ALK-negative anaplastic large cell lymphoma is sensitive to bortezomib through Noxa upregulation and release of Bax from Bcl-2

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SUPPLEMENTARY INFORMATION

Supplementary Methods

Lymphoma patient samples and cell lines

Cells from five primary systemic ALCL, which were classified according to the World Health Organization (WHO) at the Comprehensive Cancer Center of Amsterdam, were included in this study.[1] All protocols for obtaining and studying human tissues and patients' data were approved within the local ethical procedures of the institutional ethical review board at the VU University Medical Center and complied with the Code for Proper Secondary Use of Human Tissue in The Netherlands (www.federa.org). Informed consent was provided according to the Declaration of Helsinki.

Lymphoma suspensions were isolated from tissue biopsies by a combination of mechanical dissociation and collagenase/DNase digestion, and frozen until further testing.[2] Cells were thawed 1 hour prior to experimental testing and cultured in Iscove's modified Dulbecco's medium (BioWhittaker, Lonza, Belgium) supplemented with 40% fetal calf serum, 100 IU penicillin/100µg/ml streptomycin (1% P/S) at 37°C with 5% CO_2 in a humidified atmosphere.

The ALK-positive ALCL cell lines: SUDHL1, SUPM2, L82, Karpas 299 (obtained from the DSMZ cell bank, Germany) and the ALK-negative ALCL cell lines: JK, Mac1 and Mac2A (kindly provided by prof.dr. M. Kadin at the Roger Williams Medical Center, Providence, USA and by dr. J. Eberle at the Charité-University Medical Center, Berlin, Germany) were cultured in RPMI 1640 medium (BioWhittaker) containing 10% fetal calf serum and 1% P/S.

Proteasome activity assay

The chymotryptic proteolytic activity of the proteasome was measured using a Proteasome-GLO assay kit (Promega, Madison, USA) as described previously.[3] Briefly, cells were incubated with a luminogenic substrate specific for chypmotrypsin-like protease activity for 10

minutes at room temperature (RT). Subsequently, luminescence was determined using a microplate reader (Tecan, Giessen, The Netherlands).

Assessment of cell death

Primary ALCL cells and ALCL cell lines were incubated with 10, 20, 50 and 100nM bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, USA) for increasing periods of time. ALCL derived cell lines were pre-incubated with increasing concentrations of the caspase-8 inhibitor LETD-FMK, caspase-9 inhibitor LEHD-fmk or the pancaspase inhibitor z-VAD-fmk (Enzo life sciences, Lausen, Switzerland) 1 hour prior to treatment. Etoposide (Sigma, St Louis, MO, USA) was used to asses sensitivity to chemotherapy-induced apoptosis as it activates the intrinsic apoptosis pathway and therefore it is representative of the many chemotherapeutic drugs used in the treatment of ALCL.[4,5,6] ALCL cell lines and ALCL patient cells were incubated with 500nM etoposide for 24 hours. Dose-response studies demonstrated that the optimal cell death-inducing concentration of etoposide in ALCL cell lines was 500nM (data not shown).

Cell death of primary cells and ALCL cell lines was determined as described previously.[2] Briefly, cell death was detected using a standard number of fluorescent beads (Fluorospheres, Becton Dickinson, San Jose, CA, USA) in combination with 7-amino-actinomycin D (7AAD, ViaProbe, BD Pharmingen, Erembodegem, Belgium) to determine the number of viable (7AAD-negative) cells in each individual sample. Fluorescence was detected by the FACSCalibur flow cytometer and analyzed using CellQuest software (both Becton Dickinson). All tests were performed in triplicate. ALCL lymphoma cells were selected based on phenotype obtained by immunohistochemistry using antibodies against the cell-surface markers including CD3, CD25 (both BD biosciences, San Jose, CA, USA) and CD30 (DakoCytomation, Glostrup, Denmark) and on size (forward scatter, FSC).

Detection of caspase 3/7 activity

Caspase 3/7 activity was determined using a luminescent assay (Caspase-Glo^{TM} 3/7 assay, Promega Benelux, Leiden, Netherlands). Cells were lysed and incubated with the DEVD-containing substrate for 35 minutes at RT. Subsequently, luminescence was measured using a microplate luminometer (Victor2TM, Wallac, Perkin Elmer, Boston, MA, USA). Caspase activity was determined as caspase 3/7 activity levels of treated samples minus caspase 3/7 activity levels of untreated samples.

