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

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TRANSLATIONAL RELEVANCE

Cure rates for children with 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 HDACis. Proteasome inhibitors were found to increase the activity of HDACis in mature B-cell malignancies. In BCP-ALL, we identified bortezomib as a promising combination partner for HDACis 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/HDACis 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.
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 (HDACis) with a toxicity profile differing from that of conventional cytotoxic agents are needed for these extensively pre-treated patients.

Experimental Design: Anti-proliferative and pro-apoptotic effects of combined HDACi/proteasome inhibitor treatments were analyzed using BCP-ALL monocultures, cocultures with primary mesenchymal stroma cells from ALL-patients 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 (BTZ) as a promising combination partner for HDACis 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 BTZ 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 pro-apoptotic 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 to 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.
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 event free survival of 30% at ten years (1). New treatment approaches with a different toxicity profile in comparison to conventional cytotoxic agents are required for these extensively pre-treated patients. As a promising alternative, histone deacetylase inhibitors (HDACis) have been suggested (2-3). We have previously shown that HDACis are able to limit the expansion of B-cell precursor (BCP)–ALL cells in-vivo (4). The inclusion of HDACis into current ALL chemotherapy concepts requires the identification of suitable combination partners, since first clinical trials achieved only limited therapeutic responses (5-6), while establishing a good tolerance of HDACis in pediatric patients with malignant diseases. So far, only additive or even antagonistic interactions have been observed after combined treatment with HDACis and different conventional cytostatic drugs in myeloid leukemia and T-ALL cells (7). In BCP-ALL leukemia cells (LC), we have recently observed that the sequence of drug application determined synergistic or antagonistic responses to combined treatment with HDACis and methotrexate (8). Proteasome inhibitors such as bortezomib (BTZ) were found to increase substantially the activity of HDACis in different hematologic and solid malignancies (9-13). To date, no systematic studies have been performed to analyze the response of B-cell precursor leukemias to HDACi/proteasome inhibitor combinations. A well-tolerated BTZ dose level in childhood leukemia patients (14) and described additive interactions of BTZ with conventional cytostatics in-vitro indicate BTZ 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 \textit{in-vivo} and \textit{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, Germany). The cell lines were routinely tested for the expression of the corresponding surface markers and fusion genes by FACS, 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, Germany (ClinicalTrials.gov identifier: NCT00114348). Written informed consent was obtained from patients or guardians.

Substances

Bortezomib (BTZ, Janssen-Cilag International, Belgium) and valproic acid (VPA, in-vitro: Sigma-Aldrich, in-vivo: Desitin Arzneimittel, both Germany) were dissolved in isotonic saline or aqua, and Suberoylanilide hydroxamic acid (SAHA, Merck Pharmaceuticals, USA) in dimethyl sulfoxide (final concentration <0.02%). Unless not otherwise stated, the final drug concentrations were 1 mM for VPA (6), 1.2 µM for SAHA (23) and 10 nM for BTZ (14), as chosen according to clinically achievable plasma concentrations.

Gene expression analysis

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

**Mouse models**

Male NOD/SCID mice were injected either intravenously (i.v.) with LC derived from a pediatric BCP-ALL patient at first relapse (ALL-SCID2) or subcutaneously (s.c.) with Nalm6 cells (1+1 mixture with Matrigel) by transferring $1 \times 10^7$ LC/mouse (24). Animal experiments were approved by the local responsible authorities (G0221/03) and performed according to UICC guidelines (25).

**Combination effects and statistical analyses**

Student’s $t$-test, Mann-Whitney $U$-test and Kruskall-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 according to Chou and Talalay was determined (Calcusyn Software; Biosoft, Great Britain) 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 BTZ and HDACis has synergistic effects in BCP–ALL cells

To assess the antileukemic effect of combined treatments with HDACis and either established ALL-therapy elements (idarubicin, vincristine, cytarabine) or the proteasome inhibitor BTZ, BCP-ALL cell lines were exposed to the respective compounds either alone or in combination. LC proliferation and the induction of apoptosis were measured by MTS-assay and FACS analysis after Annexin-V/PI staining, respectively (Reh, Fig. 1A-B). After concomitant HDACi/BTZ treatment inhibition of proliferation and induction of apoptosis was markedly increased, as compared to BTZ alone (Fig 1A-B, left panels; Supplementary Fig. S1A). Combination index analysis identified the antileukemic activity of HDACi/BTZ combinations as synergistic (Fig. 1A-B, right panels). This synergistic effect was also observed, when serial dilutions of HDACis were combined with a constant concentration of BTZ (Supplementary Fig. S1B-D) and when apoptosis was assessed 24h and 48h after drug application (Supplementary Figure S1E).

