 cells were treated with 100 nM Bortezomib for 24 hours. Cytosol was then extracted from these cells and incubated with K562 mitochondria for 5 hours. Bax degradation was diminished by Bortezomib (Figure 4 E).** These results indicate that Bax degradation occurs at the mitochondrial level and tBid prevents Bax degradation, probably by promoting stable Bax membrane insertion. TRAIL is known to induce Bax translocation to the mitochondria⁴² and this was thought to be an essential event in the induction of apoptosis by Bax. However, our data implies that Bax degradation may be active even after translocation following TRAIL stimulation.

Stablizing Bax by proteasome inhibition contributes to Bortezomib-induced apoptosis in primary malignant B-cells

It was determined whether the proteasome inhibitor Bortezomib could induce apoptosis and Bax accumulation in primary malignant B-cells. The sensitivity of CLL cells to Bortezomib-induced apoptosis **and Bax accumulation** was determined in fresh cells from 10 patients. Susceptibility to Bortezomib-induced apoptosis was universal, with no significant differences between cases (Figure 5 A), although Bax protein levels varied and different Bax degradation activities were shown amongst these samples – 5 cases showed Bax degradation, 3 were negative and 2 were not tested (Agrawal *et al*, accompanying paper). This suggests that the differential Bax protein levels and Bax degradation activity were not sufficient to affect the sensitivity of CLL cells to Bortezomib-induced apoptosis. **Bortezomib induced Bax accumulation was observed in most**

of CLL cases tested, although the degrees of accumulation varied among these samples (Figure 5 B).

A dose-dependent Bax protein accumulation and oligomerization (dimer and trimer) were observed in Bortezomib-treated CLL cells (Figure 5. **C**, the top panel). The accumulation of both p18 and p21 Bax was seen after the treatment with Bortezomib (Figure 5. **C**, the second panel). Bax activation was initially determined by a conformational change in Bax. It was shown that Bortezomib-induced Bax conformational change was also dose-dependent (Figure 5. **C**, the third panel). Bortezomib-induced Bax activation was also confirmed by Bax translocation to mitochondria. After treatment with Bortezomib, the active Bax protein (green color) merged with the red mitochondria, becoming yellow (Figure 5. **D**). These results show that Bortezomib induces Bax protein accumulation, a change in its conformation, translocation to mitochondria and an oligomerization. We have previously shown that Bax accumulation is sufficient to trigger its activation.^{16,40} It was also observed that Bortezomib caused NOXA accumulation in CLL cells (data not shown).

Next, the effect of Bortezomib on accumulation of ubiquitinated proteins and Bax-ubiquitin conjugation was investigated. An accumulation of poly-ubiquitinated proteins was detected when CLL cells were treated with Bortezomib (Figure 6 A). Immuno-precipitation experiments showed that active Bax can be recognized by, and conjugated with, ubiquitin when proteasome-dependent degradation was blocked by Bortezomib. However, Bax-ubiquitin conjugation was also detected in the control CLL cells, but to a lesser extent compared with treated CLL cells (Figure 6 B). We therefore propose that Bax degradation in CLL cells is ubiquitin/proteasome-dependent.

We also investigated whether the Bax degradation seen with TRAIL treatment in cell lines, and the reversal of this with Bortezomib, occurred in primary malignant B cells. Fresh CLL cells treated with 500 ng/ml soluble TRAIL were also found to have reduced Bax protein levels (Figure 7 A). TRAIL-induced Bax conformational change and apoptosis were low in the CLL cells (Figure 7 **B** and **C**). 10 nmol/L Bortezomib alone did not show significant effects on cell viability in CLL cells. However, Bortezomib and TRAIL in combination had a synergistic effect on apoptosis induction and Bax activation, with prevention of TRAIL-induced Bax degradation. As with the cell lines, Bax mRNA expression in primary CLL cells was not affected during TRAIL-mediated reduction in Bax protein expression or its reversal with Bortezomib (data not shown).

Discussion

In this study, we demonstrate that Bax is a short-lived protein in malignant B-cells. Bax degradation occurs at the mitochondrial level probably prior to its stable mitochondrial membrane insertion. This affects the sensitivity of malignant B-cells to TRAIL-induced apoptosis. The proteasome inhibitor, Bortezomib, stabilizes Bax and enhances the pro-apoptotic activity of the Bax protein. Bortezomib alone, in appropriate concentrations, induces apoptosis in primary malignant B-cells by stabilizing Bax protein.

The ubiquitin/proteasome-dependent degradation pathway is a major system for short-lived protein degradation in eukaryotic cells.⁴² Many proteins must undergo subunit separation, local unfolding, or post-translational modification prior to recognition by the appropriate E3 ubiquitin ligase.^{42,43} It was reported that Bax is a short-lived protein in tumor cells and its degradation is ubiquitin/proteasome dependent, due to an increased proteasome activity in tumor cells.^{1,4} The vexing problems are what is the trigger for Bax degradation and where is Bax degraded? The native Bax is a **globular** protein with a hydrophobic core center. In the resting state, it localizes in the cytosol or is loosely attached to the mitochondria in some models. Bax translocates to the mitochondrial outer membrane in the response to apoptotic signaling. The conformational changes in Bax enable its stable membrane insertion via the $\alpha 5$ - $\alpha 6$ helices and oligomerization with Bax and/or Bcl-2. However, Bax translocation to mitochondria does not commit cells to apoptosis, because Bax translocation and membrane insertion are separate events.^{15,44} In the experiments looking at Bax degradation in the mitochondria or cytosol, a detergent such as Triton X-100 was omitted because it can trigger Bax conformational change in a cell-free system.⁴⁵ Bax protein is relatively stable in the cytosol in the absence of detergent. In the K562 mitochondria, Bax is in an inactive form and it requires the BH3-only protein for

