DEVELOPMENT OF BIOCONJUGATES AND THEIR MODUL CONSTRUCTS FOR TARGETED THERAPY OF CANCERS WITH HIGH MORTALITY

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Bortezomib has antitumor effect in melanoma cells that can be inhibited by alpha-lipoic acid

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Introduction

Bortezomib (BOZ), a targeted proteasome inhibitor, has become an unavoidable medicine in the treatment of multiple myeloma, a hematological malignancy. The U.S. Food and Drug Administration (FDA) approved BOZ for the treatment of progressive multiple myeloma in 2003. In addition, recently scientists have investigated the potential activity of BOZ and its probable combinations with the purpose of curing solid tumor malignancies, e.g. melanoma, the malignant transformation of melanocytes.[1,2,3]

BOZ is a low molecular weight, hydrophilic dipeptide-boronic acid derivative. It acts as a reversible inhibitor of the chymotrypsin-like protease activity of the proteasome.[4] It blocks the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway *via* inhibition of the degradation of the IκB protein that binds to the NF-κB and blocks its transport to the nucleus.[5,6]

In general, chemotherapy-induced peripheral neuropathy (CIPN) is a severe side-effect of various chemotherapeutic agents, such as BOZ, cisplatin or paclitaxel.[7] Unfortunately, bortezomib-induced peripheral neuropathy (BIPN) is a type of CIPN and belongs to the dose-limiting side effects of BOZ.[8,9] The symptoms of BIPN are paresthesia, allodynia, hyperalgesia.[7] These symptoms are usually presented in stocking-and-glove-shaped distribution, and thus dose reduction or discontinuation can be required.[8] To optimize the quality of life of the patients, different types of CIPN can be treated with antiepileptic agents such as gabapentin, antidepressants such as amitriptyline and neuroprotective or antioxidant vitamins such as Vitamin C.[10,11] These antioxidant vitamins are over-the-counter (OTC) products and are part of the cancer supportive care that has a growing market nowadays. However, cancer supportive care is aimed to focus on the quality of life during the treatments of cancerous illnesses, but many times it may also decrease the antitumor effect of the chemotherapeutic agent.

Other neuroprotective agents, *e.g.* alpha-lipoic acid (ALA) and Vitamin B1 (vit B1) are also applied as advantageous agents for cancer supportive care; previous studies have

shown the positive effect of these compounds mentioned above in different neurodegenerative conditions, *e.g.* diabetic neuropathy or anticancer drug-related peripheral neuropathy.[12-14]

Objectives

Based on the aforementioned findings, we hypothesized that the antioxidants may counteract the antitumor activity of BOZ. In the present work our aim was (i) to verify the anti-proliferative effects of BOZ in melanoma and myeloma cell lines, (ii) to test the influence of the ALA and vit B1 on the tumor growth inhibitory effect of BOZ, as well as (iii) to study the mechanisms of the effects of BOZ + antioxidants co-treatments.

Results

First, we determined the IC_{50} values in the 24th hour of the BOZ treatment on both cell lines (A2058 - IC_{50}: 158 nM; U266 - IC_{50}: 2.17 nM). Based on these IC_{50} values, the A2058 melanoma cell line showed a 72-fold lower sensitivity to BOZ compared with the U266 myeloma cells (Figure 1A and B). In the further experiments with the binary combination of BOZ and antioxidant vitamins, these compounds were tested only in representative concentrations (BOZ: 20, 100, 300 ng/mL; ALA: 10, 100 μg/mL and vit B1: 150, 300 nM).

Figure 1C-D shows the BOZ-induced anti-proliferation on myeloma and melanoma cells following 24 h exposure with 20, 100 and 300 ng/mL BOZ. The BOZ was anti-proliferative on the melanoma cell line A2058 in a dose-dependent manner (Figure 1C), while on the myeloma cell line U266, in all tested concentrations with the same extent (Figure 1D). It is clearly shown in Figure 1C that the number of viable melanoma cells was significantly increased following 20 ng/mL BOZ + 100 μg/mL ALA co-treatment compared to the only-BOZ treated cells. In the case of U266 cells, the antioxidants could not influence the antitumor effect of any BOZ treatment (Figure 1D).

