Synergistic Activity of Bortezomib and HDACi in Preclinical Models of B-cell Precursor Acute Lymphoblastic Leukemia via Modulation of p53, PI3K/AKT, and NF-κB

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Abstract

Purpose: Relapse of disease and subsequent resistance to established therapies remains a major challenge in the treatment of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL). New therapeutic options, such as proteasome and histone deacetylase inhibitors (HDACi) with a toxicity profile differing from that of conventional cytotoxic agents, are needed for these extensively pretreated patients.

Experimental Design: Antiproliferative and proapoptotic effects of combined HDACi/proteasome inhibitor treatments were analyzed using BCP-ALL monocultures, cocultures with primary mesenchymal stroma cells from patients with ALL, and xenograft mouse models. The underlying molecular mechanisms associated with combined treatment were determined by gene expression profiling and protein validation.

Results: We identified the proteasome inhibitor bortezomib as a promising combination partner for HDACi due to the substantial synergistic antileukemic activity in BCP-ALL cells after concomitant application. This effect was maintained or even increased in the presence of chemotherapeutic agents. The synergistic effect of combined HDACi/BTZ treatment was associated with the regulation of genes involved in cell cycle, JUN/MAPK, PI3K/AKT, p53, ubiquitin/proteasome, and NF-κB pathways. We observed an activation of NF-κB after bortezomib treatment and the induction of apoptosis-related NF-κB target genes such as TNFαRs after concomitant treatment, indicating a possible involvement of NF-κB as proapoptotic mediator. In this context, significantly lower NF-κB subunits gene expression was detected in leukemia cells from patients who developed a relapse during frontline chemotherapy, compared with those who relapsed after cessation of frontline therapy.

Conclusion: These results provide a rationale for the integration of HDACi/BTZ combinations into current childhood BCP-ALL treatment protocols. Clin Cancer Res; 19(6); 1445–57. ©2013 AACR.

Introduction

Despite continuous improvement of therapeutic options for children with acute lymphoblastic leukemia (ALL), the prognosis for patients who suffer a relapse remains poor with a probability of even-free survival of 30% at 10 years (1). New treatment approaches with a different toxicity profile in comparison to conventional cytotoxic agents are required for these extensively pretreated patients. As a promising alternative, histone deacetylase inhibitors (HDACi) have been suggested (2, 3). We have previously shown that HDACi are able to limit the expansion of B-cell precursor (BCP)-ALL cells in vitro (4). The inclusion of HDACi into current ALL chemotherapy concepts requires the identification of suitable combination partners, as first clinical trials achieved only limited therapeutic responses (5, 6) while establishing a good tolerance of HDACi in pediatric patients with malignant diseases. So far, only additive or even antagonistic interactions have been observed after combined treatment with HDACi and different conventional cytostatic drugs in myeloid leukemia and T-ALL cells (7). In BCP-ALL leukemia cells, we have recently observed that the sequence of drug application determined synergistic or antagonistic responses to combined treatment with HDACi and methotrexate (8).
**Translational Relevance**

Cure rates for children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) have continuously improved, but treatment is still associated with the risk of serious acute and late effects and the outcome of patients with relapses is poor. Therapeutic alternatives with substantial antileukemic potential and toxicity profiles differing from that of conventional chemotherapeutics are needed, as suggested for histone deacetylase inhibitors (HDACi). Proteasome inhibitors were found to increase the activity of HDACi in mature B-cell malignancies. In BCP-ALL, we identified bortezomib as a promising combination partner for HDACi due to the synergistic antileukemic activity. Our analyses indicate that active proteasomal processing and histone deacetylation function are indispensable for the survival of immature B-cell malignancies, although the underlying mechanisms differed in part from mature B-cell malignancies. The synergism of BTZ/HDACi was maintained or even increased in the presence of chemotherapeutic agents, particularly anthracyclines, establishing a rationale for the inclusion of HDACi/BTZ combinations into current chemotherapy regimens for childhood BCP-ALL.

