Effects of combination treatment of bortezomib and dexamethasone in SCCHN cell lines depend on tumor cell specificity

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Abstract. Bortezomib has recently become the new treatment standard for relapsed or refractory multiple myeloma. We previously demonstrated that bortezomib also had a significant growth-inhibiting and apoptotic effect on squamous cell carcinoma of the head and neck (SCCHN) cells *in vitro*. Preclinical evidence has provided a rationale for combining bortezomib with dexamethasone in multiple myeloma, suggesting that the therapeutic effects of the two agents might be additive. These findings are in contrast with the results achieved in solid tumor models where the addition of dexamethasone reduced the efficacy of other antineoplastic drugs. In the present study, we investigated the effect of dexamethasone in combination with bortezomib in SCCHN cell lines for the first time. The antiproliferative effect of bortezomib alone or in combination with increasing concentrations of dexamethasone was investigated in four SCCHN cell lines. Cell growth inhibition and viability were measured quantitatively using WST and LDH assays. Bortezomib alone inhibited the growth of all four SCCHN cell lines significantly ($p < 0.047$). The addition of dexamethasone leads to a clear tumor cell decline and showed a trend in enhancing the growth-inhibitory effect of bortezomib although the difference failed to reach statistical significance ($p > 0.05$). Our first results show that dexamethasone increased the cytotoxic activity of bortezomib in most SCCHN cell lines investigated. These findings might be dependent on molecular factors such as the degree of tumor cell differentiation and proliferation rate. Therefore, further studies will be required to elucidate these molecular factors to substantiate our findings from a cancer biological point of view.

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Introduction

A major concern in the use of cytotoxic chemotherapy for head and neck cancer is the potential for severe treatment-related toxicities which often necessitate interruptions of therapy and hamper quality of life of the patients (1). Another significant problem may be the development of drug resistance in patients receiving chemotherapy alone or in combination with ionizing radiation (2). Despite significant advances in the use of surgery, chemotherapy and radiation in the treatment of head and neck cancer, there has been little improvement in the prognosis of the disease during the past 30 years, and outlook for the patients remains poor (3).

We previously demonstrated that bortezomib, which has recently been approved for the treatment of relapsed or refractory multiple myeloma, had a significant growth-inhibiting and apoptotic effect on SCCHN cells *in vitro* (4). Bortezomib is a reversible small molecule inhibitor of the 26S proteasome, a large intracellular protein complex that regulates multiple cellular processes through degradation of ubiquitin-tagged proteins. Bortezomib-mediated preservation of these proteins has been shown to disrupt vital signalling pathways, thereby leading to cell death.

An increasing body of preclinical and clinical evidence has provided a sound rationale for combining bortezomib with dexamethasone in the treatment of patients with multiple myeloma, suggesting an enhanced or even additive effect of the two agents on multiple myeloma cells (5). Dexamethasone and similar glucocorticoids (GCs) were first introduced into tumor therapy on the basis of their proapoptotic effects in lymphoid cells, their effectiveness in treating tumor-related edema, inflammation, pain and electrolyte disturbances, and their stimulatory effect on appetite. In addition, GCs such as dexamethasone are often used in clinical practice as co-medication to prevent and treat nausea and several other therapy-related side effects (6). While dexamethasone has been shown to improve treatment of multiple myeloma, accumulating evidence suggests an antagonistic effect in solid tumors (2). The present study was aimed to examine whether the addition of dexamethasone has an enhancing or inhibitory effect on the cytotoxic activity of bortezomib in SCCHN cell lines.

Table I. Specific concentrations of bortezomib used for the treatment of various squamous cell carcinoma cell lines *in vitro*.

Cell line	Bortezomib concentration
Kyse 140	2.5 nmol/l
PJ 41	2.5 μmol/l
PJ 15	2.5 μmol/l
Cal-27	2.5 nmol/l

Table II. Escalating concentrations of dexamethasone used for the treatment of various squamous cell carcinoma cell lines *in vitro*.