As shown in Figure 1E, dose-escalated BOZ treatment induced remarkable p53-activation (phosphorylation of p53) even at lower concentrations on melanoma cell line compared to the control group (RFI = BOZ treated cells p53 MFI/control cells p53 MFI; RFI: ratio of the mean fluorescence intensity; MFI: mean fluorescence intensity). However, BOZ did not stimulate p53-activation in U266 cells.

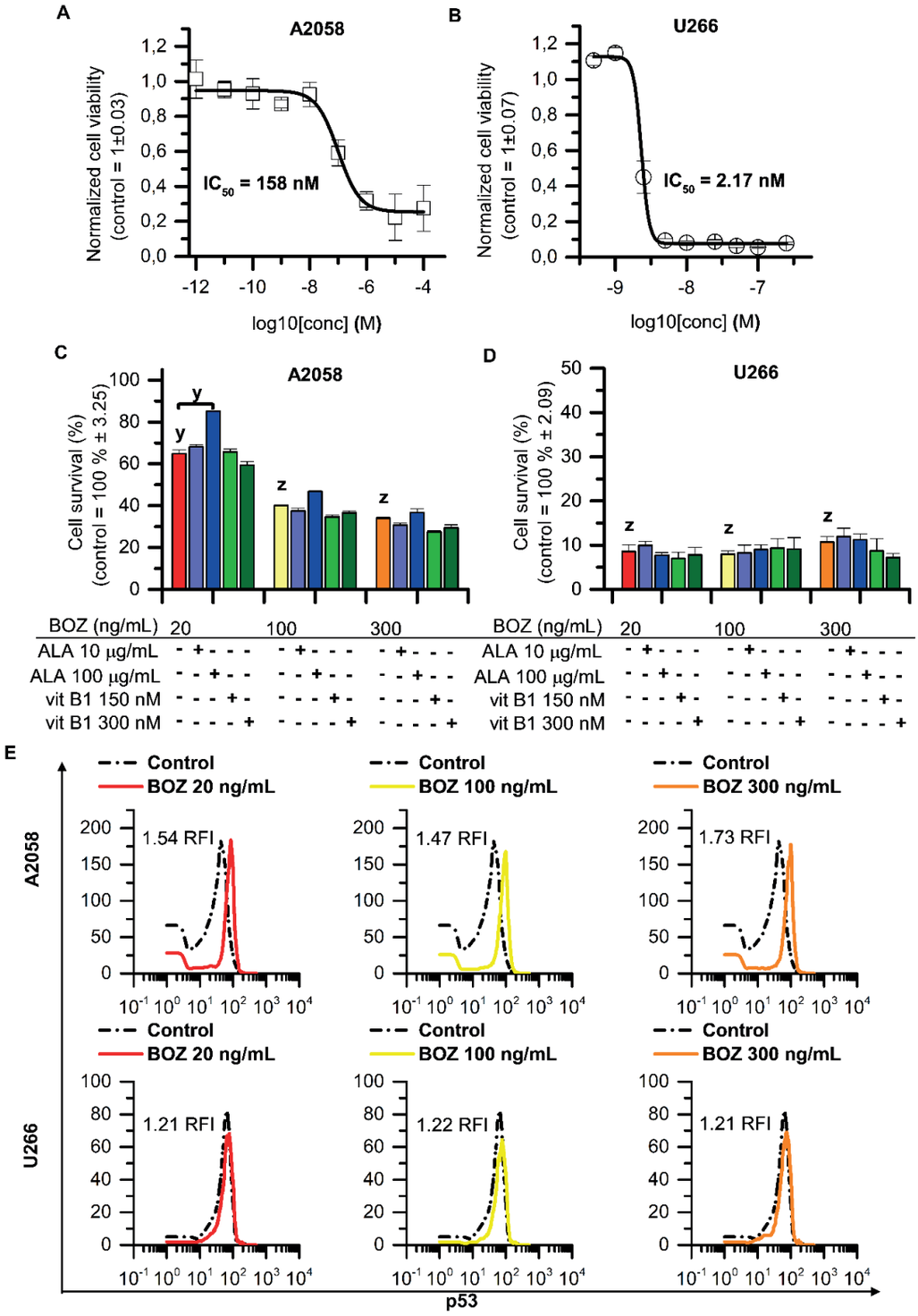

Figure 1. Concentration-response curves for (**A**) A2058 and (**B**) U266 cells treated with BOZ for 24 h. The data are normalized to the control wells. The IC_{50} value of BOZ was determined by fitting a sigmoidal dose-response curve to the data, using Origin Pro 8.0. Influence of alpha-lipoic acid and vitamin B1 on bortezomib mediated cell death on A2058 (**C**) and U266 cells (**D**) after 24 h incubation. (**E**) Analysis of the level of the activated p53 of A2058 and U266 cells after 24 h incubation with 20, 100 and 300 ng/mL bortezomib (BOZ). The levels of significance are shown as follows: x: $P < 0.05$; y: $P < 0.01$; z: $P < 0.001$, determined by the One-way ANOVA test followed by Fishers LSD *post hoc* test.

As it can be seen in Figure 2, the percentage of the Annexin V (Ax V) positive cells was increased by every BOZ treatment in both cell lines. Surprisingly, the antioxidants were able to further increase the percentage of the early apoptotic cells compared to the matching BOZ-treated control in many instances, *e.g.