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Anti-tumor activity of the combination of bendamustine with vorinostat in diffuse large B-cell lymphoma cells

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Abstract
Current standard-of-care therapy for diffuse large B-cell lymphoma (DLBCL) results in up to 40% of patients who either relapse or develop refractory disease. In this setting, further therapeutic improvements are needed. This study analyzed the in vitro effects of the combination of bendamustine with the histone deacetylase inhibitor vorinostat in DLBCL cells. This combination enhanced histone acetylation and double strand DNA breaks resulting in an additive to synergistic cytotoxic effect in both ABC- and GCB-type DLBCL cells, independently of their TP53 mutational status. These results support the rationale for considering bendamustine and vorinostat combination as a novel approach in DLBCL treatment.

Keywords: Bendamustine, vorinostat, diffuse large B-cell lymphoma, TP53, histone deacetylase inhibitor

Introduction
Diffuse large B-cell lymphoma (DLBCL) is an aggressive form of non-Hodgkin lymphoma (NHL) that comprises different entities with specific genetic alterations and heterogeneous clinical outcome. Gene expression profiling revealed three different molecular sub-types based on cell of origin: germinal center B-cell-like DLBCL (GCB-DLBCL), activated B-cell-like DLBCL (ABC-DLBCL) and mediastinal or unclassified type [1]. Although most patients respond to standard treatment with immunochemotherapy (e.g. R-CHOP), 40% remain refractory or relapse, especially within the ABC sub-group [2]. Our increasing knowledge of tumor biology and the identification of targetable pathways can enable the exploration of more effective treatment strategy approaches in DLBCL.

Bendamustine hydrochloride (BEN) was developed as a molecule with alkylator and antimetabolite structure. Compared to other conventional alkylators, such as cyclophosphamide, BEN has a unique mechanistic profile that includes induction of single and double-strand DNA (dsDNA) breaks, activation of DNA damage stress response and apoptosis, inhibition of mitotic checkpoints, and induction of mitotic catastrophe [3]. Moreover, BEN shows limited cross-resistance to other alkylating agents and has demonstrated clinical activity as single-agent therapy or in combination with other anti-neoplastic agents in relapsed indolent NHL, chronic lymphocytic leukemia (CLL) and multiple myeloma (MM), including patients refractory to alkylating or purine analog agents [4,5]. At the present time, there is limited data available on the efficacy of BEN in aggressive lymphomas, including DLBCL [6-9].

Vorinostat (VOR) is a histone deacetylase inhibitor (HDACi). HDACis regulate histone and non-histone protein acetylation, playing a critical role in the modulation of gene expression. Although their specific mechanism of action is still unclear, HDACis exert several anti-tumor effects, including induction of apoptosis and differentiation, cell cycle arrest, regulation of tumor immunology, tumor suppressor gene transcription and angiogenesis inhibition [10]. VOR has been approved for the treatment of cutaneous T-cell lymphoma [11] and is currently under study in other types of lymphoma [12-15]. Single-agent vorinostat has shown modest activity in relapsed/refractory indolent NHL (overall response rate, ORR = 29%), although higher response rates were achieved in patients with follicular histology (ORR = 47%). In aggressive B-cell lymphomas, a phase II trial has shown very modest activity for single-agent vorinostat in patients with relapsed DLBCL, with only 6% of cases responding [15]. Pre-clinical studies have found that HDACis exert their broadest activity in combination with other agents, especially when these combinations are based on the rationale of their mechanistic interaction [16-18]. Thereby, clinical trials using vorinostat
plus rituximab or other novel targeted agents have been carried out in some lymphoma types [19,20].

The present study was designed to explore in vitro the interaction of BEN and VOR in DLBCL. We hypothesized that, besides its wide anti-tumor effects, VOR would promote an open chromatin conformation that might allow extensive BEN accessibility, potentially enhancing the direct DNA damage. To assess this approach, we used two GCB-type and two ABC-type DLBCL cell lines; one of each type harbored known pathogenic TP53 mutations, which affect 20% of DLBCL patients and are an independent prognostic factor in DLBCL [21].