Proteasome inhibitors such as bortezomib (BTZ) were found to increase substantially the activity of HDACi in different hematologic and solid malignancies (9–13). To date, no systematic studies have been conducted to analyze the response of B-cell precursor leukemias to HDACi/proteasome inhibitor combinations. A well-tolerated bortezomib dose level in patients with childhood leukemia (14) and described additive interactions of bortezomib with conventional cytostatics in vitro indicate bortezomib as a promising agent for treatment of BCP-ALL (15). The synergistic induction of apoptosis after HDACi/BTZ treatments has been partially associated with an inhibition of NF-κB signaling in mature B-cell malignancies, myeloid leukemia, and T-ALL (9, 10, 13, 16). These observations are consistent with the finding that NF-κB signaling is indispensable for the homeostasis of mature B cells and contributes to the survival of mature B-cell malignancies (17, 18). The significance of NF-κB signaling for the survival and differentiation of precursor B cells is still less clear (17, 19), and its role in the leukemogenesis and treatment response of BCP-ALL remains controversial (20–22).

Therefore, we studied the effect of combined HDACi/proteasome inhibitor treatments in BCP-ALL in vitro and in vitro and analyzed the underlying molecular mechanisms.

**Material and Methods**

**Cell lines and patient samples**

The human BCP-ALL cell lines Reh, Nalm6, SD-1, 697, and SEM were purchased from the German Collection of Microorganisms and Cell cultures (DSMZ). The cell lines were routinely tested for the expression of the corresponding surface markers and fusion genes by fluorescence-activated cell sorting (FACS), quantitative real-time PCR (QRT-PCR), and FISH.

All patients were enrolled in the relapse trial ALL-REZ BFM 2002, approved by the Institutional Review Board of the Charité-Universitätsmedizin Berlin, Berlin, Germany (ClinicalTrials.gov identifier: NCT00114348). Written informed consent was obtained from patients or guardians.

**Substances**

Bortezomib (Janssen-Cilag International) and valproic acid (VPA, \textit{in-vitro}: Sigma-Aldrich, \textit{in-vivo}: Desitin Arzneimittel) were dissolved in isotonic saline or aqua and suberylanilide hydroxamic acid (SAHA, Merck Pharmaceuticals) in dimethyl sulfoxide (final concentration < 0.02%). Unless not otherwise stated, the final drug concentrations were 1 mmol/L for VPA (6), 1.2 μmol/L for SAHA (23), and 10 nmol/L for bortezomib (in Nalm6: 7 nmol/L BTZ; ref. 14), as chosen according to clinically achievable plasma concentrations.

**Gene expression analysis**

Reh cells where treated either with bortezomib, VPA, or the combination of both for 12 hours. Reh cells incubated with medium served as control. After total RNA extraction (Qiagen), samples were hybridized on Agilent Whole Human Genome Oligo Microarrays (Millenyi Biotec). Microarray normalization was conducted by Millenyi Biotec GmbH, whereas for further data analysis, the Partek Genomics Suits software, version 6.5 beta © 2009 (Partek Inc.) was used. Gene expression data are available in NCBI’s Gene Expression Omnibus data base (accession number GSE41951).

**Mouse models**

Male nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected either intravenously with leukemia cells derived from a pediatric patient with BCP-ALL at first relapse (ALL-SCID2) or subcutaneously with Nalm6 cells (1:1 mixture with Matrigel) by transferring 1 × 10^7 leukemia cells per mouse (24). Animal experiments were approved by the local responsible authorities (G0221/03) and carried out according to UICC guidelines (25).

**Combination effects and statistical analyses**

Student \(t\) test, Mann–Whitney \(U\) test, and Kruskal–Wallis test were used for the analysis of statistical significance. To analyze the effects of combined treatment with regard to synergism, additivity, or antagonism, either the combination index (CI) according to Chou and Talalay was determined (CalcuSyn software; Biosoft) or the fractional product was calculated using the method of Webb (26).

Further detailed information on the applied material and methods is provided in the Supplementary Material and Methods.
Results

Concomitant treatment with bortezomib and HDACi has synergistic effects in BCP-ALL cells

To assess the antileukemic effect of combined treatments with HDACi and either established ALL therapy elements (idarubicin, vincristine, cytarabine) or the proteasome inhibitor bortezomib, BCP-ALL cell lines were exposed to the respective compounds either alone or in combination. Leukemia cells proliferation and the induction of apoptosis were measured by MTS assay and FACS analysis after Annexin-V/propidium iodide (PI) staining, respectively (Reh, Fig. 1A and B). After concomitant HDACi/BTZ treatment inhibition of proliferation and induction of apoptosis was markedly increased, as compared with bortezomib alone (Fig. 1A and B). The ratio of human CD3+ T cells remained unchanged (Fig. 1B). Immunofluorescence analysis using CD19 and CD20 antibodies identified the antileukemic activity of HDACi/BTZ combinations as synergistic (Fig. 1A and B, left; Supplementary Fig. S1A). Combination index analysis identified the antileukemic activity of HDACi/BTZ combinations as synergistic (Fig. 1A and B, right). This synergistic effect was also observed, when serial dilutions of HDACi were combined with a constant concentration of bortezomib (Supplementary Fig. S1B–S1D) and when apoptosis was assessed 24 and 48 hours after drug application (Supplementary Fig. S1E).