Concentration 1	0.5 μmol/l
Concentration 2	2.5 μmol/l
Concentration 3	10 μmol/l
Concentration 4	20 μmol/l

Materials and methods

Four different squamous cell carcinoma cell lines were tested in this study. PJ 15 and PE/CA-PJ 41 cells were obtained from ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire, GB) and Cal-27 and Kyse-140 cells were purchased from DSMZ GmbH, Braunschweig, Germany. The fibroblast cell line was a gift from the Department of Dermatology, University Hospital, Frankfurt/Main, Germany. Bortezomib (Velcade®) was supplied by Millenium Pharmaceuticals Inc., Cambridge, MA, and Johnson & Johnson Pharmaceuticals, Raritan, NJ, USA. Dexamethasone was provided by Sigma-Aldrich, Munich, Germany. The cell lines were cultivated according to the instructions of the suppliers without antibiotics at 37°C in the cell-type specific medium Quantum 263 with L-glutamine (PAA Laboratories GmbH, Pasching, Austria). Cells were seeded in 96-multiwell plates (1x100,000 cells/ well), and after incubation for 24 h, the cells were treated with bortezomib alone, dexamethasone alone or the combination of both drugs for 24, 48, and 72 h, respectively.

In all experiments described in this publication, bortezomib was used in each cell line at a fixed, cell-line-specific, concentration that had produced maximum growth inhibition in previous systematic investigations in our laboratory (Table I), while dexamethasone was used at four increasing concentrations (Table II). The concentrations ranged from 2.5 nmol/l to 2.5 μmol/l for bortezomib, depending on the cell line treated, and from 0.5 to 20 μmol/l for dexamethasone as these concentrations are comparable with the clinically achievable tissue concentrations of the drug (7). The number of cells was determined in a Rosenthal chamber after 24, 48 and 72 h of treatment. Cell viability and cell killing were determined by a WST and lactate dehydrogenase (LDH) assay, respectively. For the WST assay, $1x10^5$ cells per well were cultivated in a 96-well plate for 24 h and then treated with different concentrations of bortezomib and dexamethasone for 24, 48 and 72 h, respectively. WST (10 μl) at 5 g/l (Roche Diagnostics, Mannheim, Germany) were added to the medium in triplicate at each dose and incubated for 1 h at 37°C. Absorbance was measured at 450 nm using a microplate

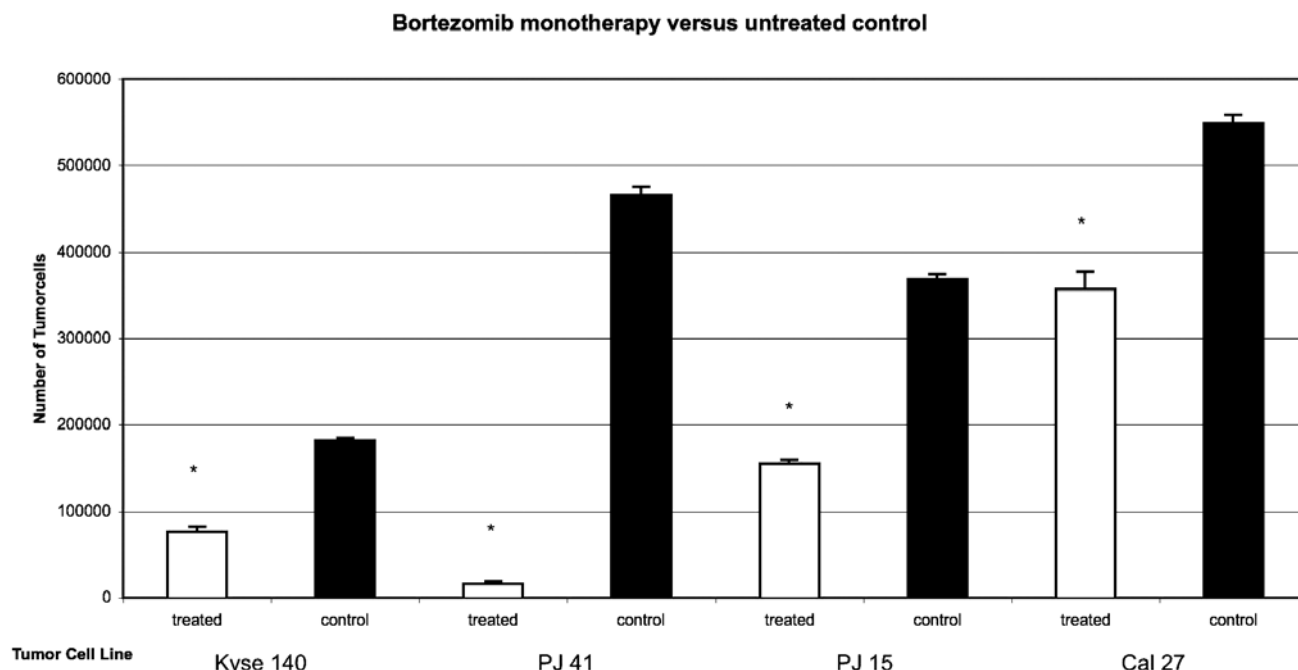**Bortezomib monotherapy versus untreated control**

