The combination of bortezomib with enzastaurin or lenalidomide enhances cytotoxicity in follicular and mantle cell lymphoma cell lines

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Abstract

We analyzed the combination of a proteasome inhibitor (bortezomib) with enzastaurin (PKC/AKT-inhibitor) or lenalidomide (immunomodulatory agent) for the inhibition of proliferation and induction of apoptosis in B-cell lymphoma cell lines and primary malignant cells. The effects of bortezomib, enzastaurin or lenalidomide, alone or in combinations, on cell viability and apoptosis were evaluated using the Cell Proliferation Kit and flow cytometry analysis. The interaction between drugs was examined by the Chou–Talalay method. Cell cycle analysis was performed by flow cytometry. The PI3K/AKT, PKC and MAPK/ERK signaling pathways were analyzed using western blot. Bortezomib with either enzastaurin or lenalidomide synergistically induced anti-proliferative and pro-apoptotic effects in B-cell lymphoma cells, even in the presence of the bone marrow microenvironment. The direct cytotoxicity is mediated by signaling events involving the PI3K/AKT, PKC and MAPK/ERK pathways leading to cell death. The significant increase of apoptosis was mediated by an increased ratio of pro-apoptotic proteins (Bax, Bad and Bim) to anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1), triggering the cleavage of caspases -3, -9, -8 and PARP. Further evaluation of the combination of bortezomib with enzastaurin or lenalidomide for the treatment of B-cell lymphoma is warranted, with the goal to improve the quality of life and survival of patients. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: apoptosis; B-cell lymphoma; bortezomib; combined therapy; enzastaurin; lenalidomide

Introduction

Treatment of patients with non-Hodgkin’s lymphomas (NHL) has significantly improved over the last decade, especially since the discovery of monoclonal antibodies and other biologic therapies [1–4]. Bortezomib is the first in class of proteasome inhibitors that targets the critical process of intracellular protein degradation or recycling [5,6]. Bortezomib has been approved by the U.S. Food and Drug Administration (FDA) for multiple myeloma (MM) [7,8] and recently, for relapsed or refractory MCL [9]. Through the inhibition of the 26S proteasome and subsequent effect on multiple key cellular pathways, bortezomib has shown an increase and/or synergistic activity with several novel target agents [10,11]. Bortezomib has been used in combination with other molecules in more than 300 clinical trials, 23 of which focused on indolent or aggressive NHL [12].

Enzastaurin is an adenosine triphosphate (ATP)-competitive inhibitor of classic (α, β, γ and δ) protein kinase C (PKC) isoforms, and has anti-proliferative activity [13,14]. Enzastaurin has many effects on different signaling pathways in cancer cells; it suppresses the PI3K/AKT/mTOR pathway [13–16], regulates the MAPK pathway [17] and seems to be involved in the interferon-regulated JAK/STAT pathway [18]. Furthermore, enzastaurin has demonstrated an activity in hematologic malignancies, in both indolent or aggressive lymphomas, with minimal hematologic toxicity in a phase II clinical trial [19,20]. Lenalidomide is an immunomodulatory drug (IMiD) that is derived from thalidomide, with enhanced potency and different toxicity, in comparison to its parent molecule. This agent has shown impressive clinical activity in patients with MM [21], and it proved to be effective in chronic lymphocytic leukemia (CLL) and T-cell lymphoma [22,23]. Preclinical models and preliminary clinical data also indicate significant antitumor activity of lenalidomide in other B-cell malignancies [24,25]. Phase I/II clinical trials are evaluating the therapeutic potential of lenalidomide alone or in combination with other antitumor drugs for the treatment of NHL. We hypothesized that combining a proteasome inhibitor with enzastaurin, an inhibitor of PI3K/AKT, or lenalidomide, an immunomodulatory agent that targets the microenvironment, will target
multiple signaling pathways in B-NHL cells, ultimately enhancing tumor cell death. Our results demonstrate that the combination of bortezomib with either enzastaurin or lenalidomide at low doses induces synergistic anti-proliferative and pro-apoptotic effects in both B-cell lymphoma cell lines and primary cells.