Materials and methods

Cell lines and primary cells

The DLBCL cell line OCI-Ly19 (GCB, TP53mut) was purchased from the DSMZ (Braunschweig, Germany). The SU-DHL-6 (GCB, TP53mut), RC-K8 (ABC, TP53mut) and U-2932 (ABC, TP53mut) were kindly provided by Dr D. Dominguez (Columbia University, New York, NY). Primary cells from a patient with GCB- and from another with ABC-DLBCL (according to Hans’ algorithm [22]) were collected by heparinized-RPMI perfusion of fresh neoplastic lymph node biopsies and were stored cryopreserved in liquid nitrogen until use. Both patients gave their signed informed consent. Biological samples were obtained from Parc de Salut MAR Biobank (MARBiobanc; Barcelona, Spain). Cell lines and primary cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μM penicillin-streptomycin (GIBCO, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Main characteristics of all cell lines and primary cells are summarized in Table I.

Chemicals

VOR (suberoylanilide hydroxamic acid, SAHA) was purchased from Selleck Chemicals (Houston, TX) and BEN was provided by Levact (Mundipharma Pharmaceuticals, Cambridge, UK). VOR was dissolved in 100% dimetil sulfoxide (DMSO) to a stock concentration of 50 mM and BEN was dissolved in physiological saline at 5 mg/mL.

Analysis of cell viability and apoptosis by flow cytometry after drug exposure

Cell lines were incubated (0.5–1 × 10⁶/mL) with different concentrations of BEN (25–100 μM) and/or VOR (0.3–5 μM).

Cell viability and apoptosis were evaluated by annexin-V staining; the 50% inhibitory concentration (IC₅₀) was defined as the concentration of drug required to reduce cell viability by 50%. Briefly, after 24 and 48 h, cells were washed with annexin binding buffer (100 mM HEPES, 280 mM NaCl and 5 mM CaCl₂, pH 7.4), resuspended in 400 μL of fresh buffer and labeled for 15 min in the dark with annexin-V-FITC (eBioscience, San Diego, CA) at a 1:200 final dilution. The DAPI nucleic acid stain (Invitrogen, Carlsbad, CA) was added (1:4000) at the time of the analysis. Each condition was assessed in duplicate and data were confirmed by at least three independent experiments.

Primary cells were analyzed at 24 h. For cell population selection, anti-CD3 (1:200) and anti-CD19 (1:40) antibodies (Becton Dickinson, Franklin Lakes, NJ) were used.

For each analysis, at least 10 000 events were recorded with a BD LSR II flow cytometer (Becton Dickinson). The double-negative population were considered viable cells, while both annexin-V-positive/DAPI-negative and annexin-V-positive/DAPI-positive populations were considered apoptotic committed cells.

Western blot analysis

Proteins were extracted in RIPA buffer (Sigma, St. Louis, MO) and quantified using Bradford assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein were separated on 10–15% SDS-PAGE gel and transferred to Immobilon-P PVDF membranes (Millipore, Hayward, CA) and incubated with primary antibodies for anti-acetyl-histone H3 (06-599; 1:20000, Millipore), anti-phospho-H2A.X (07-164, 1:500, Millipore), anti-p53 (NCL-L-p53-DO; 1:1000, Novoceastra, Newcastle, UK), anti-poli (ADP-ribose) polymerase (PARP, 11 835 238 001; 1:2000, Roche, Switzerland) and α-tubulin (CP06; 1:200, Millipore) (used as internal control). Appropriate HRP-conjugated secondary antibody (P0260 or P0448; 1:2000; DAKO, Glostrup, Denmark) was used to reveal. Signals were developed with enhanced chemiluminiscence (Pierce, Rockford, IL).