The combined treatment of HDACis with idarubicin induced mostly synergistic interactions, which were less pronounced when compared to HDACi/BTZ combinations (Fig. 1A-B). The combinations of HDACis with vincristine or cytarabine were additive to antagonistic, depending on tested concentrations and cell lines (Fig. 1A-B). Similar results were obtained in Nalm6 cells (Supplementary Fig. S2A and Supplementary Table S1-2), revealing BTZ as the most promising combination partner for HDACis.

For further validation the amount of cell death in 697, SEM and SD-1 cells was analyzed after application of two different concentrations of BTZ with and without VPA at three time points. Fractional product analysis indicated synergism for the
majority of tested conditions (Supplementary Table S3). Additionally, primary BCP-ALL cells obtained from three patients at relapse diagnosis were exposed to VPA and BTZ treatments. Fractional product and combination index analysis demonstrated that concomitant treatment induced apoptosis and reduced the amount of vital CD19\(^+\) LC in either additive or synergistic manner (Supplementary Fig. S2B-D).

In order to evaluate the effects of VPA and BTZ treatments on normal B- and T-cells, PBMCs of three healthy adults, murine splenocytes and murine BM-MNC were analyzed in–vitro (Supplementary Fig. S3-5). 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 HDACis and methotrexate (8). Therefore, we analyzed whether the sequence of application has an effect on drug responsiveness to BTZ and HDACis. Simultaneous application of both drugs was required for the synergistic induction of cell death (Fig. 1C, Supplementary Table S4). In contrast, sequential applications of BTZ and HDACis induced in most cases apoptosis in a near-additive manner, with the exception of VPA followed by BTZ, which even resulted in antagonistic interactions. Antagonism was not observed in any of the tested concomitant combinations, thus confirming a broad synergistic activity of BTZ and HDACis in BCP-ALL cells, when applied concomitantly.

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

In order 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 BTZ as well as after concomitant treatment, compared to untreated control samples. The obtained results were further validated in four cell lines. We identified 919 genes differentially expressed exclusively after concomitant treatment (Supplementary Table S7-8). 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, MAPK, ER-stress response and protein ubiquitination were identified. Heatmap analyses for seven of these pathways and validations of the corresponding genes are shown in Figures 2-4 and Supplementary Figures S6-7.

Upregulation of pro-apoptotic genes, like caspases and TNFαR’s (TNF-related apoptosis-inducing ligand receptors) and downregulation of anti-apoptotic genes like BCL2, elucidate the synergistic pro-apoptotic 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 G0/1 phase arrest after treatment with VPA, whereas after treatment with BTZ 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 LC 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-D). CDKN1A mRNA level was increased after 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 two PI3K/AKT inhibitors (LY 294002, MK-2206) on LC response to VPA/BTZ treatment were further determined. Although, the treatment with PI3K/AKT inhibitors did not induce apoptosis in LC, the triple combination with VPA/BTZ showed clearly a decrease of apoptosis compared to VPA/BTZ treatment (Supplementary Figure S7A-E), 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 LC mostly through the activation of the PI3K pathway (28-29). VLA-4 expression was validated by QRT-PCR at two time points, showing that the VLA-4 mRNA expression decreased significantly after concomitant treatments (Fig. 3B and 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 Table
S7-8), both of which we have previously found to be regulated via VLA-4 signaling in LC (29).

Gene expression profiling further revealed the modulation of the protein ubiquitination pathway after application of BTZ either alone or in combination with VPA (Fig. 3C). BTZ 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 BTZ single treatment. Ubiquitin mRNA and protein level increased after BTZ treatment, which was even more pronounced after concomitant treatment (Fig. 3C-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, Fig. S6F), indicating the activation of the ER-stress / unfolded protein response pathway.

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

The pro-apoptotic effect of BTZ 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 BTZ 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 BTZ 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 IL-6, IL-8 and TLR7. However, from the five 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 9h after drug application (Fig. 4B, above, Supplementary Fig. S7E-F). Accordingly, the activation of four NFκB subunits (p65, RelB, p50, p52) was determined in three BCP-ALL cell lines 9h after single and combined treatments using DNA-binding-ELISA based analysis (Fig. 4B, below). A significant activation of p65 was observable after BTZ and concomitant treatments, which increased even more after concomitant treatment in two out of three 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 non-canonical pathway.

