Effect of Bortezomib and Cetuximab in EGF-stimulated HNSCC

JENS WAGENBLAST[1], MEHRAN BAGHI[1], CHRISTOPH ARNOLDNER[1], SOTIRIOS BISDAS[2], WOLFGANG GSTÖTTNER[1], HANNS ACKERMANN[3], ANGELIKA MAY[1], RAINALD KNECHT[1] and MARKUS HAMBEK[1]

[1]ENT Department, [2]Department for Diagnostic and Interventional Radiology and [3]Department for Biometry and Statistical Analysis, Medical School, Goethe University, 60590 Frankfurt am Main, Germany

Abstract. *Background: Proteasome inhibition has been shown to be effective in multiple myeloma and solid tumor models. In this in vitro study, the antitumor effect of bortezomib (Velcade®) in combination with cetuximab was investigated in epidermal growth factor (EGF)-stimulated head and neck squamous cell carcinoma cell lines (HNSCC). Materials and Methods: Dose escalation studies were performed with five EGF-stimulated squamous cell carcinoma cell lines using bortezomib alone or in combination with cetuximab. Growth inhibitory and cell decline effects were measured quantitatively using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and lactate dehydrogenase (LDH) assay. Results: Bortezomib alone showed no significant antiproliferative activity in any EGF-stimulated HNSCC cell line ($p>0.05$), whereas the combination of bortezomib and cetuximab had highly significant antitumoral activity ($p<0.043$). Conclusion: Our results indicate that cetuximab increases the cytotoxic activity of bortezomib in EGF-stimulated HNSCC cell lines. A combination treatment of HNSCC with bortezomib and cetuximab may allow a therapeutical regimen to be developed that is less toxic than the conventional drugs used for these tumors.*

Head and neck cancer (HNC) accounts for about 5% of all malignancies worldwide. More than 100,000 cases are diagnosed alone in Europe each year. More than 90% of the HNC are of squamous cell origin and most patients reach hospitals with locally and regionally advanced disease (1). The most commonly used agents are cisplatin and carboplatin, often used in combination with taxanes and/or 5-fluorouracil. Despite response rates to first-line platinum-based chemotherapy of up to 90%, duration of therapy is limited because of toxicity (2). Hence new therapeutical strategies need to be found and evaluated in preclinical trials.

Several molecularly targeted approaches have been evaluated in HNC patients, aiming for a greater selectivity and toxicity. Potential targets are growth factor receptors, signal transduction proteins and proteins involved in the degradation of kinases, to mention only a few (3).

In our former investigations, we demonstrated the high growth-inhibiting and apoptotic effect of bortezomib, a small molecular weight reversible inhibitor of the intracellular 26S proteasome, a large protein complex in HNC cells (4). Although the molecular mechanisms underlying the antineoplastic effect of bortezomib are not yet fully understood, it is well known that bortezomib acts primarily by inhibition of the NFκB pathway (5) responsible for degrading specific ubiquitin-tagged proteins. *In vitro* models have demonstrated that through the selective inhibition of this pathway, proteins destined for degradation are maintained, thus disrupting signalling pathways and ultimately leading to cell death (16).

Overexpression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinoma (HNSCC) has often been correlated with poor prognosis.

The potential value of modulating EGFR signalling as a cancer treatment approach is reflected by the broad array of molecular inhibitors that have been developed in recent years. Cetuximab, a molecular inhibitor of EGFR/HER1 is under active investigation as a promising anticancer agent in general (6) and especially in HNC (7). Combining bortezomib and cetuximab in EGF-stimulated HNSCC is the primary objective of this work, raising the question whether cetuximab is able to enhance the growth-inhibition and tumor cell decline efficacy of bortezomib.

Correspondence to: Jens Wagenblast, MD, ENT Department, University Hospital, Theodor-Stern-Kai 7, 60590 Frankfurt/am Main, Germany. Tel: +49 69 63015113, Fax: +49 69 63015658, e-mail: Jens.Wagenblast@kgu.de

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Table I. *Pixel density.*

Cell line	Before EGF stimulation	After EGF stimulation
A 431	46.1±2.2	374.1±50.9
Cal 27	132.0±4.8	467.3±20.2
Kyse 140	57.5±2.3	263.4±13.4
PJ 15	16.8±1.1	177.8±15.5
PJ 41	114.0±5.4	245.5±18.1

Pixel density for the 170-kDa EGFR band in the Western blots of the 5 different squamous carcinoma cell lines before and after EGF stimulation. Mean values and standard deviation of three independent measurements are shown. Absolute pixel density was measured with a Kodak Digital Science Image Station 440 CF system (Perkin Elmer, Cologne, Germany).