Materials and methods

Cell cultures and patient samples

B-NHL cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) and included two follicular lymphoma (FL) cell lines WSU-NHL and RL and two MCL cell lines: GRANTA-519 and JEKO-1. B-NHL cell lines, with the exception of GRANTA-519, were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin. This study protocol was approved by the local ethics committee of University of Modena and Reggio Emilia.

Peripheral blood mononuclear cells (PBMCs) were obtained from three healthy volunteers, three patients with FL (CD19+/CD5+ cells), and two patients with MCL (CD19+/CD5+ cells) and were isolated using anti-CD20 magnetic microbeads (Miltenyi Biotec). Obtaining a purity of >80% was assessed by flow cytometry. Bone marrow aspirates were obtained from three patients with FL and two patients with MCL. The leukemic phase in the patients with FL (CD19+/CD5− cells) and MCL (CD19+/CD5+ cells) was diagnosed by microscopic analysis, and was confirmed at greater than 80% by flow cytometry. Bone marrow (BM) aspirates were obtained from three patients with FL. BM aspirates were isolated using anti-CD20 magnetic microbeads (Miltenyi Biotec), obtaining a purity of >90%, as assessed by flow cytometry. PBMCs and CD20+ cells were cultured in RPMI-1640 with 20% FBS and 100 U/mL penicillin and streptomycin. This study protocol was approved by the local ethics committee of University of Modena and Reggio Emilia.

Drugs

Bortezomib, enzastaurin and lenalidomide were purchased from Selleck Chemical. The drugs were dissolved in dimethylsulfoxide (DMSO), and stored at −20°C until use. In all experiments, the final concentration of DMSO did not exceed 0.1%.

Cell viability and cell cytotoxicity

Cell viability was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Cell cytotoxicity was evaluated by MTT assay (Promega). IC50 values of each drug were calculated from curves based on bortezomib concentrations (1–25 nM), enzastaurin (1–10 μM) and lenalidomide (1–100 μM) after 24 and 48 h. For the combination studies, B-NHL cell lines were cultured for 24–48 h with fixed doses of bortezomib (1, 4 and 6 nM), enzastaurin (1, 4 and 6 μM) or lenalidomide (1, 4 and 6 μM). The isobologram analysis for the combination studies was based upon the Chou–Talalay method to determine combination indices (CI) [26].

Based on the results of the MTT assay, the combination studies were performed at 24 h for all experiments using the following doses: 6 nM of bortezomib and 6 μM of enzastaurin or lenalidomide, corresponding to the concentrations at peak inhibition of proliferation.

Co-culture of B-lymphoma cell lines with bone marrow mesenchymal stem cells

BM-MSCs (5 × 10^3) were seeded in triplicate onto 96-well plates and incubated for 48 h to reach confluence. After 48 h, B-NHL cell lines were then seeded at 2 × 10^4 cells/well in the presence or absence of BM-MSCs. The next day, cells were treated with bortezomib, enzastaurin and lenalidomide, alone or in combination. Non-adherent cells were collected at 24 and 48 h after addition of the drugs, and cell viability was evaluated.

Annexin V staining and caspase-8 assay

Apoptosis was quantified using the Annexin V-FITC and propidium iodide (PI) binding assay (Miltenyi Biotec), and then analyzed by flow cytometry (Coulter Epics XL MCL) and EXPO32 ADC v.1.1 C software. Caspase-8 activity was tested by a colorimetric assay (Chemicon).

Cell cycle analysis

Cells were harvested, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol at 4°C overnight. The fixed cells were washed in PBS, resuspended in citrate buffer and stained with PI (1 mg/mL; Sigma) containing RNase A (1 mg/mL; Sigma) for 30 min at 37°C. DNA content was determined by flow cytometry (Coulter Epics XL MCL), and cell cycle distribution was calculated using EXPO32 ADC v.1.1 C software.

