Effect of Bortezomib on EGFR Expression in Head and Neck Squamous Cell Carcinoma Cell Lines

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Abstract. *Background: Overexpression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinoma (HNSCC) has often been correlated with poor prognosis. Recent investigations have shown that the proteasome inhibitor bortezomib exhibits a high antiproliferative and apoptotic activity in HNSCC cell lines. The present study investigated whether bortezomib has an effect on EGFR expression in different squamous cell carcinoma cell lines. Materials and Methods: Six EGF-stimulated or non-stimulated squamous carcinoma cell lines were treated with bortezomib. Western blots were performed to determine EGFR expression. For statistical analysis, a Wilcoxon test for matched pairs (dependent samples) was performed using SPSS 13.0 software for Windows. Results: Changes in EGFR expression after bortezomib treatment in EGF non-stimulated and EGF-stimulated squamous carcinoma cell lines failed to reach statistical significance in either experimental group ($p>0.05$). Conclusion: Given the high expression of EGFR in head and neck tumors, further investigations should address the question whether the apoptotic activity of bortezomib can be enhanced by adding an anti-EGFR antibody.*

Cancer of the head and neck is among the most common neoplastic diseases worldwide (1-3). In the last few years, more active treatment strategies with long-term beneficial effect have been sought. New antineoplastic drugs have been evaluated *in vitro* and *in vivo* (4-12). Some years ago, proteasome inhibition was shown to be an effective therapy in many tumor models, primarily in multiple myeloma, but also in solid tumors (13). The ubiquitin-proteasome pathway is the major non-lysosomal proteolytic system in the cytosol and nucleus of all eukaryotic cells, triggering degradation of proteins involved in cell cycle progression, apoptosis, inflammation and immune surveillance (14). The hierarchical nature of the ubiquitin-proteasome pathway provides a rich source of molecular targets for specific intervention and has therefore become a subject of interest for the development of novel antitumor therapies (15).

Recently, we demonstrated that bortezomib has antiproliferative and apoptotic activity in head and neck squamous cell carcinoma (HNSCC) cell lines (16). Although the molecular mechanisms underlying the antineoplastic effect of bortezomib have not yet been fully understood, it is well-known that bortezomib acts primarily by inhibiting the NF-κB pathway (17).

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 cells also express high levels of epidermal growth factor receptor (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 (18). Monoclonal antibodies and tyrosine kinase inhibitors specifically targeting EGFR have been studied most extensively and hold considerable promise for cancer treatment (7, 19, 20). Bortezomib has also demonstrated a high antiproliferative activity *in vitro* in HNSCC cell lines, but the effect of the drug on EGFR expression has not yet been reported. We therefore extended our research to investigate the effect of bortezomib on EGFR expression in six squamous cell carcinoma cell lines.

Materials and Methods

Six HNSCC cell lines were tested in this study. Detroit 562 and A 431 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). PE/CA-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 from DSMZ GmbH, Braunschweig, Germany. The fibroblast cell

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line (taken from infantile praeputium) was a gift from the Department of Dermatology, University Hospital, Frankfurt/Main, Germany. Bortezomib (Velcade®) was supplied by Millenium Pharmaceuticals Inc., Cambridge, MA, USA, and Johnson & Johnson Pharmaceuticals, Raritan, NJ, USA.

Squamous carcinoma cell lines were cultivated according to the instructions of the suppliers without antibiotics at 37ïC in the cell type-specific medium Quantum 263 with L-glutamine (PAA Laboratories GmbH, Pasching, Austria). Cells ($1.5x10^6$) were seeded in T 25 25 cm^2 cell culture flasks (Corning Inc., NY, USA), incubated for 24 h and then treated with bortezomib for another 24 h. 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. Concentrations of bortezomib ranged from 0.01 nmol/L to 2.5 μmol/L, depending on the cell line treated (Table I). The control cell lines were incubated for 24 h without bortezomib and then processed as described for the treated cell lines. All measurements were performed in triplicate.

After first experimental series with EGF unstimulated tumor cell lines, we stimulated the tumor cell lines with human epidermal growth factor (EGF) at a concentration of 10 ng/ml for 24 h at 37ïC to enhance the EGFR signalling pathway and thus tumor cell proliferation and mitosis. Subsequently, the six cell lines were treated with bortezomib as described above or remained untreated in controls.