Quantitative real-time PCR analysis of p21WAF1/CIP1 gene expression

After 48 h of treatment, total cellular RNA from 0.5 × 10⁶ cells was extracted using Trizol (Invitrogen). High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used for reverse transcription (RT) reactions following the manufacturer’s protocols. Primers and probes were

Table I. Characteristics of DLBCL cell lines and primary cells.

| DLBCL cells       | Phenotype | TP53   | Bendamustine sensitivity 48 h IC₅₀ (μM) | Vorinostat cytotoxicity 48 h 2.5 μM (%) | Bliss independencea |
|-------------------|-----------|--------|----------------------------------------|----------------------------------------|---------------------|
| OCI-Ly19          | GCB       | wtb    | 5                                      | 53.3                                   | –0.020 ± 0.188      |
| SU-DHL-6          | GCB       | p.Y234Y/C | 75                                    | 61.7                                   | 0.107 ± 0.030       |
|                  |           | p.R306L |                                        |                                        |                     |
| RC-K8             | ABC       | wt      | 100                                    | 32.5                                   | 0.072 ± 0.054       |
| U-2932            | ABC       | p.C176Y | 50                                     | 31.1                                   | 0.041 ± 0.066       |
|                  |           | p.L344L/P |                                       |                                        |                     |
| Tumor sample #1   | GCB       | wt      | 25c                                    | 41.5c                                  | –                   |
| Tumor sample #2   | ABC       | wt      | 25c                                    | 59.6c                                  | –                   |

a Difference between observed and predicted cytotoxic effect according to the Bliss model and confidence interval.

b Wild type.

c 24 h of drug exposition.
purchased from Applied Biosystems (Hs00355782_m1) and analyzed using a 7900 HT Real-Time PCR System (Applied Biosystems). GUS, β-actine and beta-2-microglobuline were used as internal controls. Relative quantification was calculated using $2^{-\Delta\Delta Ct}$.

**Cell proliferation assay**

Cells were harvested into 96-well plates ($0.1 \times 10^6$ per well) and treated with different doses of BEN (5, 10, 25, 50, 100 μM) and VOR (0.5, 1, 2, 2.5, 5 μM) alone and in all possible combinations. The CellTiter proliferation assay (Promega, Madison, WI) was used according to the manufacturer’s instructions. The absorbance was measured at 490 nm with an Infinite 200 PRO microplate reader (Tecan, Durham, NC).

**Statistical analyses**

Means were compared between two groups using a 2-sided Student t-test, using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). The proliferation and apoptosis data were shown as mean ± SEM of three independent replicates. To study the interaction between the two drugs we used the Bliss independence model. Bliss independence is the index for calculating the expected dose–response relationship for drug combination therapy as compared to monotherapy, focusing on treatment effect enhancement. Given the two drugs BEN and VOR, both inhibiting tumor growth:

BEN at dose $b$ inhibits $Y_b$ % of tumor growth and VOR at dose $v$ inhibits $Y_v$ % of tumor growth. If BEN and VOR work independently, the combined percentage inhibition $Y_{b+v}$ can be predicted using the complete additivity of probability theory as $Y_{b+v} = Y_b + Y_v - Y_{b}Y_{v}$. The interaction of each combination of the two drugs can be described by calculating the difference between the predicted percentage of growth inhibition, $Y_{b+v}$ and the observed percentage of growth inhibition, $Y_{b+v,O}$. Therefore, Bliss synergy and antagonism were concluded when $\Delta Y$ and its 95% confidence interval (CI) were >0 and <0, respectively, and in the case where the 95% CI of $\Delta Y$ would include 0, the conclusion was Bliss independence or additive interaction [23].

**Results**

**BEN and VOR individually exert a cytotoxic effect in DLBCL cell lines**

OCI-Ly19 (GCB, $TP53^{wt}$), SU-DHL-6 (GCB, $TP53^{mut}$), RC-K8 (ABC, $TP53^{wt}$) and U-2932 (ABC, $TP53^{mut}$) cells were exposed during 48 h to increasing doses of BEN or VOR. Concentrations of BEN ranging from 25–100 μM and of VOR ranging from 0.3–2.5 μM were used. IC$_{50}$ values were calculated from data obtained from the annexin-V assay performed by flow cytometry (Figure 1 and Table I). For all cell lines, both BEN and VOR alone caused a decrease in cell viability in a

