

Targeting the Proteasome as a Promising Therapeutic Strategy in Thyroid Cancer

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Background and Objectives: Targeting the ubiquitin–proteasome system by using proteasome inhibitors represents a novel approach for cancer therapy. Anaplastic thyroid cancer (ATC), a subtype of thyroid cancer (TC), fails to respond to conventional TC treatment. Here we investigated the effects of bortezomib on TC in vitro. Further, the study aimed to evaluate its potential for TC treatment in vivo.

Methods: Three anaplastic (Hth74, C643, Kat4), one follicular (FTC133), and one papillary (TPC1) TC cell lines were used. Antiproliferative, proapoptotic, and transcriptional effects of bortezomib treatment were analyzed in vitro and growth inhibition of ATC xenografts in vivo. Tumor samples were analyzed by Ki67, CD31, caspase-3, and NF- κ B immunohistochemistry.

Results: In vitro, bortezomib inhibited proliferation of TC cells (IC₅₀ 4–10 nM), increased caspase-3 activity and induced cell cycle arrest. NF- κ B activity was affected differently. In vivo, bortezomib treatment was effective in reducing tumor volume (up to 74%), accompanied by reduced proliferation (Ki67) and 57% reduced tumor vascularity.

Conclusion: Proteasome inhibition is effective in reducing cell growth and inducing apoptosis of ATC in vitro and inhibiting tumor growth and vascularity in vivo. However, the impact on nuclear transcription remains controversial. Clinical evaluation of bortezomib treatment in ATC is warranted.

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KEY WORDS: thyroid cancer; proteasome inhibitors; xenotransplantation; mitotic arrest

INTRODUCTION

Among the endocrine malignancies thyroid cancer (TC) not only represents the most common malignant tumor, but also shows an increasing incidence [1]. Standardized treatment of differentiated thyroid cancer (DTC) by surgery and radioiodine results in long term survival. In contrast dedifferentiation of a small number of these tumors is thought to give rise to anaplastic thyroid carcinoma (ATC), which is one of the most aggressive malignancies with fatal outcome [2]. Chemotherapy or radiotherapy so far serve merely as palliation [3,4], and anaplastic or poorly differentiated carcinomas exhibit an extremely aggressive behavior resulting in a median survival of only months [2,5,6]. In the face of the poor clinical outcome of ATC-patients, there is a desperate need for innovative and effective treatment options.

The increasing effort in preclinical and clinical research focussing on TC gained new insights into its development and biological behavior. Together with the development of new antitumoral compounds targeting various tumor promoting pathways, this also raised new hope of detecting an effective treatment option for ATC.

One member of the new, promising class of compounds are inhibitors of the proteasome. The multicatalytic ubiquitin–proteasome pathway is responsible for the degradation of eukaryotic cellular proteins [7–9]. This adenosine-triphosphate (ATP) dependent process is vital for normal cell cycling, function, and survival, making proteasome inhibition a novel therapeutic target in cancer. Bortezomib is the first inhibitor of the ubiquitin–proteasome pathway that reached clinical studies, especially in multiple myeloma (MM) [10], but also solid tumors [9]. Bortezomib displayed strong antitumoral activity

and was therefore approved for the treatment of MM by the US Food and Drug Administration in 2003.

Although recent evidence has displayed promising results when proteasome inhibitors were applied to TC cells in vitro, the lack of systematic in vitro and in vivo preclinical data makes its clinical use uncertain. The treatment of ATC cells with bortezomib induced caspase mediated induction of apoptosis and cytotoxic effects alone or in combination with Doxorubicin or TRAIL inhibitors. Inhibited NF-kappaB (NF- κ B) activity and increased p53, p21, and jun expression are additional possible explanations for decreased cell proliferation [11,12]. A sensitizing effect of the BH3 domain inhibitor (BH3I-1) on apoptosis induced by bortezomib was demonstrated in a single ATC cell line (FRO), indicating a synergistic mechanism [13]. Furthermore, the generation of reactive oxygen species (ROS) as an indicator of oxidative stress was shown to be implicated in cytotoxic responses of ATC cells to bortezomib. The sensitivity of TC cells to bortezomib seems to be associated with the inability to induce glutathione (GSH) synthesis and subsequent reduced intracellular GSH levels [14]. In a panel of ATC cell lines glucose-regulated protein 78 kDa (GRP78) and CCAAT/

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enhancer-binding protein homologous protein (CHOP) were shown to influence the sensitivity of ATC cells to the action of bortezomib in the sense that suppression of CHOP induction or overexpression of GRP78 partially prevents proteasome inhibition-mediated cell death. Therefore, GRP78 has been suggested as predictor of sensitivity to proteasome inhibitors in TC [15].