After completion of treatment with bortezomib (or after 48 h of incubation in control cell lines) the EGF non-stimulated and stimulated cell lines were processed for Western blotting to determine EGFR levels and to compare EGFR expression between treated and untreated cells as follows. Whole cell extracts (50 Bg/lane) were electrophoresed through a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto a PVDF transfer membrane (Perkin Elmer, Canada). Molecular weight markers (Cell Signalling Technology, Inc., Frankfurt, Germany) were included. Membranes were blocked for 60 minutes in Tris-buffered saline (TBS, pH 7.5) with 0.5% Tween-20 (TBST) and 5% non-fat dry milk. After blocking, the membranes were incubated overnight with epidermal growth factor receptor (EGFR) antibody, dilution 1:1000, (Cell Signalling). After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the membranes were developed by using the enhanced chemiluminescence (ECL) detection system (Amersham Bioscience, Piscataway, NJ, USA). Mean values of pixel density for the 170 kDa EGFR bands in the Western blots of all tumor cell samples with and without bortezomib treatment were determined. Absolute pixel density was measured with a Kodak Digital Science Image Station 440 CF system (PerkinElmer, Cologne, Germany).

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

Treatment with bortezomib resulted in reduced EGFR expression in 4 of 6 squamous carcinoma cell lines (Detroit 562, PJ 15, PJ 41 and Cal 27). In the cell lines Kyse 140 and A 431, bortezomib led to increased EGFR levels (Figure 1a, b). In contrast, in EGF-stimulated cell lines bortezomib led to an up-regulation of EGFR in 5 of 6 tumor cell lines (PJ 15, PJ 41, Kyse 140, Cal 27 and A 431). Only in Detroit 562 tumor cells did bortezomib treatment result in a decrease in EGFR expression (Figure 2a, b). These changes in EGFR expression were not statistically significant ($p>0.05$) in either experimental group.

Table I. *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
Detroit 562	0.02 nmol/L
Kyse 140	2.5 nmol/L
PJ 15	2.5 Bmol/L
PJ 41	2.5 Bmol/L

Discussion

The finding that cancer cells are more sensitive to proteasome inhibition than normal cells has led to the proposition that the proteasome could be a novel target for cancer treatment (21). Early investigations suggested that human tumors commonly exhibit enhanced proteasome expression levels (22). More recently, an analysis of transcriptional profiles from about 200 solid tumors indicated that mRNAs encoding proteasome subunits were highly co-regulated in these cancer types (23). Bortezomib is a selective and reversible proteasome inhibitor with a wide range of molecular effects, including stabilization of cell cycle regulatory proteins, inhibition of NF-κB activation, induction of apoptosis, override of Bcl-2 resistance and is antiangiogenic (21, 24). The inhibition of the NF-κB pathway by bortezomib was also described by another group (25). Signalling by the EGFR tyrosine kinase family occurs through several downstream pathways to promote cell proliferation and to inhibit apoptosis (26, 27). Lorch *et al.* (28) postulated that EGFR itself is subject to ubiquitination and subsequent proteolytic breakdown, and that this process might be affected by proteasome inhibition. They further proposed that SCC15 and SCC68 cells were able to assemble functional cell adhesion complexes in response to EGFR inhibition in the presence of bortezomib.

However, our results are inconsistent with the effects of bortezomib described for the two squamous carcinoma of the tongue cell lines SCC15 and SCC68 by Lorch *et al.* (28). We studied six squamous carcinoma cell lines and did not find any statistically significant effect of bortezomib on EGFR expression. In additional experiments, bortezomib did not affect EGF-induced receptor phosphorylation either (data not shown). These results are in keeping with the findings of Condony-Servat *et al.* in breast cancer cell lines (23).