Analysis of bcl-2 expression

Cell lines were processed for fixation and permeabilization, using the BD Cytofix/Cytoperm™ Kit (BD Biosciences). Cells were incubated with FITC-conjugated mouse anti-human
Bel-2 monoclonal antibody or FITC-conjugated mouse IgG1 monoclonal isotype control antibody (BD Biosciences), then analyzed by flow cytometry (Coulter Epics XL MCL).

Western blot analysis

B-NHL cell lines were lysed using lysis buffer (Cell Signaling), separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight with the following antibodies: AKT, p-AKT (Ser473), GSK-3β, p-GSK-3β (Ser9), p70S6, p-p70S6 (Thr421/Ser424), m-TOR, p-m-TOR (Ser2448), p-p90/RSK (Thr202/Thr204), PKC, p-PKC (Thr497), Bax, Total Bad, p-Bad (Ser112), p-Bad (Ser136), Bim, BCL-xL, Mcl-1, caspase 3 (Asp175), caspase 9 (Asp353), α-tubulin and PARP. PARP expression was also evaluated after 1 h of pre-treatment with 40uM of zVAD-fmk (Sigma), a broad inhibitor of caspases. All antibodies were purchased from Cell Signaling.

Statistical analysis

All in vitro experiments were performed in triplicate and repeated at least three times; a representative experiment was selected for the figures. Data are expressed as mean value ± standard error. Statistical differences between controls and drug-treated cells were determined by one-way analysis of variance (ANOVA). p values <0.05 were considered statistically significant. Data were analyzed using the Stata 8.2/SE package (StataCorp LP). The drug interactions were assessed using multiple effect analysis, based on the Chou–Talalay method.

Results

Cytotoxic effect of bortezomib, enzastaurin or lenalidomide in B-NHL cell lines

Bortezomib and enzastaurin each demonstrated a time- and dose-dependent inhibition of cell proliferation in all the cell lines tested. Conversely, lenalidomide induced a minor inhibition of cell proliferation even after 48h of treatment without reaching the IC50 (data not shown).

The IC50 values for each drug after 24 h of single treatment are reported in Table 1.

Bortezomib with either enzastaurin or lenalidomide inhibits B-NHL cell growth

B-NHL cell lines were cultured for 24 h with three doses of bortezomib (1, 4 and 6 nM) in combination with enzastaurin (1, 4 and 6 μM) or lenalidomide (1, 4 and 6 μM). The dose of 6nM for bortezomib and 6 μM for either enzastaurin or lenalidomide represents the peak inhibition doses that demonstrated synergistic activity of the agents with CIs <1 in all cell lines tested (Table 2 and Figure 1A).

Data were confirmed in primary lymphoma cells from patients with FL (pt #1, pt #2 and pt #3) and MCL (pt #4 and pt #5). No cytotoxicity was observed in normal PBMCs (Figure 1B). The treatment combinations also induced cytotoxicity in primary CD20+ cells, which were isolated from the BM of 3 patients with FL (Figure 1C).

Drug combinations overcome the protective effect of the bone marrow microenvironment on B-NHL cells

The synergistic activity of drug combinations against B-NHL cell lines was evaluated in the context of the bone marrow milieu. B-NHL cell lines were incubated with bortezomib (6 nM), enzastaurin (6 μM) or lenalidomide (6 μM), alone or in combination, in the presence or absence of BMSCs.

At 24 h, the drug combinations showed a cytotoxic effect on cell lines, even when cultured with BMSCs. Both combinations had minimal or no cytotoxic effect on BMSCs (Figure 1D).