Figure 1. Bendamustine and vorinostat exert cytotoxicity in GCB and ABC-DLBCL cells, independently of their $TP53$ mutational status. U-2932, RC-K8, SU-DHL-6 and OCI-Ly19 cells were treated with increasing concentrations of bendamustine (5, 25, 50, 100 μM) or vorinostat (1, 2.5, 5 μM) during 48 h. Cells were analyzed by FACS following staining with annexin-V/DAPI. The percentage of viable cells was normalized considering untreated control cells viability at 48 h as 100%.
dose-dependent manner. Each cell line showed a different sensitivity to BEN, although no association was found with DLBCL sub-types, whereas GCB-DLBCL cell lines showed a higher cytotoxic response to VOR than ABC-DLBCL sub-type ($p < 0.001$).

**BEN and VOR in combination treatment**

For the combination assays, the $IC_{50}$ of BEN and a fixed dose of VOR of 2.5 $\mu$M were used. $IC_{50}$ concentrations and achieved toxicity for each cell type are listed in Table I. Both drugs were added simultaneously. As shown in Figure 2a, the addition of VOR resulted in a significant increase of cytotoxicity compared to that obtained with BEN alone. The Bliss model for drug combination study indicated that the cytotoxic effects were slightly synergistic for the SU-DHL-6 and the RC-K8 cell lines and additive for the other two cell lines at the doses tested (Table I).

**Effect of the sequence of treatment**

To explore a potential enhanced cytotoxic effect of pretreating cells with VOR, we assessed different sequences of treatment. However, the addition of BEN 24 or 72 h after VOR did not increase the cytotoxic effect compared to the concomitant exposure to both drugs (data not shown).

**Effects in primary cells**

The effects of BEN and VOR, alone and in combination, were studied in the sub-population of lymph node B cells (CD3−, CD19+) from two DLBCL (one GCB and one ABC) patients. At 24 h, both BEN and VOR showed a dose-dependent effect on cell viability. The drug combination induced a higher cytotoxic response comparing with the individual effects of BEN and VOR alone drugs, but no significant differences in cytotoxicity were observed according to ABC/GCB sub-type (Figure 2b).

![Figure 2](image-url)
dsDNA breaks and apoptosis induction

To study the ability of BEN and VOR to induce DNA damage, H2A.X phosphorylation was analyzed as an indirect marker of dsDNA breaks. As shown in Figure 3, no signal was detected in untreated cells after 48 h incubations, whereas both BEN and VOR induced dsDNA breaks. When cells were treated with the combination of both drugs, a 1.6–5.5-fold increase in H2A.X phosphorylation was seen compared to BEN exposure alone.

The enzymatic cleavage of PARP protein, a classic marker for caspase 3-induced apoptosis, was evaluated by western blot (Figure 3). After 48 h of BEN and/or VOR treatment, the 85-kDa corresponding to cleaved PARP was detected. PARP cleavage was slightly higher after BEN treatment than after the combination treatment.

Histone H3 acetylation

Since histone H3 is one of the main substrates of HDACis, H3 acetylation was assessed by western blot. Hyperacetylation of histone H3 was observed following VOR treatment (Figure 3). Interestingly, BEN alone induced only weak detectable levels of acetylated H3 in two cell lines, whereas the combination of BEN and VOR resulted in a 2.7–5.6-fold increase of histone H3 acetylation in three out of four cell lines compared to VOR treatment alone.

p53 and p21 expression

In the western blot study, after BEN treatment, a 1.3- and a 3.3-fold increase of p53 were observed in the OCI-Ly19 and RC-K8 TP53-wild-type cells, respectively (Figure 4a), whereas VOR decreased p53 levels in all cells. After the combination
treatment, only the RC-K8 cells showed a 2.3-fold increase of p53, while the other cells showed slightly decreased p53 expression. We next examined p21WAF1/CIP1 expression by quantitative PCR. BEN treatment triggered a notable increase in p21WAF1/CIP1 in both TP53-wild-type cells OCI-Ly19 and RC-K8, matching the p53 results. VOR treatment enhanced p21WAF1/CIP1 expression in the two GCB-sub-type cell lines, OCI-Ly19 and SU-DHL-6, while it remained invariant in ABC-sub-type cells. After the BEN-plus-VOR combination treatment, p21WAF1/CIP1 increased in the OCI-Ly19 and SU-DHL-6 cells and slightly in the RC-K8 cells.

