Role of NOXA and its ubiquitination in proteasome inhibitor-induced apoptosis in chronic lymphocytic leukemia cells

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Online Supplementary Design and Methods

Lymphocyte purification and culture

Blood samples were obtained from CLL patients during routine diagnosis at the Leicester Royal Infirmary after written consent from the patient and local ethical committee approval. Only patients who had not received treatment within the preceding 6 months were included in this study. CLL cells were isolated immediately using Ficoll gradient centrifugation and then resuspended in RPMI1640 containing 10% fetal calf serum, penicillin (50 units/mL), streptomycin (50 μg/mL) and L-glutamine (5 mM). The CLL cells were left untreated (control or unstimulated) or exposed to bortezomib (1- 100 nM), MG132 (1 μM), seliciclib (10 μM), tunicamycin (10 μg/mL), brefeldin A (100 nM), or thapsigargin (1 μg/mL) for up to 16 h or 24 h as indicated. To decrease drug-induced apoptosis, CLL cells were co-cultured with CD154-expressing L cells for 16 h in the presence of interleukin-4 (15 ng/mL).[1] Where indicated, cells were pre-incubated with the broad spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD.fmk 50 μM; MP Biomedicals, London, UK) or cycloheximide (10 μM Sigma-Aldrich, Poole, UK) for 30 min before they were treated as above. After treatment, cells were prepared for tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Eugene, OR, USA) or annexin V-fluorescein isothiocyanate(FITC)/propidium iodide staining or were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of a cocktail of protease inhibitors (Roche, Burgess Hill, UK) and stored at -20°C for subsequent western blot experiments.

Reagents

RPMI1640 and fetal calf serum were from Life Technologies, Inc. (Paisley, UK). Bortezomib and MG132 were from Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA) and Calbiochem (Darmstadt, Germany), respectively. Propidium iodide and chemicals for buffers were from Sigma-Aldrich. Mouse monoclonal antibody to ubiquitin, clone FK2, was from Biomol International (Devon, UK). Anti-rabbit MCL1, and anti-p53 (D0-1 clone) antibodies were from Merck Biosciences (Nottingham, UK). The endoplasmic reticulum stress sampler kit was from Cell Signalling Technology (Hitchin, UK). Mouse anti-CHOP antibody was from Abcam (Cambridge, UK). Mouse anti-HA probe was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Assessment of apoptosis and western blot analysis

Apoptosis was quantified by either annexin V-FITC in the presence of propidium iodide (for phosphatidylserine externalization) or by TMRE (for alterations of the mitochondrial membrane potential [$\Delta\Psi$m]) and conformational changes of BAX and BAK were assessed after 4-12 h of incubation with bortezomib (100 nM) as described previously.[2] Western blotting and anti-rabbit and anti-mouse antibodies against BCL-2 family members or caspases and anti-rabbit or anti-mouse horseradish peroxidase conjugates were as previously described.[3]

Electron microscopy analysis

Cells were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 4°C overnight and post-fixed with 1% osmium tetroxide/1% potassium ferrocyanide for 1 h at room temperature. After fixation, cells were stained *en bloc* with 5% aqueous uranyl acetate overnight at room temperature, dehydrated, and embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK). Electron micrographs of ultrathin sections were recorded using a QICAM 12-bit Mono Fast 1394 digital camera and QCapture-Pro software (MAG, Pleasanton, CA, USA) in a Zeiss 902A electron microscope.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from CLL cells exposed to MG132 (1 μM) or bortezomib (10 and 100 nM) for 2-12 h using the TRI Reagent (Sigma-Aldrich). RNA (0.5-1 μg) was reverse-transcribed using the Superscript III reverse transcriptase (Invitrogen, Paisley, UK). This cDNA was prepared for real time polymerase chain reaction analysis using Fast START SYBR Green Master Mix (Roche) using 900 nM of NOXA and GAPDH primers following the manufacturer's instructions. The primers were designed using the Universal Probe Library (Roche) and their sequences were: NOXA Forward: 5'-GGAGATGCCTGGGAAGAAG -3', NOXA Reverse: 5'-CCTGAGTTGAGTAGCACACTCG-3', GAPDH Forward : 5'-AGCCACATCGCTCAGACAC -3', GAPDH Reverse: 5'- GCCCAATACGACCAAATCC-3'. The program consisted of one cycle at 95°C for 10 min and then 40 cycles of 0.15 min at 95°C, 1 min at 60°C and 0.15 min at 72°C. The fold change in *NOXA* mRNA relative to *GAPDH* was calculated according to the $2^{-\Delta\Delta Ct}$ method.