Drug combinations enhance apoptosis in B-NHL cell lines through caspase activation

At 24 h, early apoptosis ranged from 27 to 37% in WSU-NHL and GRANTA-519 cells that were treated with both

<table>
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<th>Table 1. IC50 values of the single treatments of bortezomib (1–25 nM) and enzastaurin (1–10 μM) in B-lymphoma cell lines after 24 h. IC50 values were calculated using MTT assay. Values represent three independent experiments. CI95% (confidence interval). Single agent treatment of lenalidomide (1–100 μM) did not reach IC50 after 24 h.</th>
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<tr>
<td><strong>IC50 of bortezomib (1–25 nM), enzastaurin (1–10 μM) alone in B-lymphoma cell lines</strong></td>
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<tr>
<td>B-cell lines</td>
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<tr>
<td>WSU-NHL</td>
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<td>CI95% 12.4–16.3</td>
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<td>RL</td>
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<td>CI95% 14.3–26.5</td>
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<td>GRANTA-519</td>
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<td>CI50 3.68–8.74</td>
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<td>JEKO-1</td>
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combinations (Figure 2A and 2B). Drug combinations induced a significant activation of caspase-9 and caspase-3, as observed by western blot analysis in WSU-NHL and GRANTA-519 cells (Figure 2C). Bortezomib with enzastaurin showed a stronger activation of caspase-8 compared to bortezomib with lenalidomide (Figure 2D). Activation of the apoptotic pathway was confirmed by cleavage of the PARP enzyme. To confirm that apoptosis was mediated by caspase activation, we cultured B-NHL cell lines in the presence of ZVAD-fmk, a broad caspase inhibitor.

PARP cleavage was significantly enhanced using either combinations of bortezomib/enzastaurin or bortezomib/lenalidomide compared with single agents (Figure 2E). The cleavage of PARP was abrogated by ZVAD-fmk, confirming that the apoptosis is caspase dependent (Figure 2E).

Drug combinations on the cell cycle and cell cycle-related proteins

The treatment with the single drugs did not significantly affect cell cycle distribution. In contrast, combinations of
bortezomib with enzastaurin or lenalidomide reduced the proportion of cells in the G0/G1, S and G2/M phases (Figure 3A). Furthermore, a ‘sub-G0/G1’ phase, corresponding to apoptotic cells, was detected in all cell lines that were treated with the combination of bortezomib with enzastaurin or lenalidomide (Figure 3A).

To study the effects of drug combinations on cell cycle related proteins, we evaluated p21, p27 and cyclin E. p21 and p27 are cyclin-dependent kinase inhibitors (CDKI), and they function as cell cycle regulators. Up regulation/activation of CDK inhibitors p21 and p27 prevent cyclin E activation, resulting in G1/S arrest. Bortezomib with either enzastaurin or lenalidomide augment the expression of p21 and p27, and decrease cyclin E, as demonstrated in the WSU-NHL cell line (Figure 3B). Cyclin D1 overexpression has been implicated in the pathogenesis of MCL. The treatment of GRANTA-519 and JEKO-1 cells with bortezomib and enzastaurin or lenalidomide in combination resulted in the down-regulation of cyclin D1 protein (Figure 3C).

The apoptosis induced by the drug combinations is mediated by an increased ratio of pro-apoptotic proteins (Bax, Bad and Bim) over anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1)

