SUPPLEMENTARY MATERIAL AND METHODS

Synergistic activity of bortezomib and HDACis in preclinical models of B-cell precursor acute lymphoblastic leukemia via modulation of p53, PI3K/AKT and NF-κB

Lorenz Bastian, Jana Hof, Madlen Pfau, Iduna Fichtner, Cornelia Eckert, Günter Henze, Javier Prada, Arend von Stackelberg, Karl Seeger and Shabnam Shalapour
**Detailed information on patient samples**

Primary BCP-ALL cells were collected after Ficoll density gradient centrifugation from bone marrow (BM) aspirates obtained at diagnosis of relapse (ClinicalTrials.gov identifier: NCT00114348). Stroma cells were isolated from BM aspirates (MSC) or testis biopsies (testicular MSC-like cells; T-MSC) as described previously (1). Primary PBMC were collected after Ficoll density gradient centrifugation from healthy volunteers.

**Chemotherapy Substances and PI3K/AKT inhibitors**

Idarubicin (Pharmacia, Germany), vincristine (TEVA, Germany), cytarabine (Cell pharm, Germany), mitoxantrone (Baxter, Germany), methotrexate (TEVA) and dexamethasone (Merck Serono, Germany) were dissolved either in isotonic saline or in aqua. For *in-vivo* experiments idarubicin was dissolved in 5% dextrose. LY 294002 (Gibco, USA) and MK-2206 (Selleckchem, USA) were dissolved in dimethyl sulfoxide (Sigma-Aldrich; final concentration <0.05%).

**Cell cultures and *in-vitro* treatment**

SEM cells were cultured in Iscove’s MDM medium (Biochrom, Germany). All other cell lines (Reh, 697, SD-1, Nalm6) were cultured in RPMI 1640 medium (Biochrom) in established conditions (2). For experiments, logarithmically growing cells were seeded in 12-well plates with drugs added from 100x stock solutions. For sequential treatments cells were incubated in medium containing the first drug for indicated time periods. Afterwards cells were washed two times in medium and resuspended in fresh medium containing the second drug (3). Primary LC, PBMC from healthy volunteers, murine splenocytes and murine BM-MNC were maintained for short term cultures in RMPI 1640 medium, supplemented with 20% FCS unless not otherwise
indicated and Primocin (Amaza, Germany) or 1% Penicillin/Streptomycin (Biochrom, Germany). MSC were isolated from BM aspirates and testis biopsies of four ALL-patients at different time points, as described previously (1, 4). Four MSC cultures from three ALL-patients and four T-MSC cultures (testicular MSC-like cells) from four ALL-patients were established. Coculture experiments were done as described previously (2, 5).

**Detailed information on xenograft mouse models**

As described in Material and Methods of the main text, established xenograft mouse models were applied (6). Mice were subdivided into groups of eight animals and drug treatment was initiated at day 3 in the i.v.-model or when tumors became palpable (day 17-19) in the s.c.-model (Supplementary Figure S10-S11). The drug doses and treatment schedule were established within a dose finding study (Supplementary Figure S11A) in the Nalm6 – s.c. xenograft mouse model. VPA and BTZ were dissolved in isotonic saline. Isotonic saline was used as vehicle control in both combination experiments (Supplementary Figures S10A, S11D). In the dose finding study idarubicin was dissolved in 5% dextrose. Therefore, 5% dextrose was used as vehicle control in this case (Supplementary Figure S11A). All animals were sacrificed, when mice in the control group showed signs of final disease. In the i.v.-mouse model spleens, brains and testes were isolated. BM cells from xenograft i.v. model and four WT C57BL/6 mice were obtained by flushing both femurs for analysis of LC infiltration and primary short term cultures, respectively. Spleen single cell suspensions from the xenograft i.v. model and four WT C57BL/6 mice were used for flow cytometry analyses of LC infiltration and primary short term cultures, respectively. In the s.c.-model, tumor development was observed by measuring tumor volumes using a caliper (2).
MTS assay

The MTS assay (Cell Titer 96®, Promega, Germany) was used to determine the proliferation rate of cell lines as described previously (2). Inhibitory effects were calculated from absolute cell counts which were determined using serial dilutions of cells as a standard.