triggering cytochrome *c* release.^{16,44} The mitochondrial Bax is vulnerable to degradation in the absence of the BH3-only protein tBid. The role of the BH3-only proteins on mitochondria is to promote Bax membrane insertion by engaging the multiple survival relatives guarding Bax and Bak.⁴⁶ **K562 cells have lower proteasome activity and this may be one of the reasons that K562 mitochondria maintain an abundant Bax protein. Having a higher proteasome activity, DHL-4 cytosol degrades K562 mitochondrial Bax rapidly in the absence of artificial ubiquitin. In the presence of ubiquitin, the K562 cytosol degrades mitochondrial Bax gradually and this is also inhibited by tBid. However, the sensitivity of Bax protein to degradation may depend on many factors, such as the specific conformation of Bax protein, less BH-3-only proteins, the cytosolic proteasome activity, the co-ordination of ubiquitin recognizing and conjugating with Bax. The general proteasome activity is not specific for Bax degradation.**

Using an antibody (6A7) to the active form of Bax, we detected Bax-ubiquitin conjugation after treatment with Bortezomib, suggesting that ubiquitin recognizes the active form of Bax. However, we could not rule out that ubiquitin may conjugate with the inactive Bax because none of the available antibodies that detect inactive Bax could be used for immuno-precipitation. We have recently observed (in another study) that the exposure of Bax hydrophobic domains during its conformational changes triggers ubiquitin recognition and the degradation process. The ubiquitin-sensitive domain of Bax is within its hydrophobic core (Yu *et al*, submitted). This also suggests that Bax translocation to mitochondria and subsequent conformational changes may be physical processes. This study shows that tBid **or Bortezomib** stabilize Bax and prevented mitochondrial Bax from

degradation, suggesting Bax degradation may occur prior to irreversible commitment to apoptosis.

The protein levels of Bax are determined by two opposing forces: synthesis and degradation. Cancer cells lacking Bax protein are resistant to apoptosis. Therefore, blocking Bax degradation to increase Bax protein levels is an attractive target for anti-cancer therapy. Many anti-cancer reagents, such as DNA damaging agents, induce apoptosis by an increase in Bax expression and translocation,¹⁵ indicating that rapid synthesis can overwhelm Bax degradation activity. The effect of DNA damaging agents on Bax degradation activity is unknown. TRAIL induced Bax translocation and conformational change. However, rather than increased Bax expression, TRAIL treatment was associated with enhanced Bax degradation in resistant malignant B-cells. It is known that tBid is required for TRAIL-induced Bax activation.⁴⁷ Poor coordination between Bax mitochondrial translocation and the BH3-only proteins, such as tBid, or overexpression of Bcl-2 (a common feature of malignant B-cells), would leave mitochondrial Bax vulnerable to degradation. Primary CLL cells were found to be inherently resistant to TRAIL²² and inhibition of Bcl-2 sensitized TRAIL-induced apoptosis in human leukemia and tumor cells.^{47,48} Although Bax is essential for TRAIL-induced apoptosis in certain cancer cells, the regulation of Bax in response to TRAIL is still elusive. We suggest that TRAIL-induced Bax degradation may be a consequence of insufficient levels of tBid or Bcl-2 overexpression, which cause a failure in mitochondrial membrane insertion. The proteasome inhibitor Bortezomib sensitized resistant cells to TRAIL-induced apoptosis by preventing Bax degradation and therefore maintaining levels of active Bax. These results suggest that proteasome-dependent Bax degradation is one of the mechanisms underlying the resistance of malignant B-cells to TRAIL-induced apoptosis and that it can be prevented by co-treatment with a proteasome inhibitor.

Increased degradation activity of pro-apoptotic proteins may contribute to the development of cancer, as well as resistance of cancer cells to treatment. The human leukemia CLL is currently incurable, with frequent over-expression of Bcl-2 being one of the major causes of resistance of CLL cells to therapy. Furthermore, insufficient levels of Bax protein have also been reported to be a resistance factor in CLL in response to a variety of therapies.^{3, 49,50} Reduced Bax protein expression was observed in several malignant B-cell lines, but this may not necessarily represent increased Bax degradation activity. Low Bax protein levels could also be due to a change in the balance between synthesis and degradation, namely, decreased synthesis combined with increased degradation activity.

Bortezomib induces apoptosis in CLL cells *in vitro*.^{51,52} We found there was no correlation between Bax degradation activity and the sensitivity of CLL cells to Bortezomib-induced apoptosis. Bortezomib caused Bax accumulation, a conformational change, translocation to mitochondria and oligomerization in CLL cells. Increased levels of NOXA protein were also observed when CLL cells were treated with Bortezomib. Bax up-regulation was also seen when CLL cells were treated with both Bortezomib and anti-CD20 antibody,⁵³ while co-treatment of CLL cells with Bortezomib plus cladribine and fludarabine induced an up-regulation of tBid, another short-lived pro-apoptotic protein.⁵⁴ It was reported that Bortezomib-induced apoptosis was significantly decreased in Bax-knockout colon cancer cells.⁵⁵ We propose that Bax accumulation and activation play an important role in Bortezomib -induced apoptosis.

Our results may have clinical application since inducible resistance to apoptosis occurs during TRAIL treatment and the combination of a proteasome inhibitor with TRAIL overcomes resistance to TRAIL by blocking Bax degradation. Further studies will focus on whether Bax

degradation is associated with clinical prognosis and outcome of patients with lymphoma and leukemia.