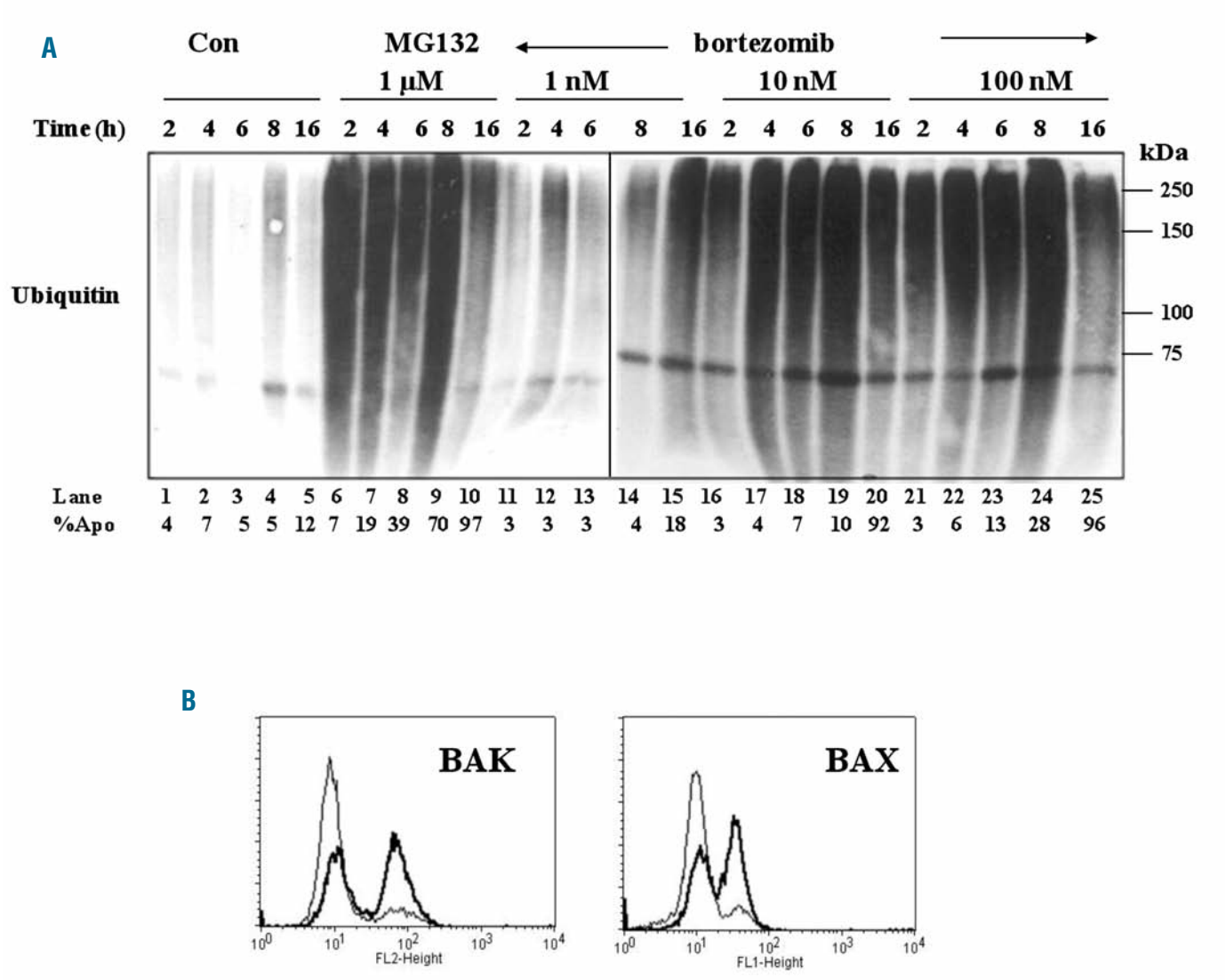
Nucleofection

CLL cells ($5x10^6$) were transfected using the B-cell nucleofector kit

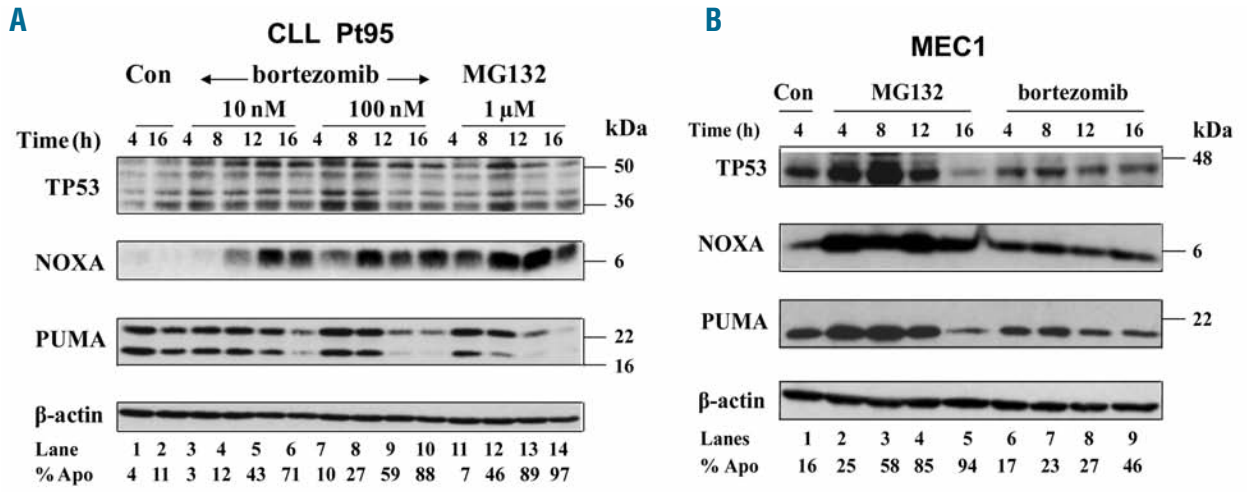
from Amaxa (Lonza Cologne, Germany) following the instructions of the manufacturer using program X-001. NOXA short interfering RNA (siRNA) (s10709 and 6019) and negative control siRNA 1 were provided by Ambion (Austin, TX, USA) and were used at 30 nM. Cells were pre-incubated for 4 h before they were exposed to bortezomib (10 nM) for 12 and 16 h. Cytotoxicity caused by nucleofection was assayed by transfecting cells with the negative control siRNA 1 as above but the cells were then left unstimulated up to 16 h. The sequences for the NOXA siRNA were: S10709: Sense: AGUCGAGUGUGCUACUCAAtt, antisense: UUGAGUAGCACA CUCGACUtc, 6019: Sense: GGAACCUGACUGCAUCAAAtt, antisense; UUUGAUGCAGUCAGGUUCCtg. At the end of the