Discussion

As a complex multi-hit disease, DLBCL shows a wide range of clinical and molecular features that result in heterogeneous clinical responses. Distinct pathogenic pathways have been described to be altered in these patients, supporting the efforts to explore new drug combination strategies [24]. Phase I and II clinical trials in DLBCL patients containing BEN [8,9,25,26] or VOR [14,15] have been reported, showing diverse responses. Despite BEN showing promising results, VOR in monotherapy has shown limited activity in DLBCL patients. However, there are no clinical trials using the combination of both drugs and the mechanisms of action of both drugs alone or in combination have been poorly explored in DLBCL [3,4,16,27–31].

In our study, we have analyzed the cytotoxic effects of BEN and VOR as single agents and in combination in DLBCL cells and we have studied the mechanisms involved in this process. At clinically achievable concentrations, both BEN and VOR exerted single-agent cell killing activity in a dose- and time-dependent manner against GCB and ABC-DLBCL cell lines as well as in patients’ cells in ex vivo cultures. Interestingly, GCB-type cell lines were more sensitive to VOR than ABC-type cells, whereas no difference in sensitivity was observed to BEN treatment. This differential response to VOR is opposed to the results obtained in a prior report using the pan-HDACi belinostat with some of the same cell lines used in our study, which found that cells’ sensitivity did not depend on the DLBCL-sub-type [32]. Hyperacetylation of NF-κB in response to HDACis has been proposed to cause an anti-apoptotic effect [10,16], which would explain the lower sensitivity to VOR in ABC-DLBCL cell lines, that are known to depend on the constitutive activation of the NF-κB pro-survival factor, in contrast to the GCB-DLBCL type [33].

Novel targeted therapies such as BCR inhibitors are known to affect ABC-DLBCL cell by affecting the NF-κB signaling pathway. For instance, ibrutinib, a BTK-inhibitor, in combination with VOR has shown a synergistic effect in mantle cell lymphoma cell lines [19]. Moreover, VOR potentiates the activity of carfilzomib, a proteasome inhibitor, in GC- and ABC-DLBCL cells, including bortezomib-resistant cells [16]. However, the activity of BEN in combination with VOR in DLBCL sub-types is limited and deserves further investigation. Other specific agents such as monoclonal antibodies against CD20 can be used to target B-cell lymphomas. Of interest, a phase 2 study using a combination of rituximab plus VOR has shown promising efficacy in patients with indolent non-Hodgkin lymphoma [20]. This combination might also be useful for patients with DLBCL, but clinical trials are still ongoing.