* A2058 cell: 100 ng/mL BOZ + 100 µg/mL ALA or 150 nM vit B1; U266 cell: 20, 100, 300 ng/mL BOZ + 10 or 100 µg/mL ALA, respectively. Both concentrations of vit B1 could reduce the percentage of Ax V positive A2058 cells, however, no significant decrease was observed in the 20 ng/mL BOZ + 100 µg/mL ALA combination compared to the 20 ng/mL BOZ-treated cells (Figure 2A).

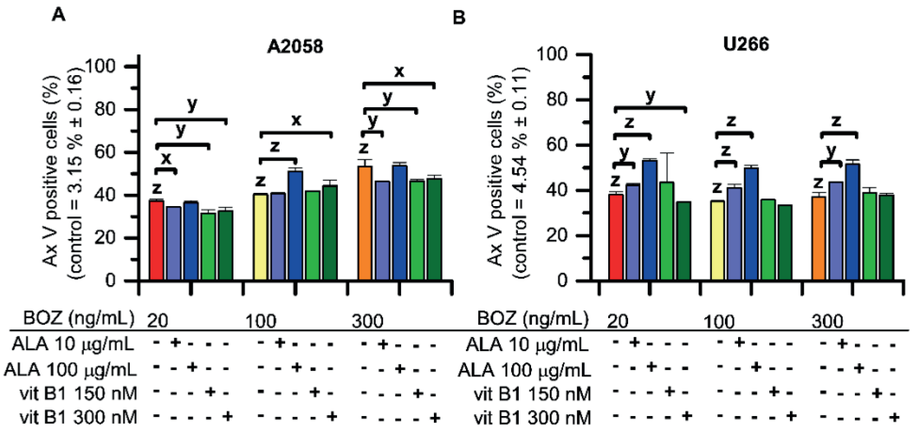

Figure 2. Apoptosis in A2058 (**A**) and in U266 (**B**) cells after 24 h long incubation with 20, 100 and 300 ng/mL bortezomib and combinations of BOZ + 10 or 100 µg/mL ALA and 150 or 300 nM vit B1 analyzed by Annexin V assay. Data are given as mean values ± standard deviation (SD) (n=2). The levels of significance are shown as follows: x: $P < 0.05$; y: $P < 0.01$; z: $P < 0.001$, determined by the One-way ANOVA test followed by Fishers LSD *post hoc* test.

We then hypothesized that BOZ may affect the cell cycle of the myeloma and melanoma cells. The cell cycle profile was evaluated after 24 h in the presence of BOZ (Figure 3). The percentage of A2058 cells in the sub G1 phase increased following the treatment with all the BOZ concentrations, along with a decrease in the proportion of the resting cells from G0/G1 phase (Fig. 3A). The treatment with 20, 100 and 300 ng/mL BOZ resulted in a tendentious increase in the proportion of cells in the S phase and G2/M phase. In contrast to our cell viability results, ALA could not significantly inhibit the effect of 20 ng/mL BOZ on the sub G1 phase (Figure 3B). The effect of the antioxidant co-treatments was insignificant on the G2/M arrest induced by 20 ng/mL BOZ treatment.