Figure 1. Growth-inhibitory effect of single-agent bortezomib at fixed cell-line-specific concentrations (see Table I) in four different squamous cell carcinoma cell lines (white columns). The corresponding untreated tumor cell lines (black columns) served as controls and were incubated only with the cell-type specific medium Quantum 263 with L-glutamine. The absolute tumor cell numbers in treated and control cell lines were determined in a Rosenthal chamber at 72 h after treatment or incubation with Quantum 263 (controls), respectively. Mean values of three independent experiments with standard deviation are shown. Significant differences versus controls are indicated by asterisks. Bortezomib inhibited cell proliferation significantly in all cell lines (p=0.047 versus untreated controls for all cell lines).
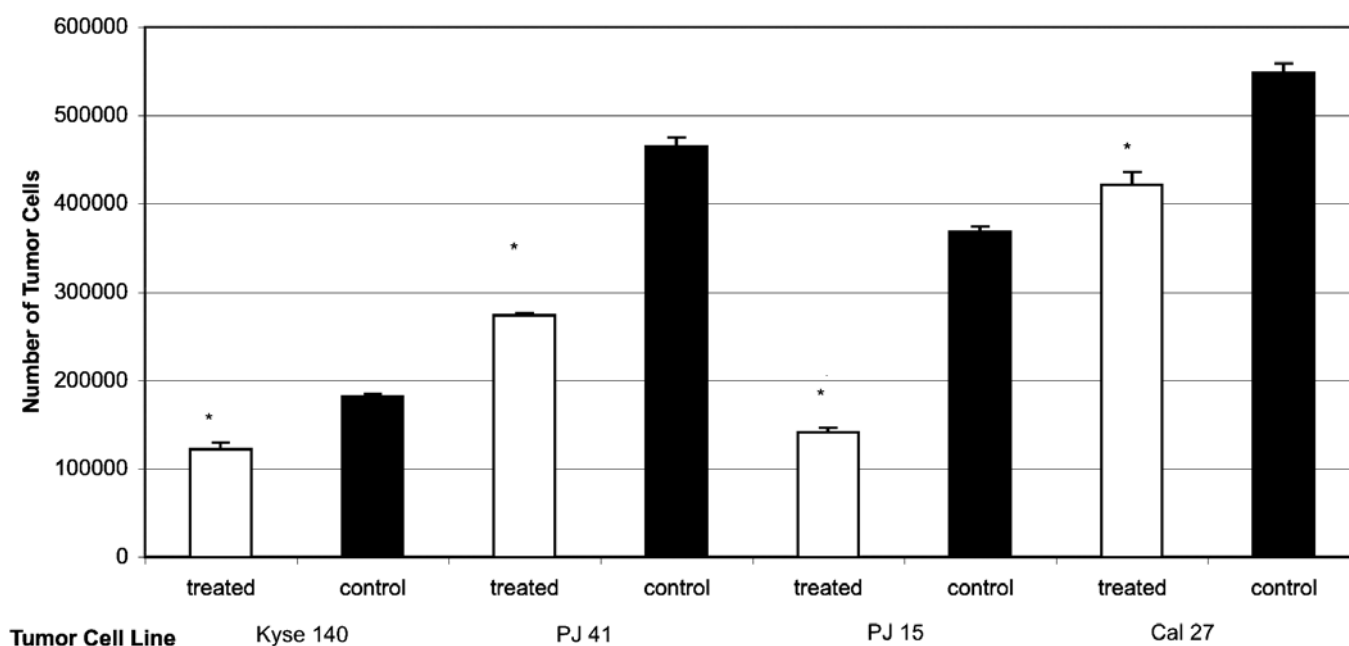
Figure 2. Growth-inhibitory effect of single-agent dexamethasone at a concentration of 20 μmol/l in four different squamous cell carcinoma cell lines (white columns). The corresponding untreated tumor cell lines (black columns) served as controls and were incubated only with the cell-type specific medium Quantum 263 with L-glutamine. The absolute tumor cell numbers in treated and control cell lines were determined in a Rosenthal chamber at 72 h after treatment or incubation with Quantum 263 (controls), respectively. Mean values of three independent experiments with standard deviation are shown. Significant differences versus controls are indicated by asterisks. Dexamethasone monotherapy inhibited cell proliferation significantly at concentrations 2 (p=0.043) and 4 (p=0.026) versus untreated controls.

reader. LDH activity in the culture medium was measured with the cytotoxicity detection kit plus purchased from Roche. Briefly, cells were incubated in a 96-well microplate (Falcon, Franklin Lakes, NJ, USA), with 5,000 cells in 200 μl seeded per well with Quantum 263 PAA.