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Figure legends

Figure 1. Bax protein instability

(A) Expression of Bcl-2, Bcl-XL, Bak and Bax proteins. 25 µg proteins/each lane were loaded to 12% SDS-PAGE. The anti-Bcl-2 (100) and anti-Bcl-XL antibody were used at 1:200 dilution; anti-Bax (2D2) antibody was used at 1:1000 dilution; anti-Bak antibody was used at 1:200 dilution; and anti-β-actin antibody was used at 1:10,000 dilution. (B) RT-PCR detection of Bax mRNA levels. (C) The lifespan of Bax protein. HRC, CRL or DoHH2 cell lines were **pre-incubated with or without 50 nmol/L Bortezomib for 2 hours and then** treated with 50 µg/ml CHX for 3 hours. **Proteins** were extracted hourly. Bax levels were evaluated by Western blotting using Bax 2D2 antibody at 1:1000 dilution. β-actin antibody was used at 1:10,000 dilution. Numbers under each pairs of blots are the ratio of Bax/β-actin.

Figure 2. Effect of TRAIL-induced Bax regulation.

(A) TRAIL-induced apoptosis was assessed with the Annexin V assay using flow cytometry. Data shown are mean ± SD from three independent experiments. (B) Proteins were extracted from HRC, CRL or DoHH2 cells, which were treated with TRAIL for up to 24 hours.

Figure 3. Bortezomib-induced Bax protein accumulation in DHL-4 cells.

DHL-4 cells were treated with 100 nmol/L Bortezomib for 24 hours. Cells were collected for protein and mRNA extraction. (A) Bax protein expression was determined with Western blotting. Anti-Bax antibody, 2D2 was used at 1:1000 dilution. Anti-β-actin antibody was used at 1:10,000 dilution. (B) Bax or actin mRNA was determined by RT-PCR. (C) TRAIL and Bortezomib-induced apoptosis in the DHL-4 cell line. After DHL-4 cells were incubated with or without 100 nmol/L Bortezomib for 24 hours, cells were incubated with (B+T) or without 500 ng/ml TRAIL (Borte) for another 24 hours. Significantly increased sensitivity ($P < 0.0001$, *t*-test)

of cells treated with Bortezomib and TRAIL was seen compared with cells treated with Bortezomib or TRAIL alone.

Figure 4. Prevention of Bax degradation by tBid at the mitochondrial level.

(A) Determination of proteasome activity. (A1) Proteasome activity was measured in both cytosol and mitochondrial fractions in the absence of ubiquitin. ‘Cyto’ and ‘Mito’ indicate cytosol and mitochondrion. ‘*’ indicates significantly different proteasome activities (P<0.0001) between DHL-4 and K562. (A2) Inhibition of proteasome activity by Bortezomib. Bortezomib (70 nmol/L) was added to the cytosol (5 µg/µl protein) one hour prior to the reaction. Data shown were from three separate experiments. ‘*’ indicates significant inhibition (P<0.0001, t-test). (B) Mitochondrial Bax degradation in K562 cytosol. K562 mitochondria were incubated with K562 cytosol in the presence of ubiquitin.

Mitochondria were mixed with cytosol according to a protein ratio of mitochondria/cytosol of 1:2 and incubated at 30°C for **3 hours. Bax degradation was monitored in the presence or absence of 10 nmol/L tBid. Bax protein levels were detected by Western blotting for both mitochondria and cytosol.** (C) **K562 mitochondria were either incubated in the degradation buffer containing ubiquitin or in the K562 cytosol without ubiquitin for 3 hours. Bax protein levels were examined in the mitochondrial fraction.** (D) Mitochondrial Bax degradation in the DHL-4 cytosol. Mitochondria were isolated from the K562 cells. Cytosol was extracted from DHL-4 cells. In this assay, ubiquitin were omitted in order to test if the DHL-4 cytosol has a higher Bax degradation activity. Mitochondria were mixed with cytosol according to a protein ratio of mitochondria/cytosol of 1:2 and incubated at 30°C for up to 60 minutes in the presence or absence of 10 nmol/L tBid. Bax protein levels were detected by Western blotting for both mitochondria and cytosol. **(E) Bortezomib prevents Bax**

degradation. DHL-4 cells were treated with 100 nmol/L Bortezomib for 24 hours and its cytosol was mixed with K562 mitochondria and incubated at 30°C for 5 hours. Mitochondrial Bax levels were then determined by Western blotting after separation from cytosol. Bax antibody (clone 2D2) was used for the Western blotting.

Figure 5. Bortezomib-induced apoptosis and Bax activation in CLL cells.

(A) Bortezomib-induced apoptosis in 10 CLL samples. Fresh CLL cells were treated with different concentrations of Bortezomib for 16 hours. Apoptotic cells were determined by the Annexin V assay. (B) Bortezomib (Borte)-induced Bax protein accumulation in 9 cases of CLL patients, (C) Bortezomib (Borte)-induced Bax protein accumulation, oligomerization and activation in 1 case of CLL patient. CLL cells were treated with Bortezomib for 16 hours. Accumulation of Bax protein was determined by Western blotting using the anti-Bax antibody, clone 2D2 at 1:1000 dilution. 30 µg protein was loaded into each lane. Bax dimers and trimers are shown in the top panel blot indicated by 2× and 3×. For detection of conformationally changed Bax, the active form of Bax was immuno-precipitated (IP) by Bax 6A7 antibody and probed by Bax 2D2 antibody. (D) Bax translocation to mitochondria. CLL cells were treated with or without 20 nmol/L Bortezomib for 16 hours. Cells were stained with 100 nmol/L MitoTracker for 15 minutes, washed for 3 times, then fixed and permeabilized. Slides were stained with anti-Bax clone 3 antibody (1:20 dilution) for 1 hour and then stained with FITC-conjugated anti-mouse IgG (1:50 dilution) for 1 hour. Finally, slides were stained with 50 ng/ml DAPI. The red color indicates the mitochondrial location and the yellow color represents the active Bax in the mitochondria.