Materials and Methods

Five different squamous carcinoma cell lines were tested in this study. A 431 cells were obtained from the American Type Culture Collection. PJ 15 and PE/CA-PJ 41 cells were obtained from the European Collection of Cell Cultures. Cal 27 and Kyse 140 cells from DSMZ GmbH, Braunschweig, Germany. The fibroblast cell line (taken from infantile preputium) 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. Cetuximab was provided by Merck (Darmstadt, Germany).

Squamous carcinoma 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).

To enhance the EGFR signalling pathway and thus tumor cell proliferation and mitosis, tumor cell lines were stimulated with human EGF at a concentration of 10 ng/ml for 24 h at 37˚C (Table I). It is evident that EGFR levels increased in all cell lines after stimulation with EGF. The cells were then seeded in 96-well plates (100,000 cells/well) and after incubation for 24 h were treated with bortezomib or cetuximab alone, or in combination for 24, 48, and 76 h, respectively. In the 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 II), while cetuximab was used at three increasing concentrations: 0.05, 0.5 and 5 μmol/l. Cell numbers were determined by counting cells in a Rosenthal chamber at 24, 48 and 72 hours after treatment. Cell viability and cell decline were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and lactate dehydrogenase (LDH) assay respectively (data not shown).

MTT assay. After incubation for 24 h, 1×10^5 cells/well in a 96-well plate, were treated with different concentrations of bortezomib and cetuximab for 24, 48 and 72 h. Ten microliters of MTT (5 g/l; Sigma Chemical Co., St. Louis, USA) were added to the medium at each dose and cells were incubated for 4 h at 37˚C. The culture media were discarded, followed by the addition of 0.2 ml dimethyl sulfoxide (DMSO) and vibration for 10 minutes. Absorbance was measured at 570 nm using a microplate reader.

Table II. *Specific concentrations of bortezomib used for the treatment of squamous cell carcinoma cell lines.*

Cell line	Bortezomib concentration
A 431	0.01 nmol/l
Cal 27	2.5 nmol/l
Kyse 140	2.5 nmol/l
PJ 15	2.5 μmol/l
PJ 41	2.5 μmol/l

LDH assay. The detection of LDH activity was performed using the Cytotoxicity Detection Kit purchased from Boehringer Mannheim, Germany, and was based on the detection of LDH activity in the culture medium. Briefly, 5,000 cells/200 μl/well were incubated in 96-well microplates (Falcon, Franklin Lakes, NJ, USA) with RPMI-1640 supplemented with 10% fetal calf serum (FCS). After 48 h, the media were removed and replaced either by a medium containing different concentrations of the drugs bortezomib and/or cetuximab or by a drug-free medium (low control). Wells for the high control had media containing 1% Triton® X-100 (Sigma Chemical Co.) added to them to determine total cellular LDH. After 24, 48 and 72 h treatment, 100 μl supernatant/well were removed and transferred to corresponding well of a fresh microplate. To determine the LDH activity of the supernatants, 100 μl kit reaction mixture were added to each well and plates were incubated for 20 minutes 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 performed in triplicate. For statistical analysis, a Wilcoxon test for matched pairs (dependent samples) was performed using SPSS 13.0 software for Windows (SPSS GmbH Software, Munich, Germany).

Results

Results considered the number of tumor cells on day 3, indicating the highest cell death rate after 72 h. Compared with the untreated control group, bortezomib had no significant ($p>0.05$) antiproliferative effect in any of the five EGF-stimulated squamous cell carcinoma cell lines. Cetuximab however was found to be significantly ($p=0.043$) active at 0.05 and 0.5 μmol/l. When single-agent bortezomib was compared with single-agent cetuximab, a clear trend indicating that the antitumoral activity of bortezomib is higher than that of cetuximab was apparent but was not statistically significant ($p>0.05$).