After 24 h, the medium was removed and replaced either by the same medium containing different concentrations of bortezomib and/or dexamethasone as specified above, or drug-free medium (low controls) or medium containing 1% Triton X-100 (Sigma Chemical Co.) to determine total cellular LDH (high controls). After 24, 48 or 72 h of treatment, 100-μl samples were removed from the wells and transferred to another well-plate, 100 μl of the LDH assay reaction mixture were added to each well, and cells were subsequently incubated for 30 min at room temperature. During the incubation period, the microplates were protected from light. The optical density of each well was determined using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA) at a wavelength of 490 nm with a reference wavelength of 630 nm. Each experiment was done in triplicate. For statistical analysis, paired t-tests were performed using SPSS 13.0 software for Windows.

Results

The following results were obtained after 72 h of treatment, when the highest cell death rate was reached. Compared with untreated controls, bortezomib had a significant (p=0.047) antiproliferative effect in all four squamous cell carcinoma cell lines (Fig. 1). Dexamethasone alone was found to be

significantly cytotoxic at concentrations 2 (p=0.043) and 4 (p=0.026) compared with untreated controls (Fig. 2). When single-agent bortezomib was compared with single-agent dexamethasone, the antiproliferative effect of bortezomib was substantially higher but the difference between the drugs failed to reach statistical significance at any concentration of dexamethasone (p>0.05).

In a next step we determined the growth-inhibitory effect of bortezomib in combination with dexamethasone administered at four different concentrations (Table II). In all experiments the combination markedly reduced the number of tumor cells compared with untreated controls (Fig. 3), but again the difference was not statistically significant (p>0.05). Similar comparisons were made for bortezomib alone versus bortezomib plus dexamethasone. The combination with dexamethasone given at 10 μmol/l (concentration 3) showed a higher antiproliferative effect than bortezomib alone in three of four tumor cell lines (Fig. 4), but the difference failed statistical significance (p>0.05).