Figure 6. Bortezomib-induced poly-ubiquitination and Bax-ubiquitin conjugation.

Fresh CLL cells were treated with Bortezomib for 16 hours. (A) Poly-ubiquitination. CLL cells **from 3 different patients** were lysed with the lysis buffer. 50 µg proteins were loaded into each lane. The anti-ubiquitin antibody was used at 1:500 dilution. Multiple ladders in the blot show an accumulation of poly-ubiquitinated proteins. (B) CLL cells were lysed with Chaps containing lysis buffer. 500 µg protein was used for immuno-precipitation with Bax 6A7 antibody overnight at 4°C and Bax-ubiquitin conjugation was probed with the anti-ubiquitin antibody at 1:500 dilution. The numbers on the left side of the blot are molecular weights of standard proteins.

Figure 7. Proteasome inhibitor facilitates TRAIL-induced Bax activation and apoptosis.

Fresh CLL cells were pre-treated with 10 nmol/L Bortezomib for 1 hour and then incubated with or without 500 ng/ml TRAIL, for 16 hours. **(A) TRAIL +/- Bortezomib induced changes of Bax protein levels in 7 cases of CLL patients.** **(B) Association between** Bax protein levels and Bax conformational change. ‘CLL’ indicates the control; ‘T’, TRAIL-treated; ‘B’, Bortezomib-treated; and ‘B+T’, treated with Bortezomib and TRAIL. The anti-Bax antibody 2D2 was used for Western blotting. The active Bax proteins were immuno-precipitated with 1 µg anti-Bax 6A7 antibody and detected by Western blotting using anti-Bax 2D2 antibody at 1:1000 dilution. **(C)** Apoptotic cell death was measured by DNA content assay. This is a one of three separate experiments. Numbers shown in the flow cytometry profiles are percentages of apoptotic cells (sub-G0/G1 population).

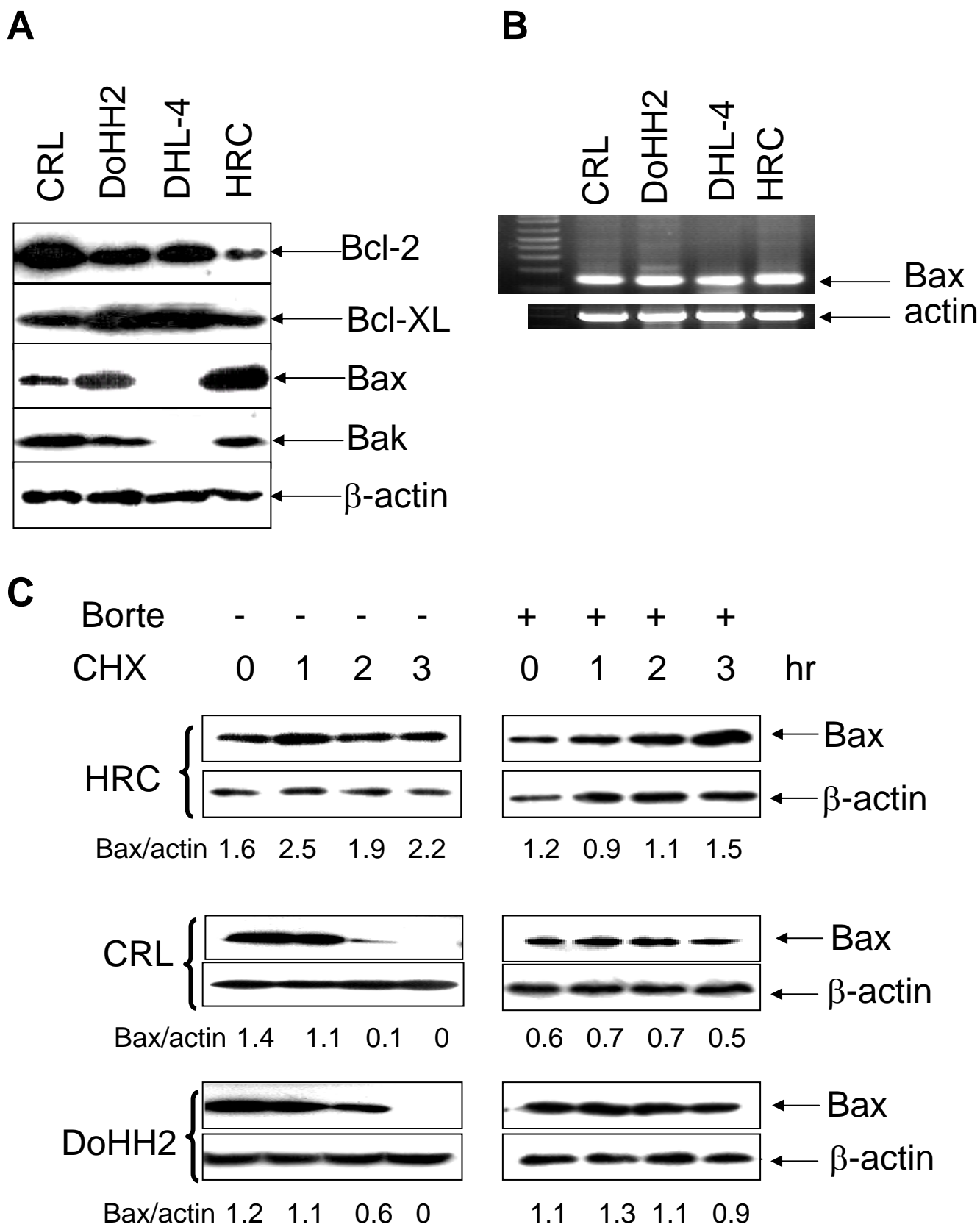
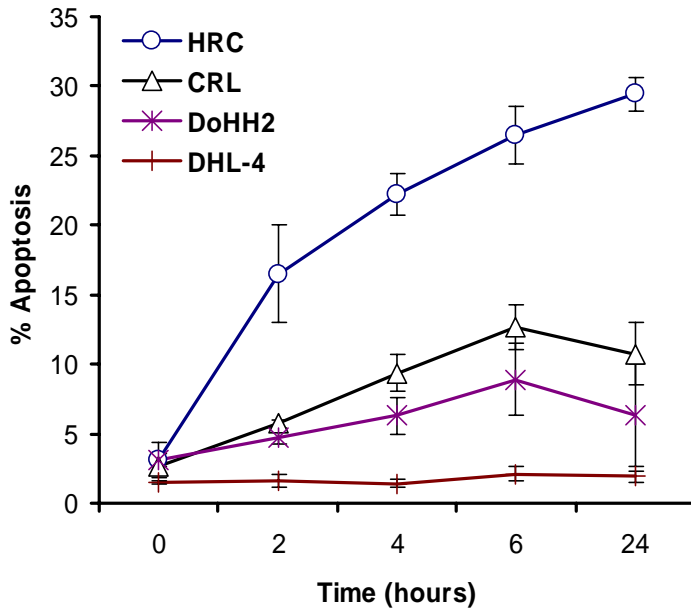