Measurement of $\Delta\psi_m$

Depolarization of the mitochondrial membrane (Ψ_m) was detected using the fluorescent probe tetramethylrhodamine ethyl ester perchlorate (TMRE, Invitrogen, Eugene, OR, USA) which accumulates in mitochondria. Briefly, cells (10^6 cells/ml) were incubated with 25nM TMRE at 37°C for 15 minutes in the dark and analyzed using FACS analysis. Mitochondrial membrane depolarization was observed as a shift to the left in the emission spectra. $\Delta\Psi_m$ was determined as the percentage unstained cells in bortezomib treated samples minus the percentage unstained cells in untreated samples.

RT-MLPA analysis

ALCL patient cells were isolated based on their phenotype using FACSsorting. Reverse Transcriptase-Multiplex Ligation-dependent Probe Amplification (RT-MLPA) was performed on total RNA as described previously.[7,8] For detailed description of the design of the 40 probes, (including 34 genes implicated in the intrinsic apoptosis pathway): www.mrc-holland.com. Data were analyzed using GeneMapper (Applied Biosystems, Warrington, UK) and Coffalyser 7 (MRC-Holland, Amsterdam, the Netherlands) software. The ß-glucuronidase (*GUS-B*) housekeeping gene was used as internal reference.

siRNA analysis

ALCL cells were electroporated using an Easyjet Plus electroporator (Equibio, Ashford, UK). Cells were resuspended in 200µl Optimix electrobuffer (Cell Projects Ltd, Harrietsham, UK) containing 2µM control nontargeting siRNA pool or Noxa specific siRNA (both provided by Dharmacon, Lafayette, CO, USA) and electroporated at 300V and 150µF. Cells were harvested in fresh medium and transferred to culture plates and cultured for 3 hours at 37 °C. After low-speed centrifugation to remove dead cells, viable cells ($1x10^6$ cells/ml) were incubated for 3 hours at 37°C followed by treatment with bortezomib for 16 hours.

Western blotting and immunoprecipitation

Western blot analysis was performed as described previously.[9] Antibodies against the following proteins were used: Bax, Bak, Bik, Puma, MCL1, Bcl-X_L (all Cell Signaling Technology, Boston, MA, USA), Noxa (Calbiochem, San Diego, CA, USA), and Bcl-2 (Millipore, Temecula, CA, USA). Proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Cellular α-tubulin (clone DM1a, Abcam, Cambridge, UK) and β-actin (BD biosciences) were used as loading controls for each sample.

ImmunoCruz™ IP/WB Optima E System (sc-45042, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for performing the co-IP experiments that included IP with anti-MCL1 or anti-Bcl-2 and detection by anti-Noxa or anti-Bax, respectively. Lysates were incubated overnight at 4°C with mouse MCL1 (clone 22, Santa Cruz Biotechnology) or mouse Bcl-2 (clone 124, Dako, Glostrup, Denmark) antibody or the control antibody complexed with an immunoprecipitation matrix, according to manufacturer's instructions. The matrix was washed and resuspended in loading buffer, heated to 95°C for 5 minutes and centrifuged. Supernatants with the immunoprecipitated proteins were subjected to western blot analysis.

Statistical analysis

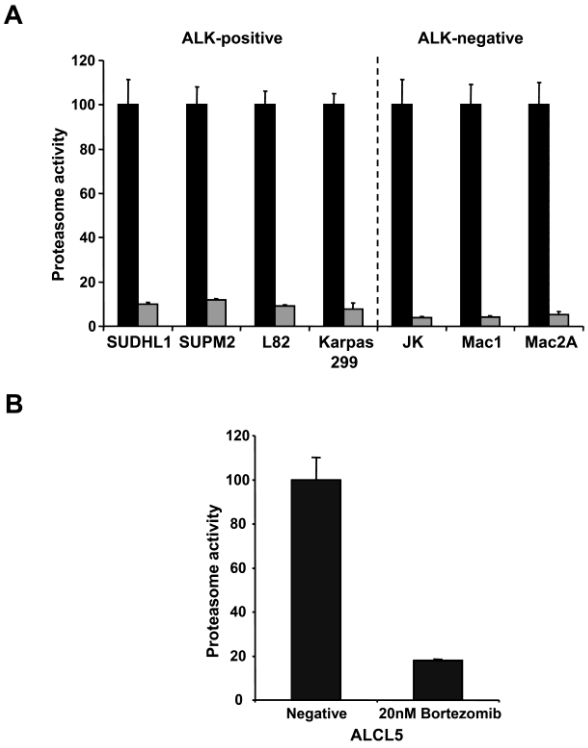
Comparisons of differences in continuous variables between two groups were performed using unpaired Student's *t*-tests. *P*-values of <0.05 were considered significant.