The combined treatment of HDACi with idarubicin induced mostly synergistic interactions, which were less pronounced when compared with HDACi/BTZ combinations (Fig. 1A and B). The combinations of HDACi with vincristine or cytarabine were additive to antagonistic, depending on tested concentrations and cell lines (Fig. 1A and B). Similar results were obtained in Nalm6 cells (Supplementary Fig. S2A and Supplementary Table S1 and S2), revealing bortezomib as the most promising combination partner for HDACi.

For further validation the amount of cell death in 697, SEM and SD-1 cells were analyzed after application of 2 different concentrations of bortezomib with and without VPA at 3 time points. Fractional product analysis indicated synergism for the majority of tested conditions (Supplementary Table S3). In addition, primary BCP-ALL cells obtained from 3 patients at relapse diagnosis were exposed to VPA and bortezomib treatments. Fractional product and combination index analysis showed that concomitant treatment induced apoptosis and reduced the amount of vital CD19+ leukemia cells in either additive or synergistic manner (Supplementary Fig. S2B–S2D).

To evaluate the effects of VPA and bortezomib treatments on normal B and T cells, PBMCs of 3 healthy adults, murine splenocytes and murine BM-MNC were analyzed in vitro (Supplementary Figs. S3–S5). Analyses of subpopulations revealed a significant reduction of CD19+ B cells after single and concomitant treatment. The proportion of human CD3+ T cells remained unchanged in vitro.

Changes in the order of application can substantially alter the effect of drug combinations, as we have previously shown for the combination of HDACi and methotrexate (8). Therefore, we analyzed whether the sequence of application has an effect on drug responsive-ness to bortezomib and HDACi. Simultaneous application of both drugs was required for the synergistic induction of cell death (Fig. 1C; Supplementary Table S4). In contrast, sequential applications of bortezomib and HDACi induced in most cases apoptosis in a near-additive manner, with the exception of VPA followed by bortezomib, which even resulted in antagonistic interactions. Antagonism was not observed in any of the tested concomitant combinations, thus confirming a broad synergistic activity of bortezomib and HDACi in BCP-ALL cells, when applied concomitantly.

The synergistic induction of apoptosis involves the regulation of cell cycle, death receptor, and p53 signaling pathways

To determine the molecular mechanisms involved and/or regulated by the synergistic activity of VPA/BTZ treatment, we sought to identify genes whose expression changed after combined treatments. Gene expression profiles were analyzed from the cell line Reh after single treatment with either VPA or bortezomib as well as after concomitant treatment, compared with untreated control samples. The obtained results were further validated in four cell lines. We identified 919 genes differentially expressed exclusively after concomitant treatment (Supplementary Tables S7 and S8). Pathway analyses confirmed, as was observed by aforementioned in vitro tests, the involvement of genes related to cell cycle, apoptosis, and death receptor signaling. Furthermore, changes in the signaling pathways involving retinoic acid, NF-κB, p53, TLR/TREM1, PI3K/AKT/mTOR, mitogen-activated protein kinase (MAPK), endoplasmic reticulum (ER) stress response, and protein ubiquitination were identified. Heatmap analyses for 7 of these pathways and validations of the corresponding genes are shown in Figs. 2–4 and Supplementary Figs. S6 and S7.

Upregulation of proapoptotic genes, such as caspases and TNIaR (TNF-related apoptosis-inducing ligand receptors), and downregulation of antiapoptotic genes, such as BCL2, elucidate the synergistic proapoptotic effect of concomitant treatment (Fig. 2A). The increase of DR-5 (TNFRSF-10B; Fig. 2B) and cleaved caspase-3 (Supplementary Fig. S6A) was confirmed by flow cytometry. Analysis of cell-cycle distributions indicated a G0–G1 phase arrest after treatment with VPA, whereas after treatment with bortezomib or the combination of VPA/BTZ no relevant changes in the cell-cycle phases were observed, despite the synergistic induction of apoptosis after combined treatment (Supplementary Fig. S6B). The gene expression signature of concomitant treatment revealed the modulation of the p53 pathway, which we have identified to be associated with the response to therapy of relapsed ALL (27). Key genes of the p53 pathway such as MDM2 and CDKN1A were differentially expressed in leukemia cells after concomitant treatment (Fig. 2C). These genes were further validated by immunocytochemistry (Fig. 2D) and QRT-PCR after simultaneous and sequential exposure to both drugs (Supplementary Fig. S6C and S6D). CDKN1A mRNA level was increased after