It has been assumed that tumor cells activate NF-κB signaling in response to chemotherapy as a pro-survival factor (34). In contrast, new findings (35-37) and our results indicate that NF-κB has also a tumor suppressive function. In ALL, 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 LC of BCP-
ALL patients 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 \( NF-\kappa B \) subunits in relevant clinical and biological subgroups of ALL-relapse (Supplementary Table S5). \( NF-\kappa B \) subunits expression (\( RelA \), \( RelB \), \( NFKB2 \), \( NFKB1 \)) in LC 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 \( NF-\kappa B \) 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 LC cocultures with MSC**

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

**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 LC 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), BTZ, VPA or VPA/BTZ, respectively (Supplementary Fig. S10A). Dosages and treatment schedules for xenograft experiments were determined within a prior dose finding study (Supplementary Figure S11A-C). 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). In order to study the ALL cell burden in the whole body, the amount of LC was analyzed in different lymphatic tissues (spleen and BM) by flow cytometry and in extramedullary non-lymphatic tissues (brain and testis) by QRT-PCR. Quantification of the LC burden in BM and spleen showed a significant reduction of the proportion of hCD19⁺hCD10⁺LC after combined treatment when compared to controls or to the single drug treatments (Fig. 5B, Supplementary Fig. S10D).

Treatment with BTZ alone had no significant impact on testis and brain involvement (Supplementary Fig. S10E-F). LC infiltration increased significantly after VPA treatment in testis and slightly in brain (Fig. S10E-F). This is in accordance with recently published results, showing, that HDACi treatments augment the migration and metastasis in different tumor models (41). The combined treatment significantly
reduced LC infiltration in testis, only when compared to VPA (Fig. S10E). Most importantly, the combined treatment significantly decreased the LC infiltration in brain, when compared to controls and to single VPA treatment (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 Figure 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 BTZ or VPA, which was more pronounced after combination treatment (Supplementary Fig. S12A). Body weight development indicated a significant weight loss in the BTZ and the VPA/BTZ treated groups compared to control, which resolved after completion of treatment (Supplementary Fig. S11E). Analysis of complete blood counts after three days of treatment revealed a thrombocytopenia after BTZ and VPA/BTZ treatment, while leukocyte counts and hemoglobin levels remained unaffected (Supplementary Fig. S11F). This is in accordance with the known clinical risk profile of BTZ. No significant differences in body weight and hematotoxicity data were observed between BTZ 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 (LC) (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 LC-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 to 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, HDACis and BTZ were combined with established ALL chemotherapeutic elements at a moderately effective dose level (Fig. 6, Supplementary Fig. S13A-B). These combinations were analyzed in three 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, 6D) 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 (Fig. 1B and 6A-B). Combination of VPA/BTZ with vincristine or methotrexate significantly increased the antileukemic efficacy of single chemotherapeutic treatments, while 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 demonstrates that HDACis and the proteasome inhibitor BTZ 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 LC 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 HDACis 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 G0/1 phase cell cycle arrest, while 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 anti-neoplastic 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
like BTZ 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 BTZ 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 demonstrating
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, BCP-ALL patients who suffered a relapse during
frontline treatment, which is generally associated with a poor outcome, showed lower
expression of NF-κB subunits in LC. Nevertheless, we do not want to exclude a
tumor promoting role of NF-κB in BCP-ALL, depending on the context of its activation
(49).
Only minor responses have previously been observed after single treatments with either BTZ (14) or HDACis (5-6) in phase-1 studies, revealing thereby a good tolerability. BTZ has recently shown favorable results in the treatment of children with relapsed ALL when combined with conventional chemotherapy (50). HDACis are promising candidates for combination with conventional chemotherapy since 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 biological 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 BTZ and HDACis as therapeutic alternative in childhood BCP-ALL.
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FIGURE LEGENDS

Figure 1. Concomitant combinations of HDACis and BTZ synergistically inhibit proliferation and induce apoptosis in BCP-ALL cell lines. A-B, Reh cells were treated for 72h with BTZ or chemotherapeutic agents alone or in combination with HDACis (VPA, SAHA). Shown are A, the inhibition of proliferation (MTS-assay) and B, the induction of apoptosis (Annexin-V/PI FACS), together with combination index analyses (right). Duplicates ±SD of 2-3 experiments. C, Induction of apoptosis in Reh cells after treatment with single drugs for 36h followed by 36h in fresh media or sequential combinations of 36h with the first and 36h with the second drug or concomitant combinations of both drugs for 72h. Triplicates ±SD of one experiment representative for Reh and Nalm6. ***-p<0.001, n.s.–not significant (Student’s t-test).

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 12h, 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. Shown are also the expression levels of those genes after single treatments. B, FACS analyses of DR-5 (TNFRSF10B) expression on Reh cells after treatments. Representative for two 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 DAPI. (magnification 400x). Shown are the corrected total cell fluorescence measurements of MDM2 expression in 30 nuclei ±SD and the presence of nuclear p53 after counting of 40-70 nuclei. **-p<0.01 (Mann-Whitney U-test).
Figure 3. Differential regulation of genes involved in PI3K/AKT/mTOR/MAPK and protein ubiquitination pathway after concomitant treatment. A, heatmap depicts differentially expressed genes involved in the PI3K/AKT/mTOR/MAPK–pathway. B, relative ITGA4 (VLA-4) mRNA expression in Reh cells after treatments. C, heatmap depicts differentially expressed genes involved in the protein ubiquitination pathway. D, above, SD-1 cells were stained with mAb against ubiquitin (green) and DAPI after treatment for 12 h (magnification 400x). graph: corrected total cell fluorescence measurements of ubiquitin expression in 30 nuclei ±SD; below, relative HSPA1A mRNA expression in Reh cells after treatment for 12h. Triplicates ±SD of 1-2 experiments, *-p<0.05, **-p<0.01, ***-p<0.001 (Student’s t-test). ABL1 was used as housekeeping gene for both QRT-PCR experiments.