Measurement of NF-κB activation

Nuclear cell extracts were prepared using the Nuclear Extract Kit and analyzed with the TransAM NF-κB Family Transcription Factor Assay Kit according to the manufacturer’s instructions (both Active Motif, Belgium).

Flow cytometry

Annexin-V FITC (IQ Products, The Netherlands) / propidium iodide (PI, Sigma Aldrich, Germany) were used following standard protocols (2). PI stained cells were analyzed for cell cycle distributions as described previously (3). For intracellular staining cells were fixed with paraformaldehyde (4%) and permeabilized with ice cold methanol (90%) according to standard protocols. Single cell suspensions from mouse bone marrow and spleen were analyzed using hCD19 and hCD10 antibodies for determination of the proportion of BCP-ALL cells in corresponding mouse organs. Samples were measured on a FACSCalibur flow cytometer (Beckton Dickinson, USA) and analyzed with CellQuest software (Beckton Dickinson) or FlowJo.8 software (Tree Star, USA).
Antibodies and Dyes

Antibodies specific for the following antigens were used: Death Receptor 5-PE (TNFRSF10B, Biolegend, USA), hCD10-APC, hCD19-FITC, hCD19-APC, hCD3-PE, hCD45-APC, hIgG-FITC/-PE/-APC isotype controls (all Beckton Dickinson), mIgM-FITC (G53-238), mCD19-APC (1D3), mCD3-FITC (145-2C11), mCD11b-APC (M1/70), mIgG-FITC/-PE/-APC isotype controls (all Beckton Dickinson), Cleaved Caspase-3 mAb Apoptosis Kit (Beckton Dickinson), mAb rabbit anti-hMDM2 (clone 154-167; Acris Antibodies, Germany), mAb rabbit anti-hp53 (7F5; Cell signaling, USA), mAb mouse anti-hUbiquitin (PAD1; Santa Cruz Biotechnology, USA), mAb rabbit anti-NF-κB p65 (C22B4), mAb rabbit anti-Phospho-NF-κB p65 (Ser536) (93H1), mAb rabbit anti-hPhospho-Akt (Ser473) (193H12) (all Cell signaling), mAb mouse anti-hprolyl-4-hydroxylase beta (Acris, Germany), mAb rabbit anti-hcleaved Caspase-3 (5A1E; Cell signaling), mAb rat anti-mCD31(MEC13,3; Beckton Dickinson), Ab rat anti-mERTR7 (Santa Cruz Biotechnology). The following Alexa 594-, Alexa 647- and Alexa 488-conjugated secondary antibodies were used: donkey anti-rat IgG, donkey anti-rabbit IgG, goat anti-rat IgG, donkey anti-mouse IgG (all Molecular Probes, Invitrogen, Germany). Further secondary reagents were biotin-conjugated mouse anti-hCD10 (eBio CB-CALLA, eBioscience, USA), Streptavidin-conjugated Alexa 594 (Molecular Probes). For LC separation, CD19-beads (Milteny, Germany) were used. For the long-term observation of LC in-vitro, especially in cocultures, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester [SE (CFSE)] was used for labeling LC (PromoCell GmbH, Germany).
Additional information on gene expression analysis

The “12 h” time point was chosen for gene expression analysis since upstream events related to gene expression such as NFκB activation (Figure 4B) revealed clearly detectable changes 12 h after treatment, while treatment-induced apoptosis was negligible but increased in the following period to 24 h (Supplementary Figure S1E). A fold-change above two and a p-value < 0.001 were considered as reliable result of differentially expressed genes. Pathway analysis of the differentially expressed genes was performed with Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

Gene expression data of RelA, RelB, NFkB1 and NFkB2 genes were obtained by analyzing the available RNA hybridization results from 52 bone marrow samples of BCP-ALL patients at first relapse enrolled in the relapse trial ALL-REZ BFM 2002 on the Affymetrix HG-U133A microarray (7).