Figure 1.

A



B

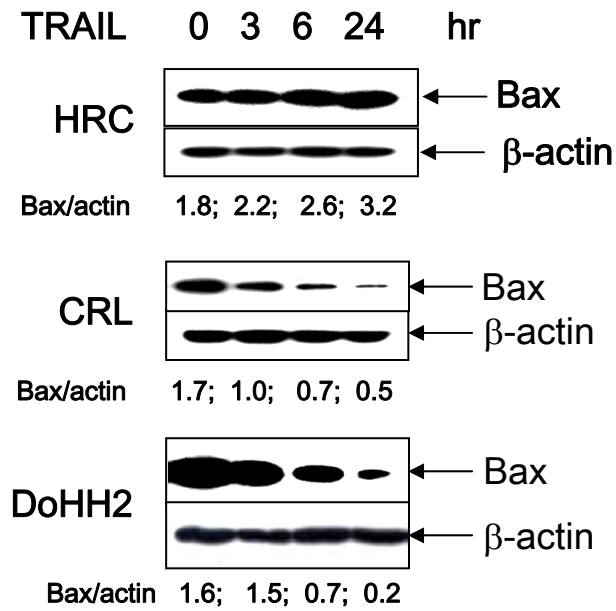
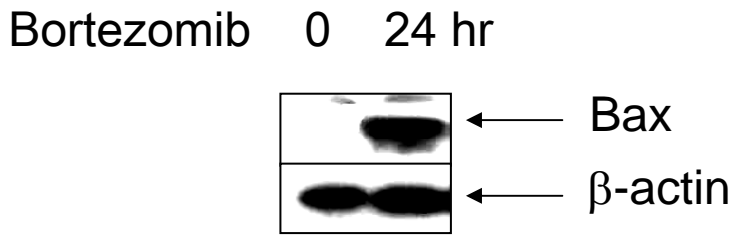
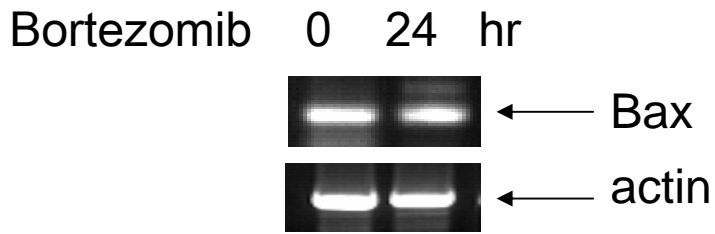


Figure 2

A



B



C

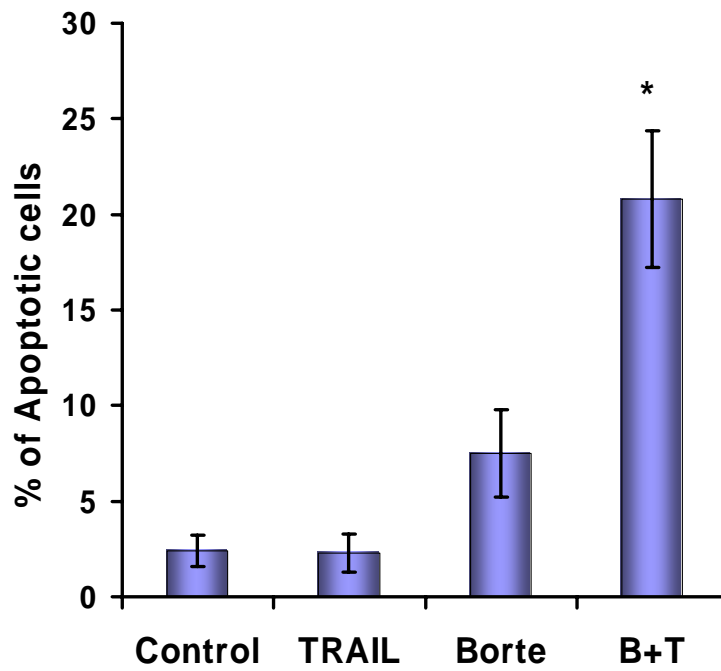


Figure 3.