We then compared the growth-inhibitory effect of bortezomib combined with cetuximab at 3 different concentrations. In all experiments, the combination therapy was more effective compared to the untreated control group and was statistically significant ($p=0.043$) at 0.5 and 5.0 μmol/l cetuximab (Figure 1). Similar comparisons were made for

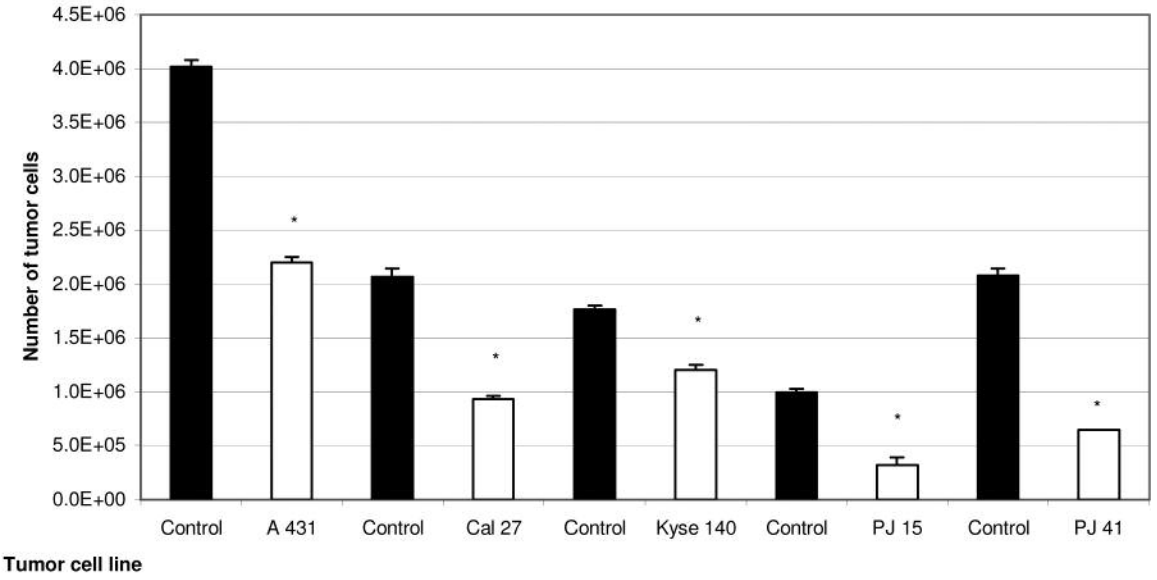

Figure 1. *Growth-inhibitory effect of bortezomib plus cetuximab (open columns) versus untreated control group (closed columns) in five different squamous cell carcinoma cell lines. Bortezomib was used at fixed cell line-specific concentrations (see Table II) and cetuximab at three different concentrations. The absolute tumor cell numbers were determined in a Rosenthal chamber at 72 hours after treatment. Mean values of three independent experiments with standard deviation are shown. Significant differences between combination therapy and untreated control are indicated by asterisks. Only the results achieved with cetuximab at 5 μmol/l are shown. The growth-inhibitory effect of bortezomib/cetuximab was significantly greater than in the untreated control group at a cetuximab concentration of 0.5 μmol/l and 5 μmol/l (p=0.043).*

bortezomib alone *versus* bortezomib plus cetuximab. The combination was significantly more active than bortezomib alone at a cetuximab concentration of 5.0 μmol/l (p=0.043) (Figure 2).

In the last series of experiments, it was shown that the addition of bortezomib to cetuximab increased the growth-inhibitory effect of the combinational setting with regard to cetuximab monotherapy in 4 out of 5 EGF-stimulated tumor cell lines for all cetuximab concentrations but this effect failed to reach statistical significance (p>0.05).

Discussion

Growth factors play an important role in normal cell proliferation by stimulating specific receptors located on the cell surface. Tumor cells express high levels of growth factor receptors which might serve as molecular targets for anticancer treatment. HNSCC tumors also express high levels of EGFR. Overexpression of EGFR in epithelial tumors, including those of the head and neck, and other solid tumors, has frequently been correlated with poor prognosis. This finding has stimulated efforts to develop new cancer therapies that target EGFR (8).

Numerous investigations have demonstrated that bortezomib is a prime candidate for drug development in hematological malignancies such as multiple myeloma, but also in solid tumors including HNSCC (9). Bortezomib inhibits a proteasome involved in the ubiquitin-proteasome pathway, thereby resulting in cell cycle disruption, inhibition of tumor cell proliferation and induction of apoptosis (10, 11). Being the first proteasome inhibitor approved for clinical use, bortezomib has an interesting antiproliferative activity in many tumor cell lines, both as a single agent and in combination with other chemotherapeutic drugs (12-16). In our former investigations, we demonstrated the high antitumoral effect of bortezomib in HNSCC (4), while this effect had no apparent consequence on EGFR expression (17).

In the present study, tumor cell lines were stimulated with EGF to enhance the EGFR signalling pathway and thus tumor cell proliferation and mitosis. Considering the apoptotic effect of bortezomib in HNSCC with native EGFR expression, in the stimulated tumor cells it had no significant apoptotic effect. The finding that the combination of bortezomib and cetuximab led to a significant tumor cell decline suggests that bortezomib admittedly has no effect on EGFR expression but is by itself manifestly dependent on the EGFR level, which is reduced by anti-EGFR agents such as cetuximab. Bortezomib efficacy may thus be enhanced by combining it with anti-EGFR agents.