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Figure 1. Concomitant combinations of HDACi and BTZ synergistically inhibit proliferation and induce apoptosis in BCP-ALL cell lines. A and B, Reh cells were treated for 72 hours with BTZ or chemotherapeutic agents alone or in combination with HDACi (VPA, SAHA). A, the inhibition of proliferation (MTS assay). B, the induction of apoptosis (Annexin-V/PI FACS), together with CI analyses (right). Duplicates ± SD of 2 to 3 experiments. C, induction of apoptosis in Reh cells after treatment with single drugs for 36 hours followed by 36 hours in fresh media or sequential combinations of 36 hours with the first and 36 hours with the second drug or concomitant combinations of both drugs for 72 hours. Triplicates ± SD of one experiment representative for Reh and Nalm6.

***, P < 0.001 (Student t test).
VPA single, concomitant, and sequential treatments. The MDM2 mRNA and protein level decreased significantly only after concomitant treatment, as confirmed by QRT-PCR and immunocytochemistry. In line with this, only after concomitant treatment the upregulation and nuclear localization of p53 protein was detectable (Fig. 2D), revealing the involvement of the p53 pathway in the synergistic induction of apoptosis.

Concomitant treatment involves the modulation of PI3K/AKT/mTOR, MAPK, and protein ubiquitination signaling pathways

The gene expression signature of concomitant treatment revealed a differential regulation of genes involved in PI3K/AKT/mTOR (e.g., ITGA4, BCL-2) and MAPK signaling pathways (e.g., MAP2K3, CREB5; Fig. 3A). The effects of 2 PI3K/AKT inhibitors (LY294002, MK-2206) on leukemia cell

Figure 2. Induction of cell-cycle, death receptor, and p53 pathways after concomitant treatment. Reh cells were treated with either BTZ or VPA alone or in concomitant combination. After 12 hours, mRNA was isolated for gene expression analyses. A, heatmap depicts differentially expressed genes in Reh cells after concomitant VPA/BTZ treatment, which regulate cell-cycle and death receptor signaling. Expression levels of those genes after single treatments. B, FACS analyses of DR-5 (TNFRSF10B) expression on Reh cells after treatments. Representative for 2 experiments. C, heatmap depicts differentially expressed genes involved in the p53 pathway. D, Reh cells after treatments were stained with mAbs against MDM2 (green) or p53 (red) and 4,6-diamidino-2-phenylindole (DAPI). Magnification, ×400. Shown are the corrected total cell fluorescence measurements of MDM2 expression in 30 nuclei ± SD and the presence of nuclear p53 after counting of 40 to 70 nuclei. **, P < 0.01 (Mann–Whitney U test).
response to VPA/BTZ treatment were further determined. Although the treatment with PI3K/AKT inhibitors did not induce apoptosis in leukemia cells, the triple combination with VPA/BTZ showed clearly a decrease of apoptosis compared to VPA/BTZ treatment (Supplementary Fig. S7A–S7E), revealing the involvement of the PI3K/AKT pathway in the synergistic induction of apoptosis.

Moreover, ITGA-4 (VLA-4)-mediated signaling during the interaction with stroma cells has been shown to support the survival of leukemia cells mostly through the activation of the PI3K pathway (28, 29). VLA-4 expression was validated by QRT-PCR at 2 time points, showing that the VLA-4 mRNA expression decreased significantly after concomitant treatments (Fig. 3B; Supplementary Fig. S6E). Furthermore, concomitant treatment downregulated the expression of NLRXL, an inhibitor of NF-κB activation, and upregulated CRY1, a circadian clock gene (Supplementary Tables S7 and S8), both of which we have previously found to be regulated via VLA-4 signaling in leukemia cells (29).