In order to explore the contribution of an HDACi to BEN mode of action, a fixed dose of VOR was added. The addition of a 2.5 μM dose of VOR [34] to the BEN treatment of DLBCL cell lines resulted in a significantly higher toxicity compared with BEN treatment as a single-agent (Figure 1). This increased toxic effect was synergistic in two cell lines (SU-DHL6 and RC-K8), while it was more likely to be independent or additive in the others, according to the Bliss independence model. Apart from their single-agent antitumor effects, HDACis act in remodeling chromatin to a loosen conformation [10], potentially making the DNA more accessible to damaging agents. Here, we show a striking increase of phosphorylated-H2A.X (P-H2A.X) levels when co-treating cells with BEN and VOR, thus demonstrating enhanced DNA damage [35]. Regarding this, prior reports have provided evidence about the importance of the sequence of treatment when combining HDACis with DNA-damaging agents [36–39], but in our study VOR pre-treatment exposure did not enhance the BEN cytotoxic effect compared to the addition of both drugs concomitantly, in accordance with a previous study in leukemia cells of our group [40]. On the other hand, in this study, we have demonstrated that the ability of VOR to promote acetylation was enhanced when cells were treated together with BEN, even though BEN alone showed a very weak acetylation effect. A recent study reported the synergistic histone acetylation when treating lymphoma cells with bendamustine and the HDACi romidepsin together, leading to an increased toxicity, but the underlying mechanisms behind this effect are still unknown [41]. Common alterations affecting genes that play a role in epigenetic regulation have recently been described in DLBCL, such as those affecting the HATs CREBBP and EP300, that are known to be mutated in 32% DLBCLs [42–44]. Mutant forms of these two genes have been shown to cause an impaired acetylation that may contribute to lymphomagenesis by over-expression of the main DLBCL-related oncogene BCL6 and inactivation of p53 [45,46]. Inactivating mutations in EP300 and/or CREBBP are known to affect the HAT function of SU-DHL-6 and OCI-Ly19 cells, whereas RC-K8 presents a well characterized rearrangement in EP300 [45,47]. As we have demonstrated in this study, VOR and, more powerfully, BEN plus VOR combination enhanced histone acetylation in these defective cells. Thus, the use of VOR and BEN combined could potentially revert this situation as part of their antilymphoma mechanisms.

One fifth of all DLBCL patients harbor somatic mutations in the TP53 gene, which correlate with poor survival to R-CHOP standard treatment, even within GCB and ABC subtypes [21]. The p53 protein is a crucial tumor suppressor that mediates cell-cycle arrest, DNA repair, apoptosis, senescence and autophagy in response to cellular stress [48]. In order to explore the role of the TP53 mutational status in the BEN and VOR combination treatment, two out of the four cell lines used in this study carried deleterious TP53 mutations. In our case, TP53 mutational status did not correlate with differences in viability and apoptosis after BEN and VOR exposure,
which implies that both drugs are effective in DLBCL cells independently of the TP53 mutational status. However, p53 protein levels were differentially modulated according to TP53 status and to the cytotoxic treatment, revealing that different mechanisms of apoptosis may be driving the cytotoxic effect in each situation. BEN exposure resulted in an increase of p53 protein levels and p21 upregulation only in the TP53-wild type cell lines, meaning that the cytotoxicity observed in cells with a non-functional p53 involved p53-independent mechanisms, such as the previously postulated mitochondrial oxidative stress or mitotic catastrophe [3,49]. On the other hand, it is well described that wild type p53 is not necessary for HDACis induced apoptosis [50], since cell cycle arrest through p53-independent induction of p21 is a major mechanism of VOR induced cytotoxicity [10,34,51]. In our experiments, VOR decreased the expression p53 in all cases, but only GCB cells had increased p21 expression, further correlating with the higher cytotoxic achieved effects. Intermediate amounts of p53 and cleaved PARP were seen in the BEN plus VOR combination treatment, corroborating that HDACis and p53-activating agents may cause p53 antagonistic regulation in wild type-p53 cells, rather than increase the antitumor efficacy [50]. However, increased levels of p21 expression were maintained and DNA damage levels, histone acetylation and apoptosis were enhanced, meaning that opposed regulation of p53 is overcome by these and potentially other mechanisms. Published data indicate that sensitivity of NHL cells to treatment with HDACis is dependent on the complex regulation of BCL-2 antiapoptotic family members, that includes the accumulation of MCL-1 as well as other inhibitors of apoptosis and the downregulation of BCL-X, among others [31,52,53]. The accumulation of antiapoptotic regulators would cause a decrease in the apoptosis levels, whereas the drop of BCL-X after HDACi treatment could explain the reduction of p53 and cleaved PARP without compromising cytotoxic effects.

In summary, we have demonstrated that the pan-HDAC inhibitor VOR potentiates the BEN-induced cytotoxicity in both GCB and ABC-DLBCL cells, independently on their TP53-mutational status. Given the favorable toxicity profiles of both drugs, their distinctive mechanism of action and the favorable perspective of new drug combination approaches, the combination of BEN and VOR may be promising for the management of DLBCL patients with relapsed or refractory disease, even those with TP53 mutations.

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