Discussion

For almost half a century physicians have relied on GCs (Glucocorticoids) to treat several types of malignant diseases. Since GCs can kill lymphoid cancer cells, they play an important role in the treatment of hematologic malignancies (8). Based on a variety of other beneficial effects, GCs are also widely used in combination with chemotherapy and radiation for the treatment of patients with solid tumors. It is well established that GCs reduce nausea and vomiting, protect
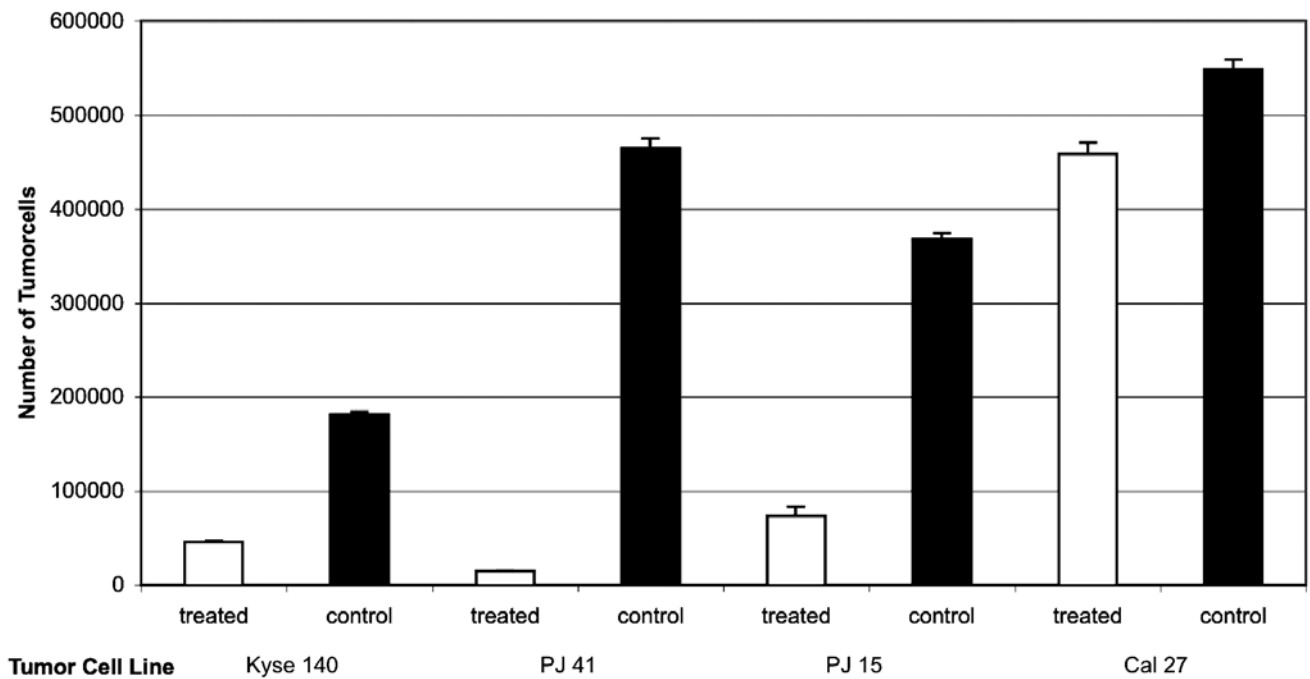
Figure 3. Growth-inhibitory effect of bortezomib plus dexamethasone (white columns) versus untreated controls (black columns) in four different squamous cell carcinoma cell lines. Bortezomib was used at fixed cell-line-specific concentrations (see Table I) and dexamethasone at 4 increasing concentrations (see Table II). The absolute tumor cell numbers were determined in a Rosenthal chamber at 72 h after treatment. Mean values of three independent experiments with standard deviation are shown. Only the results achieved with dexamethasone at concentration 3 are shown in the figure. Bortezomib/dexamethasone inhibited cell proliferation markedly but not significantly compared with untreated controls ($p>0.05$).
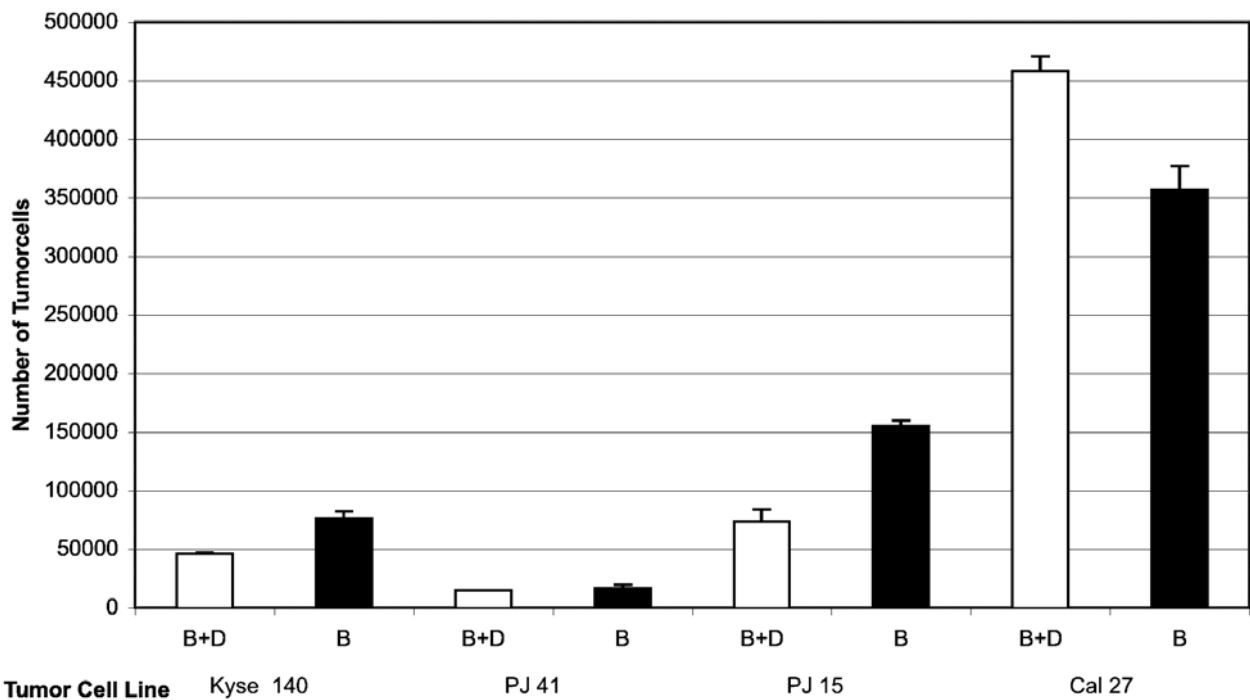