incubation period, apoptosis was measured by TMRE or annexin-propidium iodide staining as described above or cells were lysed in RIPA buffer for subsequent western blot analysis.

References

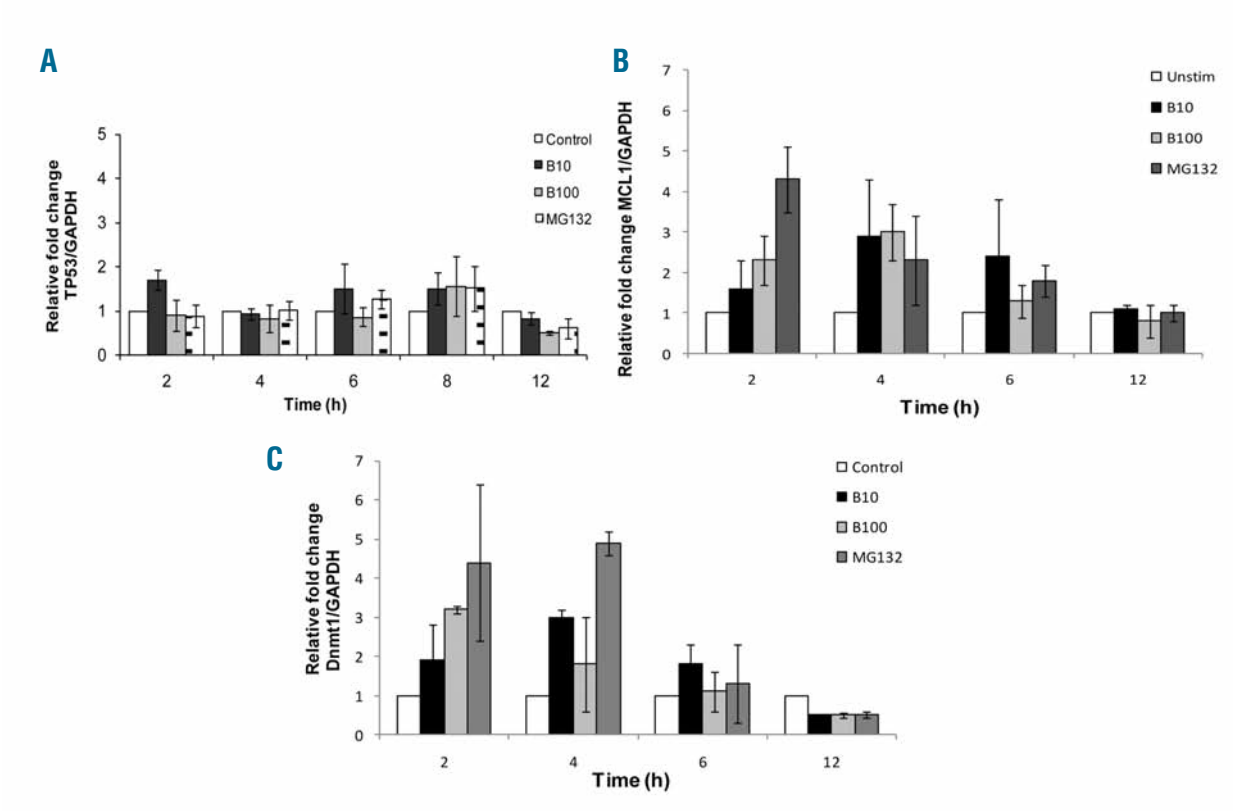
1. Vogler M, Butterworth M, Majid A, Walewska RJ, Sun XM, Dyer MJ, et al. Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. Blood. 2009;113(18):4403-13.
2. Vogler M, Dinsdale D, Sun XM, Young KW, Butterworth M, Nicotera P, et al. A novel paradigm for rapid ABT-737-induced apoptosis involving outer mitochondrial membrane rupture in primary leukemia and lymphoma cells. Cell Death Differ. 2008;15(5):820-30.
3. Dewson G, Snowden RT, Almond JB, Dyer MJ, Cohen GM. Conformational change and mitochondrial translocation of Bax accompany proteasome inhibitor-induced apoptosis of chronic lymphocytic leukemic cells. Oncogene. 2003; 22(17):2643-54.