The evaluation of Bcl-2 expression showed similar results with both single drugs and their combination in all B-NHL cell lines used. Therefore, based on similar response to single treatment and their combination, we decided to show results of WSU-NHL and GRANTA-519 cells.
In GRANTA-519 and JEKO-1 cells, the flow cytometry analysis showed that 24-h treatment with bortezomib, enzastaurin or lenalidomide alone did not alter the protein levels of Bcl-2. In WSU-NHL and RL cells, the protein levels of Bcl-2 decreased after treatment with bortezomib alone (Figure 4A). However, in comparison to the effects of the single treatments, the drug combinations reduced Bcl-2 expression. Bortezomib with either enzastaurin or lenalidomide induced a greater down-regulation of Bcl-2 in FL cells, in comparison to MCL cells (Figure 4A) and decreased the expression of some antiapoptotic proteins, such as Bcl-xL and Mcl-1, in WSU-NHL cells (Figure 4B). The drug combinations increased the levels of Bax and decreased the levels of p-Bad\textsuperscript{136} and p-Bad\textsuperscript{112}. Bad has pro-apoptotic activity when dephosphorylated at Ser\textsuperscript{136} and Ser\textsuperscript{112}. Moreover, the drug combinations, but not single agents, up-regulated Bim (Figure 4B). Bim plays a role in the initiation of the mitochondrial apoptotic signaling cascade via cytochrome-c release and caspase-9 activation [27]. These results indicate that the apoptosis induced by the drug combinations is mediated by the increased ratio of pro-apoptotic proteins (Bax, Bad and Bim) over anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1).

Drug combinations on the PI3K/AKT, PKC and MAPK/ERK signaling pathways

While the drugs alone did not significantly affect AKT, the drug combinations decreased the phosphorylation of AKT (Ser\textsuperscript{473}), as well as the down-stream factors, GSK3-\(\beta\), p70S6, mTOR and 4EBP1 (Figure 5A). The PKC and MAPK/ERK pathways interact with the PI3K/AKT pathway in hematological malignancies [28]. The drugs alone did not affect the phosphorylation of PKC, MAPK/ERK or c-Myc; in contrast, the drug combinations inhibited the PKC, MAPK/ERK pathway (Figure 5B) and decreased the expression of the proto-oncogene, c-Myc (Figure 5C).

Discussion

PI3K/AKT and MAPK/ERK pathways have been implicated in the tumorigenesis of many cancers, including B-cell lymphoma [28,29]. Intensive research efforts, which focused on better understanding the molecular pathogenesis of lymphoma, have paved the way toward identifying and testing targeted therapies for clinical investigation [25,30]. Bortezomib represents the first proteasome inhibitor with...
Synergism of bortezomib with either enzastaurin or lenalidomide

Figure 4. Bortezomib, combined with either enzastaurin or lenalidomide, tilts the balance of Bcl-2 family members toward apoptosis. (A) The bar graph shows the representative data (%) of Bcl-2 levels in WSU-NHL and GRANTA-519 cells, evaluated by flow cytometry. *p < 0.001 compared to control. (B) Western blot of cellular extracts from WSU-NHL cells probed with antibodies against Bcl-xL, Mcl-1, Bim, Bax, Total Bad, p-Bad<sub>136</sub> or p-Bad<sub>112</sub>. Tubulin was used to normalize protein loading.

results are in agreement with recently published data [40], suggesting that the effects of lenalidomide do not directly target neoplastic cells [41]. Other studies have reported that lenalidomide can induce apoptosis as a single agent [42,43]. We demonstrated a synergistic effect of bortezomib with either enzastaurin or lenalidomide, at concentrations lower than their IC<sub>50</sub> values, including inhibition of proliferation, induction of apoptosis, activation of caspases and altered expression of Bcl-2 family proteins. The Bcl-2 pathway remains of major interest, due to its overexpression in FL and implications in lymphomagenesis [44]. The overexpression of Bcl-2 is common in many tumor cells, and can increase cell viability through the abrogation of normal mechanisms of programmed cell death.