Supplemental Table I: Summary of expression of apoptosis regulating genes after treatment with bortezomib in ALK-positive and ALK-negative cells.

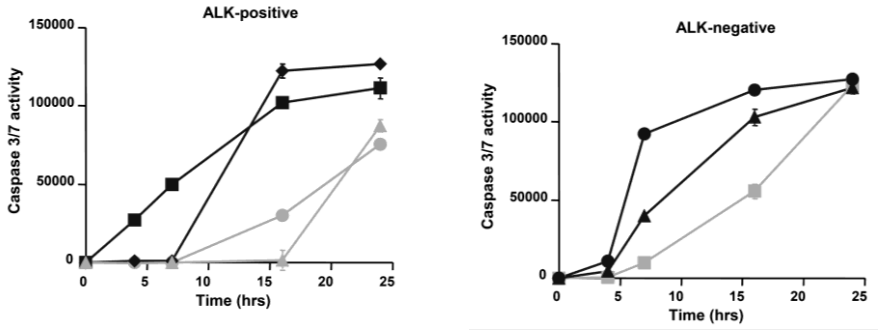
Genes	ALK-negative ALCL cells	ALK-positive ALCL cells
Pro-apoptotic genes		
BH3-only		
Noxa	**+++**	**+++**
Bad	**+**	**+**
Bim	**++**	**++**
Bid	**+**	**+**
Puma	**++**	**+/++**
Bik	**+**	**+**
BMF	**-**	**-**
BNIP3	**+/++**	**+/++**
BNIP3L	**+/++**	**+/++**
MCL1-short	**+**	**+**
Bax-like		
Bax-short	**++**	**-/+**
Bax-long	**+++**	**-/++**
Bak	**++**	**++**
Bcl-Rambo	**-/+**	**-**
Assorted		
AIF	**++**	**+/++**
Apaf-1	**-**	**-**
Apaf-1L	**-**	**-**
Apaf-1XL	**-**	**-**
Smac	**++**	**+/++**
Anti-apoptotic genes		
Bcl-2 family		
Bcl-W	**-**	**-**
Bcl-X_L	**--/-**	**--/-**
Bcl-2	**-**	**-**
Bcl-2A1	**-**	**-**
MCL1-long	**++**	**++/+++**
IAP family		
NAIP	**-**	**-**
cIAP2	**-**	**-**
XIAP	**-**	**-**
Survivin	**--/-**	**-**
Apollon	**-**	**-**
Assorted		
c-Flip	**-**	**-/+**
Pi-9	**-**	**-**

Expression of Bcl-G, Harakiri, cIAP1 and BIRC7 were undetectable in the majority of cases and were excluded from further analysis.
--, weak decrease; -, equal; +, weak increase; ++, moderate increase; +++, strong increase; in expression after exposure to bortezomib.
/, variable expression between different cell lines and/or patient samples

Supplemental Figures:



Supplemental Figure 1. Inhibition of proteasome activity by bortezomib in ALCL cells. (**A**) ALCL cell lines were treated with 20nM bortezomib for 1 hour and chymotrypsin-like activity of the proteasome was analyzed. (**B**) ALK-negative ALCL patient cells L5 were selected and isolated using FACS sorting. After 1 hour of incubation with 20nM bortezomib, the chymotrypsin-like proteasome activity was measured.



Supplemental Figure 2. Bortezomib induced caspase-3 activity in ALK-positive and ALK-negative ALCL cell lines SUDHL1 (●), SUPM2 (■), L82 (♦), Karpas 299 (▲), JK (■), Mac1 (▲) and Mac2A (●) after exposure to 20nM bortezomib for increasing periods of time.

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