Gene expression profiling further revealed the modulation of the protein ubiquitination pathway after application of bortezomib either alone or in combination with VPA (Fig. 3C). Bortezomib treatment markedly increased the expression of regulatory proteasome subunits (19S) as well as structural and catalytic subunits (20S). After concomitant treatment the upregulation of 19S and 20S subunits was less pronounced and the immunoproteasome subunit β5i was downregulated. Thus, concomitant treatment with VPA shifted the regulation of proteasome subunits, which was induced by bortezomib single treatment. Ubiquitin mRNA and protein level increased after bortezomib treatment, which was even more pronounced after concomitant treatment (Fig. 3C and D; Supplementary Fig. S6G). This is consistent with previously published results reporting the accumulation...
of polyubiquitinated proteins as a consequence of reduced proteasome activity (11). Furthermore, gene expression analysis showed an upregulation of different heat shock protein mRNAs (Fig. 3C; e.g., HSPA1A and HSPA5). The highest induction of HSPA1A was observed after concomitant treatment, as validated by QRT-PCR (Fig. 3D; Supplementary Fig. S6F), indicating the activation of the ER stress/unfolded protein response pathway.

**Concomitant treatment activates the NF-κB signaling pathway**

The proapoptotic effect of bortezomib in different malignancies has been attributed, in part, to the inhibition of the constitutive and induced activation of NF-κB signaling pathway (9, 13, 30, 31). In contrast, an activation of the NF-κB pathway after bortezomib treatment has also been described in multiple myeloma (32) and endometrial carcinoma cells (33).

In the tested BCP-ALL model, gene expression profiles clearly showed a modulation of NF-κB signaling pathway genes after bortezomib single and concomitant treatments (Fig. 4A). Importantly, the gene expression signature of NF-κB pathway varied between single and concomitant treatments, for example, regarding the expression of interleukin (IL)-6, IL-8, and TLR7. However, from the 5 NF-κB subunits, only RelB mRNA expression significantly increased after concomitant treatment. We assessed the NF-κB activation by FACS and Western blot analysis of p65 (RelA) phosphorylation in Reh and 697 cells at multiple time points after VPA/ BTZ treatment, revealing a marked p-p65 increase 9 hours after drug application (Fig. 4B, above; Supplementary Fig. S7E and S7F). Accordingly, the activation of 4 NF-κB subunits (p65, RelB, p50, p52) was determined in 3 BCP-ALL cell lines 9 hours after single and combined treatments using DNA-binding ELISA-based analysis (Fig. 4B, below). A significant activation of p65 was observable after bortezomib and concomitant treatments, which increased even more after concomitant treatment in 2 of 3 cell lines (Fig. 4B). Immunocytochemical analysis confirmed the nuclear translocation of p65 after concomitant treatment (Fig. 4C; Supplementary Fig. S9B). These results indicated an activation of the canonical NF-κB pathway. Furthermore, DNA-binding activity of RelB subunits increased slightly after concomitant treatment in 697 and Reh cells, suggesting also a possible involvement of the noncanonical pathway.

It has been assumed that tumor cells activate NF-κB signaling in response to chemotherapy as a prosurvival factor (34). In contrast, new findings (35–37) and our results indicate that NF-κB has also a tumor-suppressive function. In ALLs, a constitutive activation of NF-κB as a survival factor but also an involvement of NF-κB signaling in chemotherapy-induced apoptosis was reported (20, 37, 38). A clear correlation between NF-κB expression and BCP-ALL pathophysiology remains to be established. Therefore, the contribution of NF-κB subunit expression in leukemia cells of patients with BCP-ALL at first relapse was analyzed by the gene expression profiling data available for a total cohort of 52 patients (39). We compared the expression levels of NF-κB subunits in relevant clinical and biologic subgroups of ALL relapse (Supplementary Table S5). NF-κB subunits expression (RelA, RelB, NFKB2, NFKB1) in leukemia cells correlated significantly with the time point of relapse. Furthermore, patients who suffered a relapse of ALL during the frontline chemotherapy (relapse on treatment) had significantly lower NF-κB subunits expression than those who suffered a relapse after cessation of frontline therapy (relapse off-treatment; Fig. 4D; Supplementary Table S5).