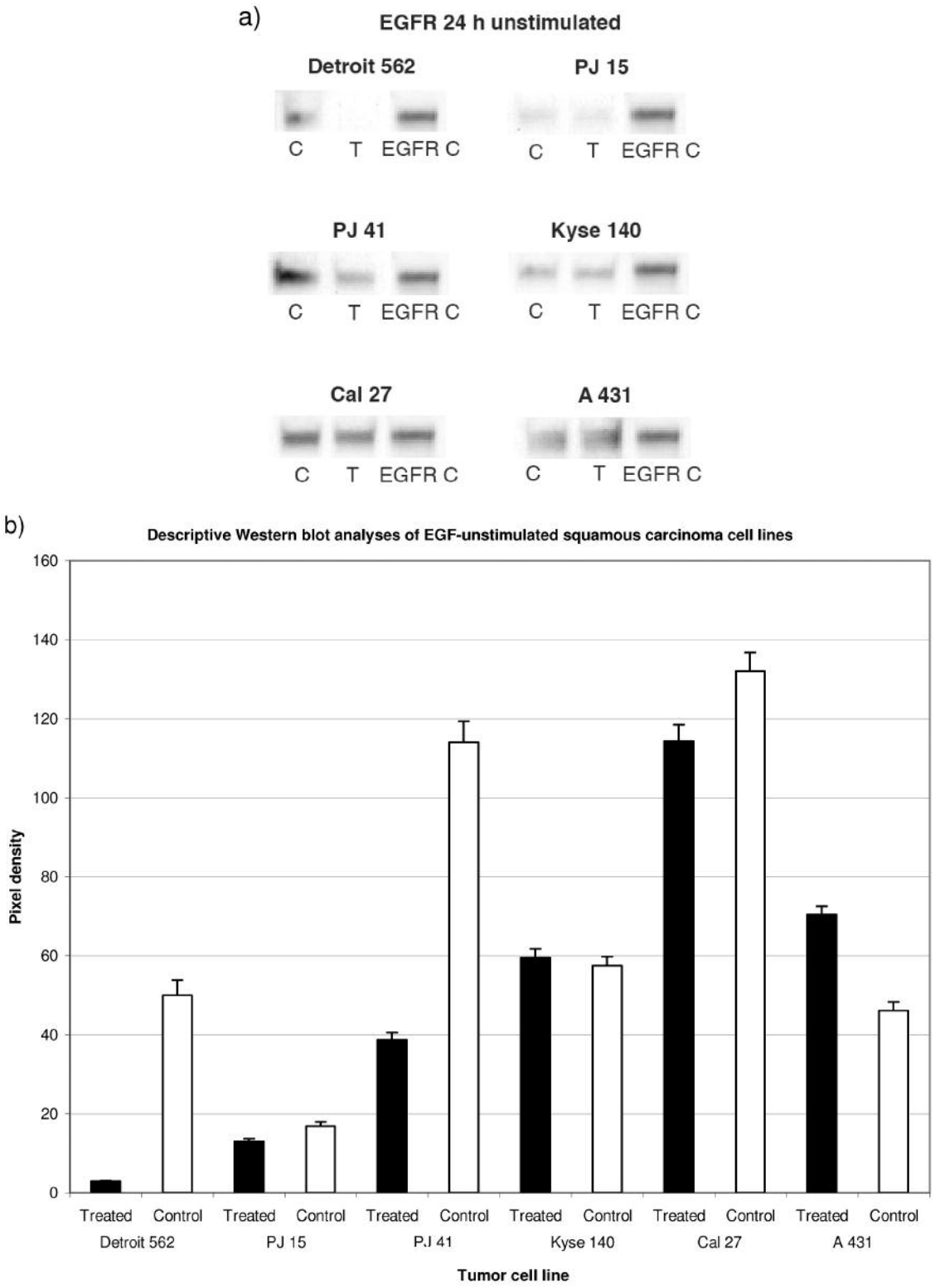

Figure 1. *a) Detection of EGFR protein by Western blotting in native squamous carcinoma cell lines either treated with bortezomib (T) or not (C). EGFR produced typical bands (EGFR-C) of 170 kDA (calibration band). b) Pixel density for the 170-kDa EGFR band in the Western blots of six different squamous carcinoma cell lines either treated for 24 h with bortezomib or not (controls). Mean value and standard deviation of three independent measurements are shown. There was no significant difference in EGFR expression ($p>0.05$ for comparison vs. control). Absolute pixel density was measured with a Kodak Digital Science Image Station 440 CF system.*