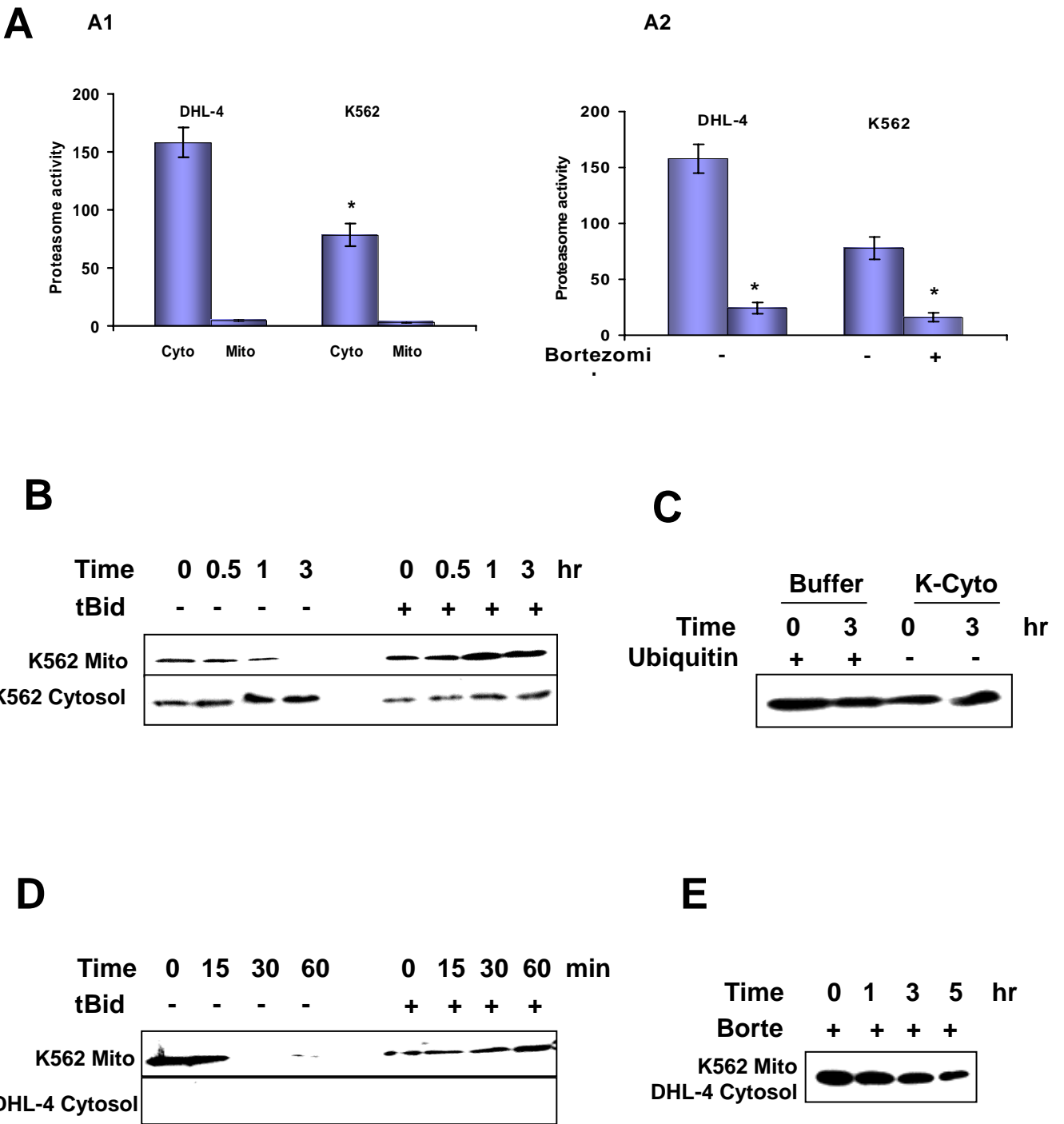


Figure 4

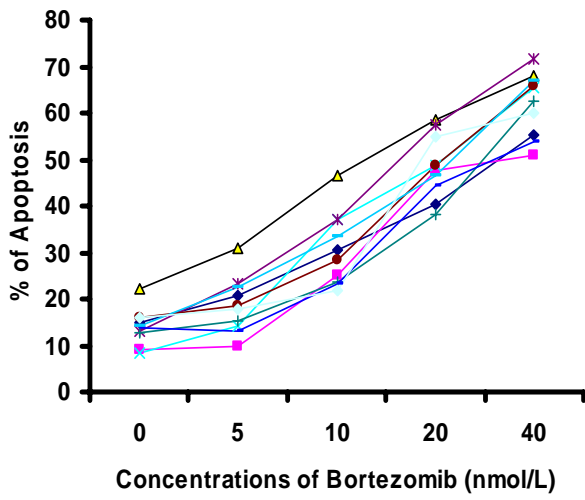
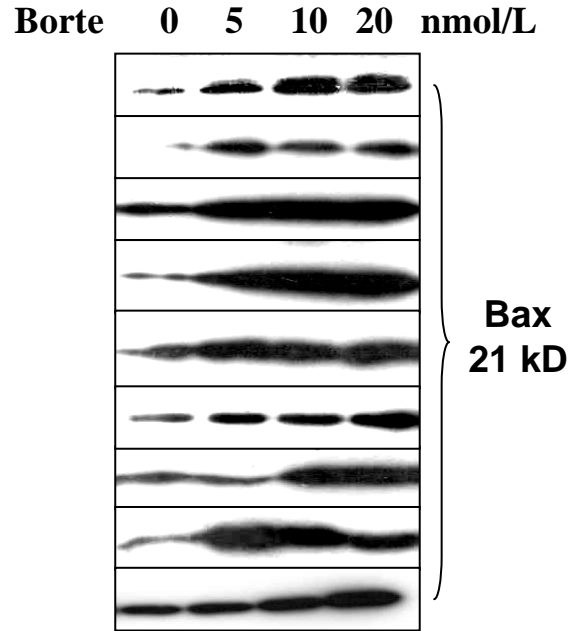