**Concomitant treatment has synergistic effects in leukemia cell cocultures with mesenchymal stroma cells**

To ascertain, whether the stroma might exert a cytoprotective effect on leukemia cells, as it was observed when treated with some chemotherapeutics (29, 40), the effects of combined VPA/BTZ treatments were analyzed in cocultures of leukemia cells with primary stroma cells. Mesenchymal stroma cells (MSC) were isolated from bone marrow (MSC; n = 4) or testis (T-MSC; n = 4) of patients with BCP-ALL. Both organs represent major sites of leukemia cells infiltration in relapsed ALLs. Reh and 697 cells were cocultured with MSCs and treated with VPA and bortezomib as described above. The amount of cell death was analyzed by flow cytometry. Combined treatment induced synergistic cell death in leukemia cells both in monocultures and in cocultures (Fig. 5A; Supplementary Fig. S8A and S8B). No significant induction of cell death in primary MSCs and T-MSCs was observed both in monocultures and cocultures (Supplementary Fig. S8C and S8D, S8G and S8H). However, antileukemic activity of combined treatment in cocultures was reduced about 15% to 20% when compared with monocultures (Fig. 5A). Moreover, culturing primary leukemia cells with MSCs confirmed the prosurvival effect of MSCs, whereas combined treatment was able to overcome the protective effect of MSCs (Supplementary Fig. S8E and S8F). In accordance with the aforementioned results, we observed an increase of RelA mRNA expression, translocation of p65 in nuclei and decrease of MDM2 in leukemia cells after combined treatment in cocultures (Supplementary Fig. S9A–S9C).

**Combined treatment reduces leukemia cell burden in a BCP-ALL i.v. xenograft mouse model**

To assess the effect of combined VPA/BTZ treatment on leukemia cells survival and engraftment in vivo, an established i.v. xenograft mouse model of chemotherapy-resistant childhood BCP-ALL was used (24). The protocol included four groups, which were treated with isotonic saline (control), bortezomib, VPA or VPA/BTZ, respectively (Supplementary Fig. S10A). Dosages and treatment schedules for xenograft experiments were determined within a prior dose finding study (Supplementary Fig. S11A–S11C). No significant weight loss was observed in any of the treatment groups (Supplementary Fig. S10B). Also, spleen...
weights did not differ significantly between treatment groups (Supplementary Fig. S10C). To study the ALL cell burden in the whole body, the amount of leukemia cells was analyzed in different lymphatic tissues (spleen and bone marrow) by flow cytometry and in extramedullary nonlymphatic tissues (brain and testis) by QRT-PCR. Quantification of the leukemia cells burden in bone marrow and spleen showed a significant reduction of the proportion of hCD19+ hCD10+ leukemia cells after combined treatment when compared to controls or to the single drug treatments (Fig. 5B; Supplementary Fig. S10D).

Treatment with bortezomib alone had no significant impact on tests or brain involvement (Supplementary Fig. S10E and S10F). Leukemia cell infiltration increased significantly after VPA treatment in tests and slightly in brain (Supplementary Fig. S10E and S10F). This is in accordance with recently published results, showing that HDACi treatments augment the migration and metastasis in

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**Figure 4.** Analysis of NF-κB signaling pathway in response to concomitant treatment and in primary BCP-ALL cells from patients at first relapse. A, heatmap depicts differentially expressed genes involved in the NF-κB pathway. B, analysis of NF-κB activation by FACS in Reh and 697 cells with mAbs against phospho-p65 (blue line, control; red line, VPA + BTZ; right: bars, mean fluorescence intensity) at indicated time points. Results from Reh cells are shown. DNA-binding activity of NF-κB subunits after treatments for 9 hours. Measurements from 1 to 2 experiments in each cell line with group medians. Median fold change >1.5 and \( P < 0.05 \) was considered statistically significant (Mann-Whitney U test). C, Nalm6 cells were stained with a mAb against p65 and 4’,6-diamino-2-phenylindole (DAPI) after treatments for 12 hours. Arrow indicates nuclear translocation of p65 (magnification, \( \times 400 \)). Insert, mean proportions of nuclear p65+ cells after counting of 40 to 80 nuclei. D, RelA and RelB mRNA expression levels in BCP-ALL cells from patients at first relapse (\( n = 52 \)) as obtained by microarray analysis. The patient cohort was subdivided according to the time point of relapse, during the frontline chemotherapy (on treatment) or off treatment. Signal intensity (SI) of gene expression in individual patients with group medians. *, \( P < 0.05 \); **, \( P < 0.01 \) (Mann-Whitney U test).
different tumor models (41). The combined treatment significantly reduced leukemia cell infiltration in testis, only when compared with VPA (Supplementary Fig. S10E). Most importantly, the combined treatment significantly decreased the leukemia cells infiltration in brain, when compared with controls and single VPA treatment (Supplementary Fig. S10F).