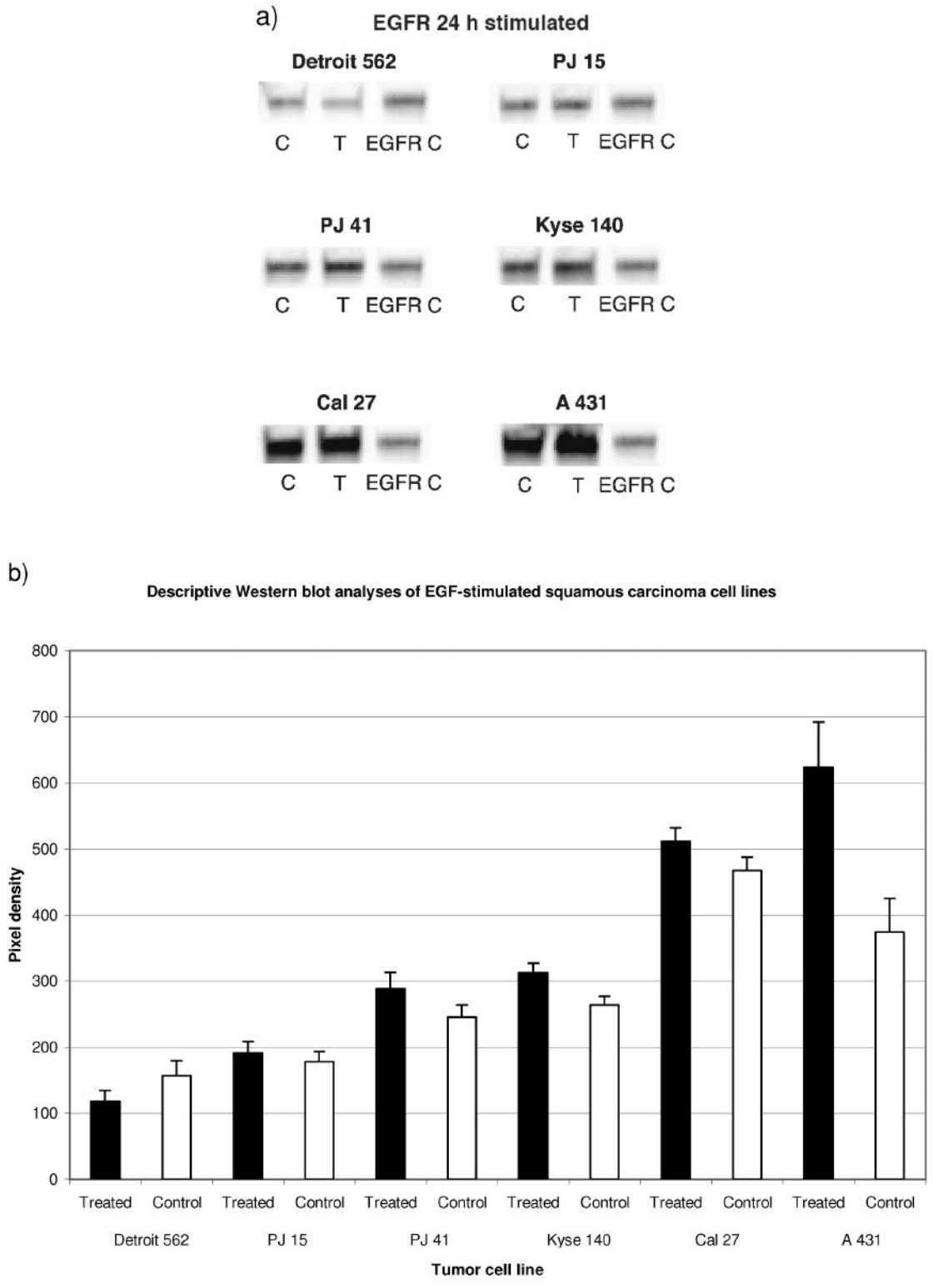

Figure 2. *a) Detection of EGFR protein by Western blotting in EGF-stimulated squamous carcinoma cell lines either treated with bortezomib (T) or not (C). EGFR produced typical bands (EGFR-C) of 170 kDA (calibration band). b) Pixel density for the 170-kDa EGFR band in the Western blots of six different EGF-stimulated squamous carcinoma cell lines either treated for 24 h with bortezomib or not (controls). Mean values and standard deviation of three independent measurements are shown. There was no significant difference in EGFR-expression ($p>0.05$ for comparison vs. control). Absolute pixel density was measured with a Kodak Digital Science Image Station 440 CF system.*

The fact that bortezomib induces apoptosis in head and neck cancer cells without affecting EGFR expression, primarily by inhibiting the NF-κB pathway, seems to be only part of the truth. Signalling through the EGFR has been shown to result in NF-κB activation (29), thus illustrating the complex mechanism of bortezomib with regard to EGFR action. Given the high expression of EGFR in head and neck tumors, it appears possible that the apoptotic effect of bortezomib may be enhanced by adding an EGFR antibody, such as cetuximab. Based on these considerations we are planning to embark on further investigations in HNSCC cell lines to evaluate the effect of this combined molecular treatment approach.

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