Conclusion

Under the concept of targeted therapy, bortezomib and cetuximab are two promising drugs. Based on our *in vitro* results, it is likely that the antitumoral efficacy of bortezomib

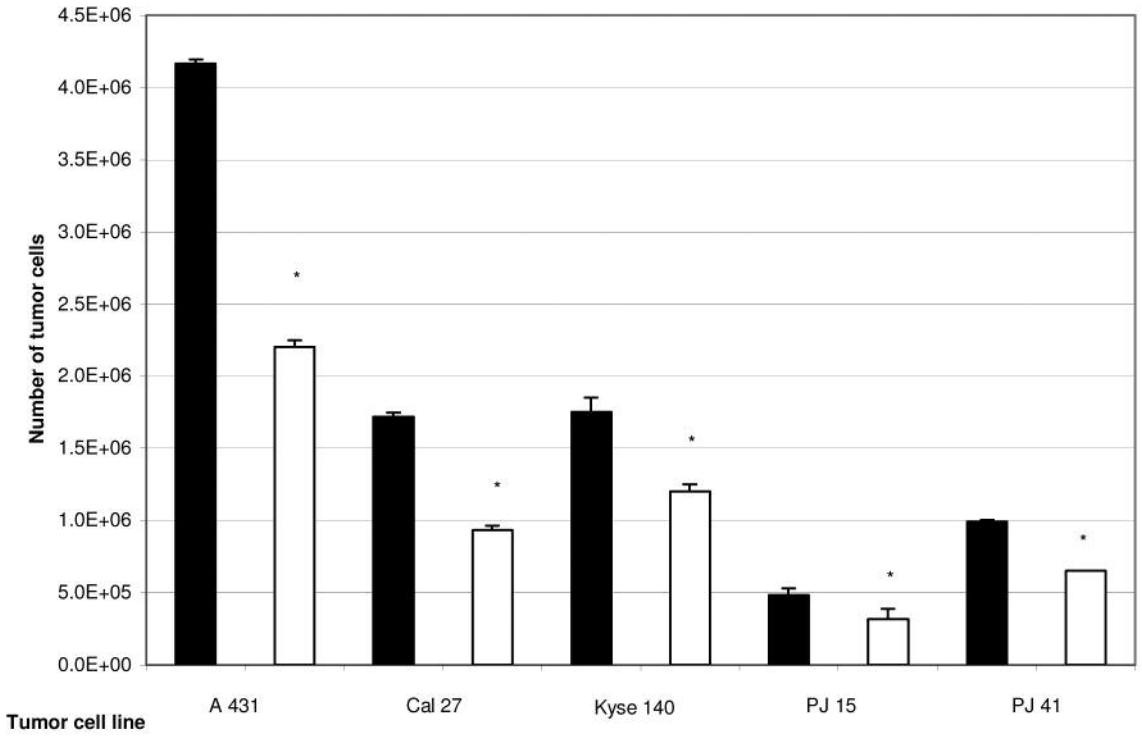

Figure 2. *Growth-inhibitory effect of bortezomib plus cetuximab (open columns) versus bortezomib monotherapy (closed columns) in five different squamous cell carcinoma cell lines. Bortezomib was used at fixed cell line-specific concentrations (see Table I) and cetuximab at 3 different concentrations. The absolute tumor cell numbers were determined in a Rosenthal chamber at 72 hours after treatment. Mean values of three independent experiments with standard deviation are shown. Significant differences between combination therapy and bortezomib monotherapy are indicated by asterisks. Only the results achieved with cetuximab at 5 μmol/l are shown. The growth-inhibitory effect of combination treatment with bortezomib and cetuximab at a concentration of 5 μmol/l was significantly greater than that with bortezomib monotherapy (p=0.043).*

shows a strong dependency on EGFR expression, although bortezomib itself does not influence EGFR expression. Cetuximab appears to enable bortezomib to exert its apoptotic effect in high EGFR expression HNSCC.

Taken together, the addition of cetuximab to bortezomib may improve the efficacy of treatment in HNSCC patients with a high expression of EGFR without adding severe systemic toxicities.

As experimental findings cannot be translated easily into clinical practice, clinical trials should be initiated to investigate the feasibility and optimal dosage of combination regimens including bortezomib and anti-EGFR agents.

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