Combined treatment reduces tumor volumes in a BCP-ALL s.c. xenograft mouse model through induction of apoptosis and changes the stroma microenvironment

The interaction of tumor cells with their microenvironment plays an important role in mediating the effect of antineoplastic drugs (29, 40, 42). To assess to which extent induction of apoptosis might contribute to the reduced ALL cell burden observed in the i.v. xenograft mouse model and to analyze the impact of combined treatment on angiogenesis and tumor-associated fibroblasts, we used an s.c. xenograft mouse model bearing Nalm6 cells. The treatment protocol is provided in Supplementary Fig. S11D. Tumor volumes were significantly reduced after treatment with VPA (\(P = 0.015\)) or after combined treatment with VPA/BTZ (\(P = 0.008\)) in comparison to untreated controls (Fig. 5C). Furthermore, tumor growth curves showed a delay of tumor growth after single treatments with bortezomib or VPA, which was more pronounced after combination treatment (Supplementary Fig. S12A). Body weight development indicated a significant weight loss in the bortezomib- and the VPA/BTZ-treated groups compared with control, which resolved after completion of treatment.
(Supplementary Fig. S11E). Analysis of complete blood counts after 3 days of treatment revealed a thrombocytopenia after bortezomib and VPA/BTZ treatment, whereas leukocyte counts and hemoglobin levels remained unaffected (Supplementary Fig. S11F). This is in accordance with the known clinical risk profile of bortezomib. No significant differences in body weight and hematoxicity data were observed between bortezomib and combined treatment groups. Overall statistical analysis of events (death) indicated no significant differences between treatment and control groups (Supplementary Table S6), showing an enhancement of the therapeutic efficacy for the combined treatment without an increase in toxic effects in vivo.

Furthermore, tumor sections were stained for the expression of cleaved caspase-3 and hCD10 (leukemia cells; Fig. 5D; Supplementary Fig. S12B). After combined VPA/BTZ treatment, a significant increase in cleaved caspase-3 amount was observed, whereas application of single drugs showed no significant changes (Fig. 5D). To examine a possible effect on angiogenesis, the density of CD31+ microvessels (MVD) was quantified in tumor sections (Fig. 5D) (40). A significant decrease of MVD was observed after single treatment with VPA, which was more pronounced after combined treatment (Fig. 5D). The analysis of leukemia cell–associated fibroblasts indicated a decreased infiltration of ER-TR7 fibroblastoid cells in tumor tissue, as well as changes in reticular structures and extracellular matrix after combined treatment when compared with control samples (Supplementary Fig. S12C).

The synergistic effect of concomitant HDACi/BTZ treatment is maintained or even enhanced in the presence of chemotherapeutic agents

To establish a rationale for the integration of HDACi/BTZ combinations into current BCP-ALL treatment protocols, HDACi and bortezomib were combined with established ALL chemotherapeutic elements at a moderately effective dose level (Fig. 6; Supplementary Fig. S13A and S13B). These combinations were analyzed in 3 BCP-ALL cell lines, and the induction of cell death was measured by FACS. Combination of VPA/BTZ with the anthracyclines (mitoxantrone, idarubicin; Fig. 6B), cytarabine (Fig. 6C), or dexamethasone (Fig. 6D) significantly increased the antileukemic efficacy of the VPA/BTZ combination (Fig. 6A and D) as well as of single chemotherapeutic treatments. The induction of cell death was more pronounced in combination with anthracyclines, possibly due to the synergistic potential of the corresponding double combinations (Figs. 1B and 6A and B). Combination of VPA/BTZ with vincristine or methotrexate significantly increased the antileukemic efficacy of single chemotherapeutic treatments whereas the effect of VPA/BTZ remained unchanged (Fig. 6C). To further exclude possible adverse effects of VPA/BTZ on the efficacy of chemotherapeutic treatments, submaximal effective concentrations of cytostatic agents were combined with VPA/BTZ, showing no reduction and mostly a slight increase of cytostatic treatment efficacy (Supplementary Fig. S13C).

Discussion

This study shows that HDACi and the proteasome inhibitor bortezomib interact in a synergistic manner to induce apoptosis in BCP-ALL cells and to inhibit the expansion of leukemia cells in monocultures and cocultures with stroma cells. In addition, leukemia disease was significantly reduced upon combined treatment in vivo, which was associated with a decrease of leukemia cell infiltration in extramedullary tissues, reduction of angiogenesis and changes in the supportive stroma microenvironment. Synergistic induction of cell death required simultaneous application of both drugs, which is well applicable in the clinical setting.