Quantitative Real time PCR (qRT-PCR)

mRNA isolation, cDNA reverse transcription and quantitative real-time PCR (QRT-PCR) were performed as described previously (2, 8). QRT-PCR was performed using TaqMan gene expression assays for CDKN1A (Hs00355782_m1), for MDM2 (Hs00234753_m1), for HSPA1A1 (Hs00359163_s1), for RelA (Hs01042010_m1), and for ABL1 (Hs01104728_m1, all from Applied Biosystems, USA) on a Fast real-time PCR system (Applied Biosystems). QRT-PCR analyses for VLA-4 (ITGA4) were performed as described previously (5). Means of triplicates of one to two experiments are shown in bar graphs.

For the quantification of BCP-ALL cell infiltration in testis and brain isolated from the s.c.-xenograft mouse model, DNA was isolated from both tissues and QRT-PCR was
performed using human specific \( \beta \)-globin TaqMan-probes, and mouse specific \( \beta \)-globin TaqMan-probes. Serial dilutions of DNA from Nalm6 cells in NOD/SCID mouse DNA were used for standard curve preparation in order to determine efficiency and sensitivity of the assay in each run. The relative amount of \( h\beta \)-globin was measured and normalized to the amount of \( m\beta \)-globin. The fold difference (as relative amount) was calculated by the comparative CT method (\( \Delta \)CT).

\[
\begin{align*}
\text{m\beta-globin-fw:} & \quad 5\prime\text{-}TTC\ TGA\ CAT\ AGT\ TGT\ GTT\ GAC\ TCA\ C \\
\text{m\beta-globin-rev:} & \quad 5\prime\text{-}TTC\ TTG\ TGA\ GCT\ GCC\ TTG\ TA \\
\text{m\beta-globin TagMan-probes (FAM/TAMRA):} & \quad ACC\ ACC\ AAC\ TTC\ ATC\ GGA\ GT\ X\ TC\ ACC\ TT\ P \\
\text{h\beta-globin-fw:} & \quad 5\prime\text{-}CTG\ ACA\ CAA\ CTG\ TGT\ TCA\ CTA\ GC \\
\text{h\beta-globin-rev:} & \quad 5\prime\text{-}CCA\ CAT\ GCC\ CAG\ TTT\ CTA\ TTG \\
\text{h\beta-globin TagMan-probes (FAM/TAMRA):} & \quad CCT\ GAG\ GAG\ AAG\ TCT\ GCC\ GT\ X\ TAC\ TGC\ P
\end{align*}
\]

**Immunohistochemistry**

Immunohisto-/cytochemistry and H&E analyses were performed on either frozen or paraffin embedded tissue sections or cytospins as described previously and examined using an Axioplan 200 microscope with AxioVision Release 4.5 software (Zeiss, Germany) (2, 8). For the analyses of nuclear p53 and p65, 40-100 nuclei were counted. For quantification of protein expression on IHC-sections, the fluorescence intensity of single cells was determined by ImageJ software analysis (9). The corrected total cell fluorescence was calculated by subtracting background signal from the fluorescence of single cells as has been described (10). The shown microphotographs are representative for two to three independent experiments.

**Western blot analysis**

Reh cells were lysed using SDS buffer (Tris-HCL, Glycerol, SDS 1%, EDTA, Pefabloc, Vanadat, DTT, Protease Inhibitor). Equal protein amounts were separated
by 8-10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Buckinghamshire, United Kingdom). The membranes were probed with rabbit antibodies against human NFκB-p65, p-NFκB-p65, p-AKT (Cell Signaling), followed by horseradish peroxidase (HRP)–linked secondary antibodies. Blots were subsequently treated with anti–beta actin (Sigma-Aldrich) Abs as control for equal loading. The bands were visualized using Super Signal West Dura (Thermo Scientific).

**Normalization of apoptosis and cell death measurements**

Apoptosis and cell death measurements were normalized to adjust for spontaneous apoptosis or cell death prior to further combination effect analysis (11).
SUPPLEMENTARY REFERENCES