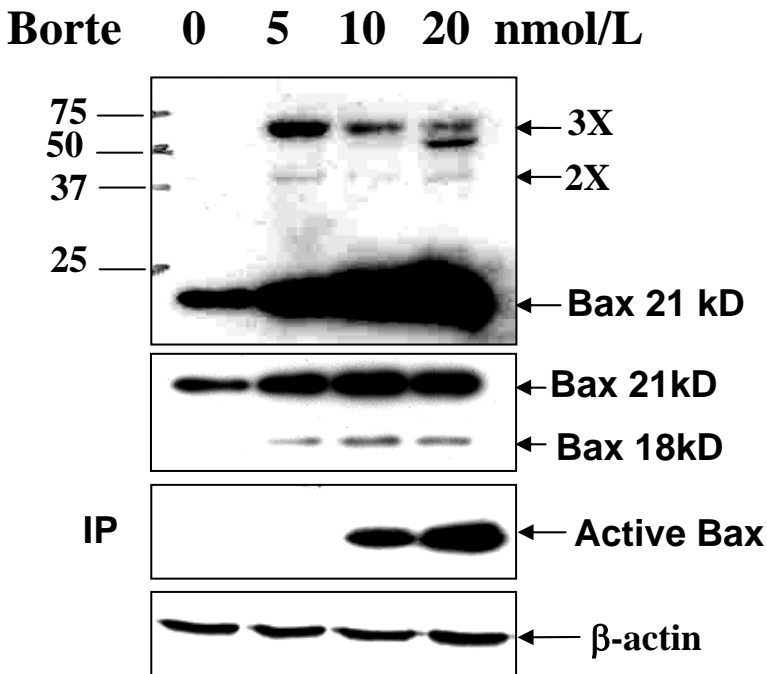
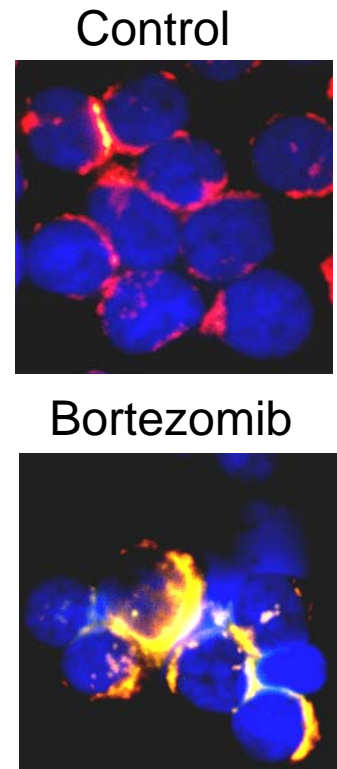
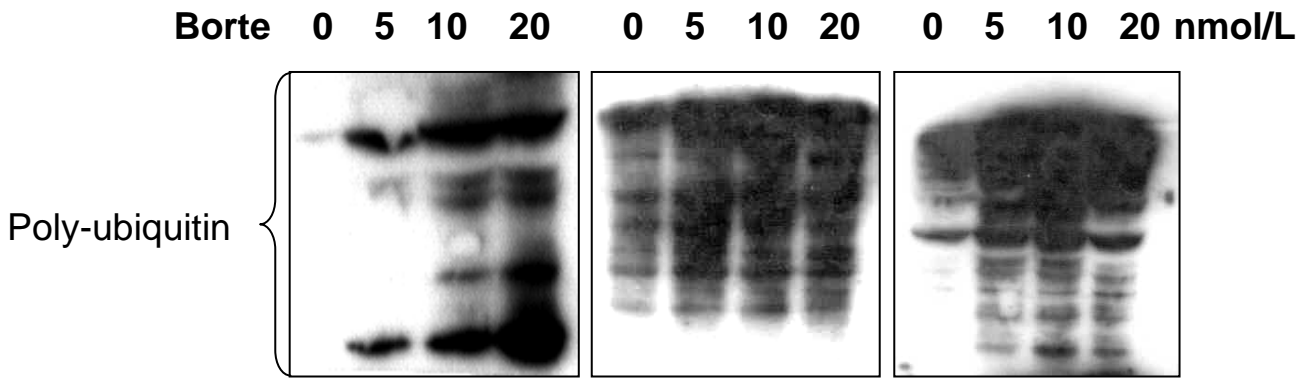
A**B****C****D**