Despite the relatively uniform synergistic induction of apoptosis by HDACi/proteasome inhibitors, the molecular pathways involved in the response to combined treatment are still not fully understood and vary between different malignancies (9, 10, 13). The synergistic induction of apoptosis in our models of BCP-ALL was associated with modulation of cell cycle, death receptor, JUN/MAPK, and PI3K/AKT pathways. These pathways have also been implicated either in combined or single agent activity of HDACi and proteasome inhibitors in other malignancies (9, 13, 16, 43, 44).

The differential regulation of p53 pathway genes such as BBC3 (PUMA), MDM2, CDKN1A (p21), and nuclear p53 accumulation indicated that concomitant treatment induced p53-dependent apoptosis. However, treatment with VPA alone induced CDKN1A gene expression and a G0–G1 phase cell-cycle arrest, whereas levels of p53 and its inhibitor MDM2 remained unchanged. This indicates a p53-independent CDKN1A regulation after VPA single treatment, which has also been reported (45). These findings are supported by previous reports showing that CDKN1A-induced cell-cycle arrest can result either in senescence or in apoptosis and thereby influences the therapeutic efficacy of antineoplastic treatments (45, 46). A possible explanation for the regulation of p53 and MDM2 only after concomitant treatment might be found in the ubiquitin/proteasome system, which plays a central role in the protein homeostasis of MDM2, p53, and various other-cell cycle regulators (47). Accordingly, after concomitant treatment, we observed complex changes in the gene expression of proteasome subunits, which might contribute to the stabilization of p53, MDM2, and regulation of NF-κB pathway (30). Moreover, the observed induction of ER stress signaling is in accordance with previous results, showing its involvement in p53 activation (48) and the synergistic induction of apoptosis after HDACi/proteasome inhibitor treatment (11, 12).

NF-κB activation has frequently been found to promote cancer cell survival and resistance to apoptosis (18, 30). Thus, the clinical activity of proteasome inhibitors such as bortezomib has, at least in part, been attributed to the inhibition of NF-κB activity (30). The differential regulation
of NF-κB target genes in our model indicated that this pathway is also involved in the treatment response of BCP-ALL cells. However, we observed an activation of particular NF-κB subunits (e.g., RelA) both after bortezomib single and concomitant treatment, although the differential regulation of apoptosis-related genes and synergistic induction of apoptosis was only observed after concomitant treatment. Our findings are consistent with recently published studies showing the tumor-suppressive role of the NF-κB signaling pathway, especially during chemotherapy-induced senescence that contributes to the outcome of cancer therapy (35–37). In our study, patients with BCP-

Figure 6. The synergistic effect of VPA/BTZ treatment is maintained or increased in combination with established ALL therapy elements. A, proportions of dead cells 48 hours after treatments as determined by FACS analysis of PI-stained cells. B to D, proportions of dead cells 48 hours after treatment either with 1 of 6 established ALL therapeutics at moderately effective concentrations, or double or triple combinations with VPA and BTZ. Results obtained after VPA/BTZ treatment, as shown in A, are presented in B and C as gray data points. Combined results from cell lines with similar sensitivity to chemotherapeutic agents are shown. Analyses from cell lines with different sensitivities and Annexin-V/PI FACS results after 24-hour single and triple combination treatments are provided in Supplementary Fig. S13A and B. Measurements from 1 to 2 independent experiments in each cell line with group medians. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (Mann–Whitney U test).
ALL who suffered a relapse during frontline treatment, which is generally associated with a poor outcome, showed lower expression of NF-kB subunits in leukemia cells. Nevertheless, we do not want to exclude a tumor-promoting role of NF-kB in BCP-ALL, depending on the context of its activation (49).

Only minor responses have previously been observed after single treatments with either bortezomib (14) or HDACi (5, 6) in phase I studies, revealing thereby a good tolerability. Bortezomib has recently shown favorable results in the treatment of children with relapsed ALL when combined with conventional chemotherapy (50). HDACi are promising candidates for combination with conventional chemotherapy, as they have a moderate and completely different toxicity profile. Our analyses revealed that the synergistic antileukemic effect of BTZ/VPA combination indeed was maintained or even increased in the presence of particular chemotherapeutic agents. However, these need to be carefully selected to avoid antagonistic effects of single agents such as methotrexate or PI3K/AKT inhibitors. The presented findings establish a biologic basis for the clinical evaluation of concomitant HDACi/BTZ treatment not only due to the synergistic potential of this combination but also because this enhanced therapeutic efficacy was achieved without an increase of toxic effects. In summary, our results provide new perspectives for considering combinations of bortezomib and HDACi as therapeutic alternative in childhood BCP-ALL.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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