The Bcl-2 family of proteins are central regulators of mitochondrial apoptosis. The anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL and Mcl-1, are associated with the outer mitochondrial membrane and protect cells against a variety of apoptotic stimuli; Bcl-2 prevents the release of cytochrome c from mitochondria, a pivotal event in the apoptotic pathway [45]. The pro-apoptotic members of this family, such as Bax, Bad and Bim, induce mitochondrial outer membrane permeabilization, which results in the release of cytochrome c from the intermembrane space of the mitochondrion into the cytosol. Bim is a pro-apoptotic protein of the Bcl-2 family that plays a role in initiating the mitochondrial apoptotic signaling cascade via cytochrome c release and caspase-9 activation. Three major isoforms of Bim are generated by alternative splicing: Bim<sub>EL</sub>, Bim<sub>L</sub> and Bim<sub>S</sub>. The shortest form, Bim<sub>S</sub>, is the most cytotoxic and is generally only transiently expressed during apoptosis[27]. The combined treatments up-regulated the expression of the Bim<sub>S</sub> isoform without detectable changes in the other two isoforms. Bad is a pro-apoptotic Bcl-2 family member that is inactivated by phosphorylation at two critical sites, Ser<sup>112</sup> and Ser<sup>136</sup>. When phosphorylated at Ser<sup>112</sup> or Ser<sup>136</sup>, Bad is complexed to the cytosolic 14-3-3 protein, and fails to interact with the anti-apoptotic Bcl-xL protein, thus favoring cell survival [46]. In our experiments, Bad activation (dephosphorylation) was confirmed in the drug combinations.

Drug combinations reduced the proportion of cells in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases after 24 h. This effect is associated with the up-regulation of cell cycle regulating proteins p21, p27 and cyclin E. The CDK inhibitors, p21 and p27, have been described as regulators of the cell cycle in normal and malignant lymphocytes [47]. p21 induces growth arrest by inhibiting the ability of the cyclin-CDK complex to phosphorylate the cell cycle regulator, retinoblastoma protein (Rb). p27 can produce cell cycle arrest in response to inhibitory stimuli. Cyclin E/CDK2 regulates multiple cellular processes by phosphorylating numerous downstream proteins, including p21 and p27, and plays a critical role in the G<sub>1</sub>-S phase transition. Cell cycle arrest can be also mediated by the modulation of
cyclin D1, a protein involved in cell cycle progression, which is highly expressed in MCL. MCL cell tumorigenicity is correlated with the nuclear accumulation of cyclin D1 and p27. Recently, Moros et al. have shown that lenalidomide alone, in REC-1 cells, (MCL cell line), disrupts cyclin D1/p27 complexes inducing apoptosis [48]. In addition, the cyclin D1 has been reported to be down-regulated by enzastaurin alone in different cancer type [49,50]. Our results showed, in Figure 3C, a modulation of cyclin D1 by lenalidomide and enzastaurin as single agents in GRANTA-519 cells. No alteration on cyclin D1 expression was observed in JEKO-1 cells. (MCL cell line), disrupts cyclin D1/p27 complexes inducing apoptosis [48].

In addition, the cyclin D1 has been reported to be down-regulated by enzastaurin alone in different cancer type [49,50]. Our results showed, in Figure 3C, a modulation of cyclin D1 by lenalidomide and enzastaurin as single agents in GRANTA-519 cells. No alteration on cyclin D1 expression was observed in JEKO-1 cells. Our in vitro results are partly in agreement with previous data obtained by Podar et al. [14], and Moreau et al. [15], which reported a synergistic effect of the combination of enzastaurin and bortezomib in MM cells and additive cytotoxicity in Waldenström Macroglobulinemia. However, the effects of the combination treatment on cell cycle, apoptosis and signaling pathways were not completely analyzed.

In conclusion, although there has been improvement in survival outcome in NHL patients, there is a clear clinical need for novel agents to offer new options in resistant disease, and to potentially improve outcomes, even in curative settings. Enzastaurin and lenalidomide are compounds with favorable toxicity profiles. They are not classic chemotherapeutic agents that cause severe side effects; they could be considered examples of a new innovative attempt at an anti-cancer ‘chemotherapy free, soft treatment’. The combination of bortezomib with enzastaurin or lenalidomide could play a role in FL and MCL treatment in the future.

Conflict of interest
The authors have no competing interest.

Acknowledgement
We are grateful to the Associazione ‘Angela Serra’ per la Ricerca sul Cancro for financial support.

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