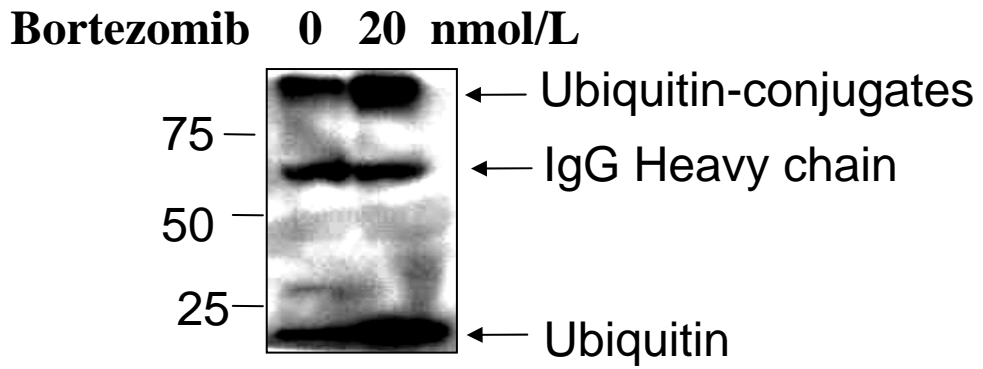
Figure 5.

A



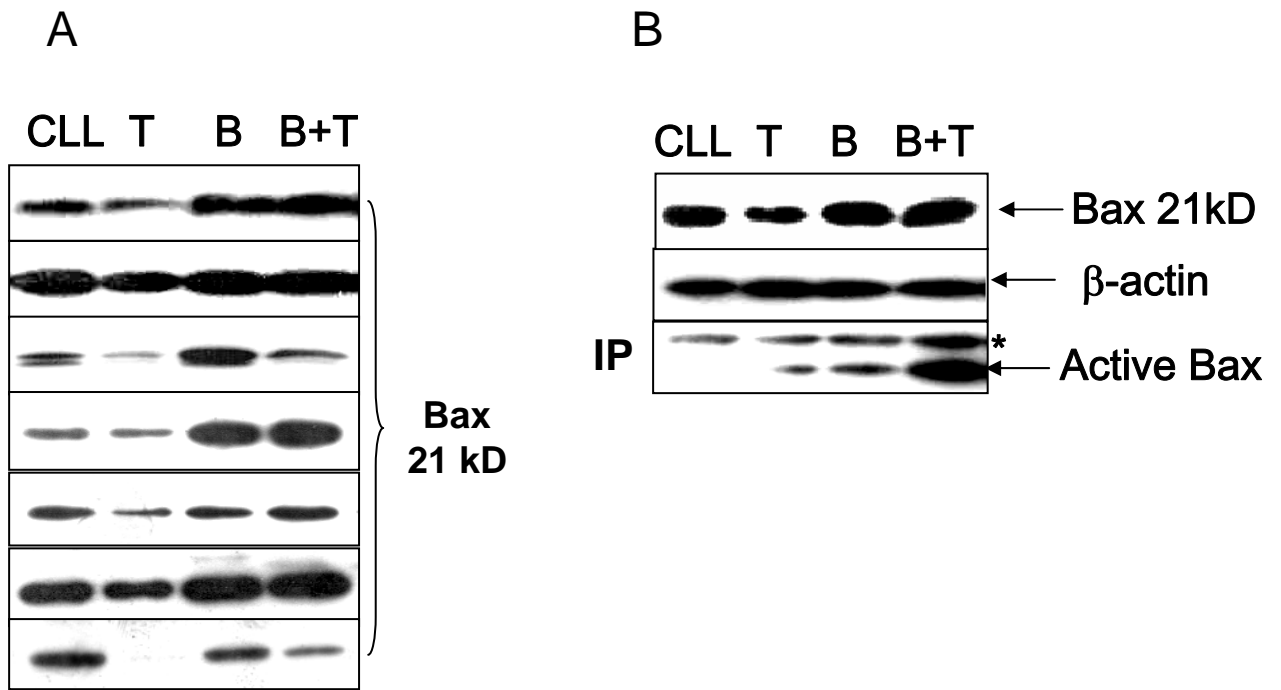
WB: Ubiquitin

B



IP: Bax; WB: Ubiquitin

Figure 6



C

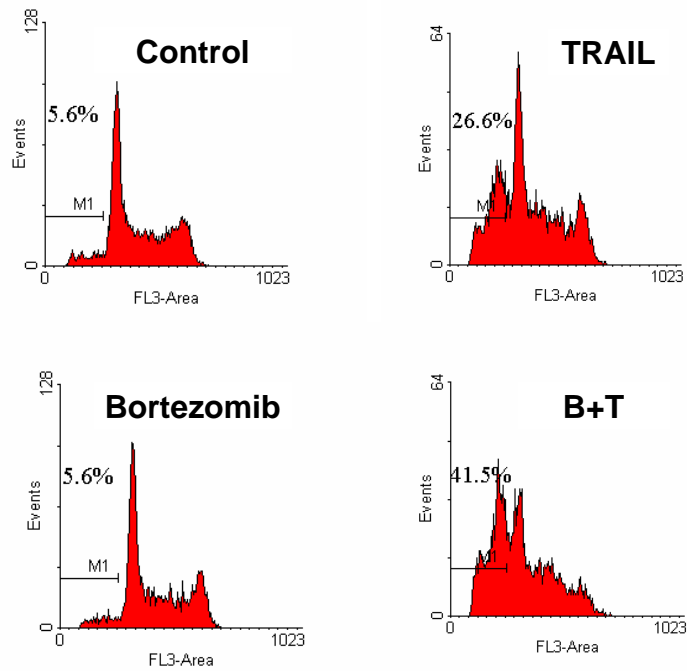


Figure 7.