Figure 4. Growth-inhibitory effect of bortezomib plus dexamethasone (B+D) (white columns) versus bortezomib alone (B) (black columns) in four different squamous cell carcinoma cell lines. Bortezomib was used at fixed cell-line-specific concentrations (see Table I) and dexamethasone at 4 increasing concentrations. The absolute tumor cell numbers were determined in a Rosenthal chamber at 72 h after treatment. Mean values of three independent experiments with standard deviation are shown. Only the results achieved with dexamethasone at concentration 3 (10 μmol/l) are shown in the figure. Although combination therapy had a greater antiproliferative effect in three of four tumor cell lines compared with bortezomib alone, the results were not statistically significant.

normal tissues from cytotoxic side effects, and diminish the inflammatory tissue response to invasive malignant growth (6). However, while the therapeutic benefit of GCs is indisputable in hematologic malignancies, concerns have long been raised about the common adjunctive use of these agents in the treatment of solid tumors (10). Certain studies have described a detrimental effect of GCs on treatment outcome (9), including the finding of an increased rate of metastases in breast cancer patients (11) and an increased risk of skin cancer and non-Hodgkin's lymphomas among users of systemic GCs (12).

The present *in vitro* study has shown for the first time that dexamethasone may enhance the efficacy of bortezomib to inhibit tumor cell growth in SCCHN. The cytotoxic effect of dexamethasone in SCCHN cell lines was evident both for the single agent and the combination with bortezomib although the results failed statistical significance. These results are in contrast to the findings of Zhang *et al* (13) who reported corticosteroid-induced resistance to chemotherapy in the majority of solid tumor cell lines (bone, brain, breast, cervix, melanoma and neuroblastoma) treated with conventional agents such as cisplatin or 5-fluorouracil (5 FU). Similarly, Gassler *et al* (2) described dexamethasone-induced resistance to cisplatin and gemcitabine in lung cancer samples. Considering our opposing findings, it seems possible that the effect of dexamethasone varies both with the type of anti-neoplastic drug and the type of cancer. With regard to the tumor cell lines focused in our investigation, Cal-27, one of four tumor cell lines, showed no higher tumor cell decline treated with dexamethasone and bortezomib compared to bortezomib single therapy (Fig. 4). From our point of view it might be possible that complex resistance mechanisms, cell differentiation features and proliferation properties of each tumor cell line may influence the apoptotic effects of dexamethasone and bortezomib respectively as a sign of subspezialisation within SCCHN.

Many studies in animal models have shown that administration of dexamethasone results in immuno-suppression that may exacerbate metastatic spread and accelerate tumor growth (14). This could explain why GCs inhibit the effect of conventional chemotherapy with cisplatin and 5-FU in certain solid tumor models, while they are beneficial as an adjunct to bortezomib in the treatment of hematologic malignancies (5).

New active drugs are urgently needed to devise anti-neoplastic treatment regimens with improved efficacy and a favorable toxicity profile. Our *in vitro* results suggest that bortezomib could become a useful treatment option for patients with SCCHN. Moreover, our findings suggest a trend of dexamethasone to enhance the efficacy of bortezomib. However, results obtained *in vitro* cannot be easily translated to the clinical setting, and we are planning further experimental *in vivo* studies in our laboratory to define the effect of bortezomib in combination with dexamethasone on SCCHN in animal models.

Our *in vitro* results suggest a positive interaction of dexamethasone and bortezomib, resulting in enhanced cytotoxicity against SCCHN cell lines compared with either agent given alone. Considering the other beneficial effects of dexamethasone as a cancer co-medication, the combination with bortezomib appears promising. Thus, further investigations are warranted to substantiate our preliminary results, to explain the contradictory findings obtained with dexamethasone and conventional cytotoxic agents in the treatment of other solid tumors and finally to elucidate the complex resistance mechanisms, cell differentiation features and proliferation properties of each tumor cell line that may influence the apoptotic effects of dexamethasone and bortezomib respectively as a sign of subspezialisation within SCCHN.

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