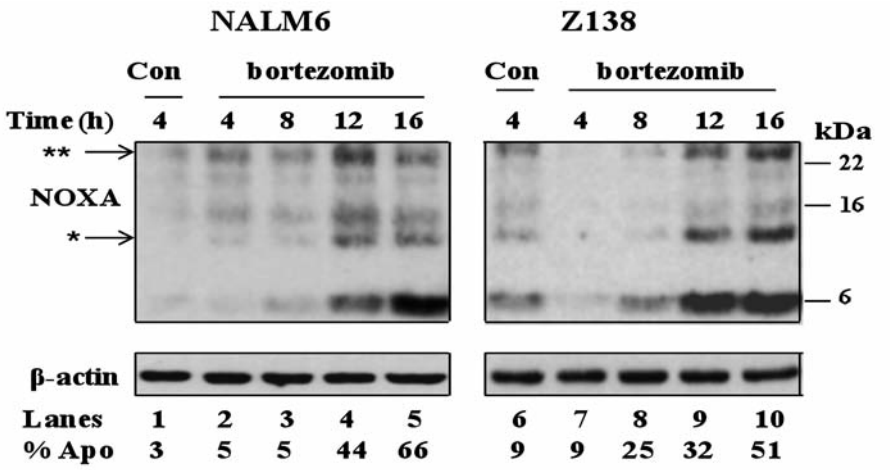
Online Supplementary Figure S1. (A) Proteasome inhibitors cause the accumulation of ubiquitinated proteins in CLL cells. Western blot analysis using a mouse monoclonal antibody against ubiquitin in lysates prepared from CLL cells exposed to bortezomib and MG132 at the concentrations indicated for 2- 16 h. The accumulation of ubiquitinated proteins indicates the efficiency of the proteasome inhibitors in inhibiting protein degradation. The percentage of phosphatidylserine-positive cells is shown below the plot. (B) CLL cells from one patient were exposed to bortezomib (100 nM) for 12 h and monitored for conformational change in BAK (black thick line, left panel) and BAX (black thick line, right panel) protein compared to unstimulated cells (black thin line in both panels) using conformation-specific monoclonal antibodies for BAK and BAX and FACS analysis.