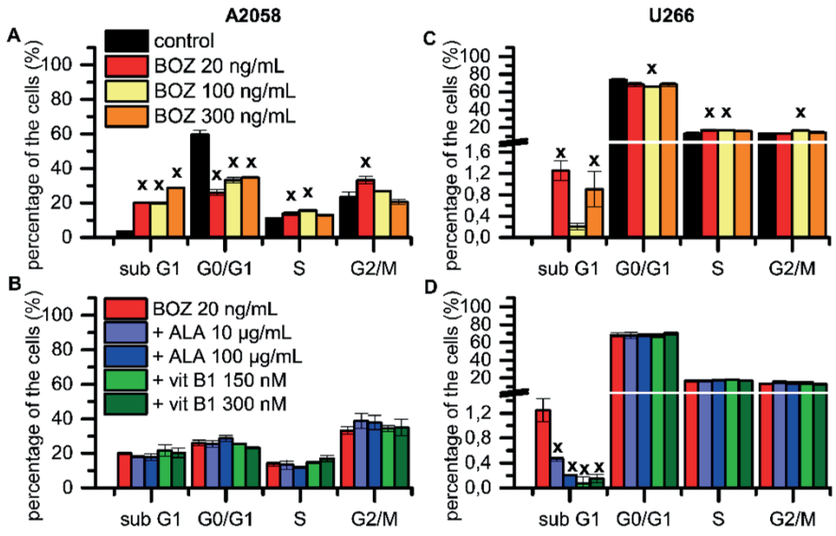

Figure 3. Cell cycle analysis of A2058 (**A-B**) and U266 (**C-D**) cell lines after 24 h long incubation with 20, 100 and 300 ng/mL bortezomib (BOZ) and combinations of 20 ng/mL BOZ + 10 or 100 µg/mL alpha-lipoic acid (ALA) and 150 or 300 nM vitamin B1 (vit B1) analyzed by NucleoCounter. The levels of significance are shown as follows: x: $P < 0.05$; y: $P < 0.01$; z: $P < 0.001$, determined by the One-way ANOVA test followed by Fishers LSD *post hoc* test.

Conclusions

Bortezomib, the first FDA approved proteasome inhibitor drug, is very important for the first-line therapy of multiple myeloma.[15] There is a large volume of studies attempting to explain the effects of BOZ on U266 myeloma cells.[16-18] As Chen pointed out, BOZ increased Ax V positivity and inhibited the growth of the U266 cells. In 2013, Selimovic *et al.* published a paper in which they described BOZ induced apoptosis- and autophagy-related pathways in melanoma cells, too.[19]

According to Larsson *et al.*,[20] the IC_{50} values of drugs are suitable drug response metrics to predict the sensitivity of cells. In the present work, we report that U266 myeloma cells were (IC_{50}: 2.17 nM) more sensitive to BOZ than A2058 melanoma cells (IC_{50}: 158 nM). Our data demonstrate a tumor-specific antitumor effect of BOZ, because p53 activation was observed only on melanoma but not on myeloma cell lines. These results are consistent with other studies and suggest that, depending on the tumor type, BOZ may act independently of p53 phosphorylation.[21]

The detection of the early apoptotic cells developed by the BOZ treatments confirms our initial results on the cell viability of both cell lines. Dose-dependent apoptotic effect of BOZ was observed in the melanoma cells, whereas dose-dependency could not be detected in the myeloma cells. In contrast, other experiments carried out in this field,[22,23] we could detect

a cell cycle arresting effect of BOZ on myeloma cells in the S-phase, while our results showed a G_2/M-phase arrest on melanoma.

This work calls the attention that BOZ may alter different pathways in the investigated melanoma and myeloma cell lines *in vitro*. Further data collection is required to unfold the dose-dependent correlation, how ALA affects the antitumor effect of BOZ. To sum up, the fact that 100 μg/mL ALA was able to disrupt the antineoplastic effect of 20 ng/mL BOZ in melanoma cells *in vitro*, should point towards the proper use of cancer supportive care that must be in accordance with evidence-based medicine and must be under medical control.

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