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 mAB against phospho-p65 (bars: mean fluorescence intensity) at indicated time points. Results from Reh cells are shown. DNA-Binding activity of NF-κB subunits after treatments for 9h. Measurements from 1-2 experiments in each cell line with group medians. Median fold change >1.5 and p<0.05 was considered as statistically significant (Mann-Whitney U-test). C, Nalm6 cells were stained with a mAb against p65 and DAPI after treatments for 12h. Arrow indicates nuclear translocation of p65 (magnification 400x). Insert: mean proportions of nuclear p65+ cells after counting of 40-80 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).

**Figure 5.** Anti-leukemic activity of combined treatment in LC/MSC cocultures and BCP-ALL xenograft mouse models. A, cell death in Reh cells was analyzed by FACS in mono- and cocultures after treatments. MSC were isolated either from BM \( (n=3) \) or from testis \( (T\text{-MSC, } n=1) \) of BCP-ALL patients (details: Supplementary Figure S8). Results from independent experiments with group means (Student’s \( t \)-test). B, FACS analysis of hCD19\(^+\)CD10\(^+\)LC in whole spleen and BM of the i.v.-xenograft mouse model (details: Supplementary Figure S10). C, tumor volumes in the s.c.-xenograft mouse model on the final day of the experiment (details: Supplementary Figure S11-12). D, tumor sections were stained with mAbs either against cleaved caspase-3 and CD10 and DAPI or CD31 and DAPI (magnification 400x), apoptosis was measured as detailed in Supplementary Figure S12 and the MVD was quantified by counting CD31\(^+\) microvessels within representative 400x magnification fields. Measurements of individual mice with group medians. (Mann-Whitney \( U \)-test). \( *-p<0.05, **-p<0.01, ***-p<0.01 \).

**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 48h after treatments as determined by FACS analysis of PI stained cells. B-D, proportions of dead cells 48h after treatment either with one of six 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 panel A, are presented in panel B-C as grey 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 24h single and triple combination treatments are provided in Supplementary Fig. S13A-B. Measurements from 1-2 independent experiments in each cell line with group medians, *-p<0.05, **-p<0.01, ***-p<0.01 (Mann Whitney-U test).
### A: Cell cycle regulation and death receptor pathway

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### B: DR-5 (TNFRSF-10B) fluorescence intensity

- Control (MFI: 8.42)
- VPA (MFI: 7.65)
- BTZ (MFI: 9.39)
- VPA+BTZ (MFI: 13.45)

### C: p53 - signaling

### D: MDM2 expression (CTFC)

- Control
- VPA + BTZ

### E: p53+ cells (% of total)

- Control
- VPA + BTZ

**Significance:**

- **: p < 0.05
- ***: p < 0.01

**Note:**

- Blue color indicates lower expression, red color indicates higher expression.
- Cell cycle regulation and death receptor pathway images show differential expression patterns.
- p53 signaling images illustrate specific signaling pathways in different treatments.
Bastian et al. Figure 3

PI3K/AKT/mTOR- and MAPK - pathway

Control

BTZ

VPA

BTZ + VPA

relative expression of HSPA1A mRNA

relative expression of VLA-4 mRNA

protein ubiquitination pathway

D

control

BTZ + SAHA

UBE2C

CDC20

PSMB8

UCHL1

USP6

HSPA6

HSPA8

PSMB2

PSMC6

PSMA1

PSMC1

PSMD12

PSMA3

PSMD7

PSMD4

PSMB4

UBC

UBE4B

USP45

HSPA5

DNAJB6

DNAJC3

UBE2H

USP30

IFNGR1

DNAJB9

DNAJC18

HSPA1A
A. **NF-κB - signaling**

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| *p65 (RelA) mean fluorescence intensity (MFI)*

B. **Relapse on treatment**

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C. **Control vs VPA + BTZ**

D. **RelA (p65)** and **RelB** mRNA expression

- **RelA (p65)**: **p**
- **RelB**: *
Figure 5

A) MSC-1, MSC-2, MSC-3, T-MSC-3

B) i.v. - mouse model

C) s.c. - mouse model

D) control, BTZ + VPA

cl. Casp-3
CD10
DAPI

CD31
DAPI
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, et al.

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