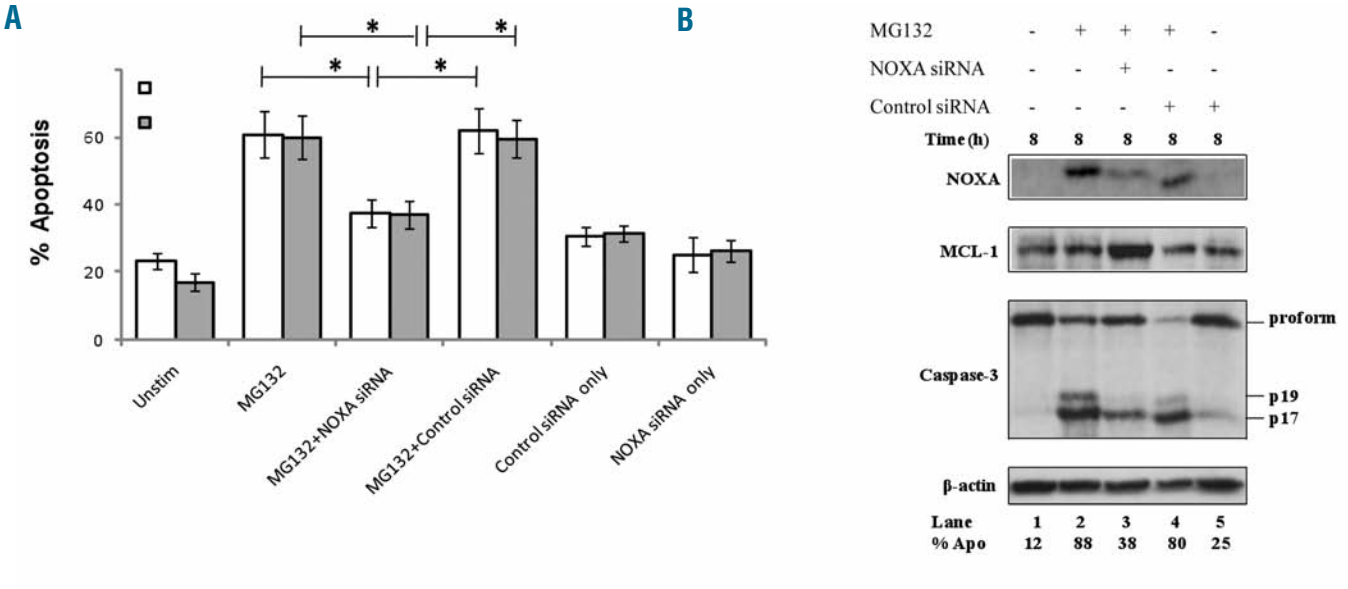
Online Supplementary Figure S2. Induction of TP53 following proteasomal inhibition in CLL cells. (A) CLL cells, from a patient with a deletion in one and mutation in the second *TP53* allele, were exposed to MG132 or bortezomib at the indicated concentrations for 4-16 h. Stabilization of TP53 protein was monitored by western blot analysis using a monoclonal antibody recognizing residues 11-25 (clone: DO-1). NOXA protein was significantly induced in these CLL cells. The percentage of apoptotic cells, as assessed by phosphatidylserine externalization (PS^+ cells), is shown below the plot. (B) The MEC1 cell line bearing a frameshift deletion in *TP53* was exposed to MG132 or bortezomib (10 nM) for 4-16 h. TP53 levels were monitored as in (A). NOXA protein was also significantly induced in these cells. The percentage of PS^+ cells is shown below the plot.



Online Supplementary Figure S3. Bortezomib induces an increase in mRNA for *MCL1* and *Dnmt1* but not *TP53*. Quantitative real time polymerase chain reaction results for *TP53* (A), *MCL1L* (B), and *Dnmt1* (C) mRNA levels from CLL cells, from up to seven patients, which were exposed to either MG132 or bortezomib for 2-12 h. The results shown represent the mean ± sem of the relative to *GAPDH* mRNA fold change in either *TP53* (n=7), *MCL1L* (n=4) or *Dnmt1* (n=5) mRNA levels as calculated by the $2^{-\Delta\Delta Ct}$ method.



Online Supplementary Figure S4. Z138 and NALM6 cell lines were exposed to bortezomib (10 nM) for 4-16 h and examined for immunoreactive NOXA bands. These experiments were performed in triplicate.



Online Supplementary Figure S5. (A) CLL cells from nine patients were transfected with either NOXA siRNA or the negative control siRNA 1 before exposure to MG132 (1 μM) for 8 h. Apoptosis was measured by TMRE and annexin V-propidium iodide staining and the results shown are the mean ± sem. Statistics were performed using a one-way-ANOVA followed by Bonferroni's multiple test or paired Student's t test and * denotes statistical significance with a *P* value <0.05 compared to either MG132 or MG132 plus control siRNA exposed cells (B) CLL cells from one patient were exposed as in (A) and were processed for western blot analysis. The proform and cleaved caspase-3 subunits are indicated.