In an experimental setting an ATC cell line (MB1), freshly established from a patient suffering from ATC, was shown to respond to bortezomib treatment. Second-line treatment with bortezomib induced necrosis in the relapsed tumor mass of the patient as demonstrated by imaging and fine-needle biopsy [16].

Overall very few data focussing on the role of proteasome inhibitors in ATC are yet available and the clinical impact remains somewhat unclear. We conducted a systematic analysis of antitumoral effects induced by bortezomib on ATC cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Culture and Reagents

Five TC cell lines, one papillary (TPC1 [17], one follicular (FTC133 [18] and three anaplastic (Hth74, C643 [19], Kat4.1 [5]) were used. Cells were maintained in full growth medium (FGM) as described previously [20]. During experiments FGM was changed to serum-starved conditions (2%FCS). Cell viability was assayed by trypan blue exclusion.

Drugs

Bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA). *In vitro* experiments bortezomib stock solutions of 2.5 mM were prepared in DMSO. For *in vivo* experiments bortezomib was dissolved in PBS.

Cell Growth Inhibition Studies

The antiproliferative activity of bortezomib was evaluated using the MTT assay. Briefly, cells were plated at a density of 1×10^4 cells/well in 96-well plates, changed to serum starved conditions after 24 hr and treated with increasing concentrations of bortezomib (1–20 nM) for up to 144 hr. After 24, 72, and 144 hr cell density was determined and IC_{50} values were calculated. All experiments were repeated three times with triplicates each.

NF- κ B DNA-Binding Activity

The DNA-binding activity of NF- κ B was quantified by ELISA using the trans-AM NF- κ B p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA) according to manufacturer instructions. Briefly, cells were incubated with and without bortezomib (10–100 nM) for 24 hr, harvested, and then nuclear extracts were prepared using a nuclear extraction kit (Active Motif). Protein content was quantified by BCA protein assay (Pierce, Rockford, IL). Nuclear extracts (10 μ g) were incubated in 96-well plates pre-coated with an oligonucleotide containing a consensus binding site for the p65 subunit of NF- κ B. Binding of NF- κ B was detected by incubation with an antibody specific for the activated form of the p65 subunit, followed by development of the ELISA using an anti-IgG HRP conjugate and a developing solution provided by the kit. OD was determined at 450 nm with a reference wavelength of 670 nm. Experiments were repeated twice with duplicate estimations for each condition.

Apoptosis Array Analysis

A human apoptosis array kit (ARY009, RnDSystems, Minneapolis, MN) was used to evaluate the effect of bortezomib on apoptosis-

related proteins in TC cell lines. Briefly; 1×10^7 cells were incubated with and without bortezomib (100 nM) for 24 hr. Then cell lysates were prepared using lysis buffer provided by the kit and protein content quantified by BCA protein assay. Array membranes were incubated with lysates (500 μ g protein each) for 16 hr at 4°C, washed and incubated with the detection antibody cocktail, followed by Streptavidin–HRP conjugate, both provided by the kit. For development a chemiluminescent reagent was used. Relative expression levels of the different proteins were estimated by placing the transparent overlay on the array image.

Caspase-3 Activity

For analysis of caspase-3 activity cells were plated into triplicate wells of 96-well plates (1×10^4 cells/well), switched to 2%FCS and incubated with and without bortezomib (5, 10, 50, and 100 nM) for 24 hr. Caspase-3 activity was assessed by a luminescence based caspase assay (Caspase Glo™ 3/7 Assay, GloMax Multimode Reader (Promega, Mannheim, Germany)) according to manufacturer instructions. Experiments were repeated twice.

Flow Cytometry and Cell Cycle Analysis

Cells were incubated with or without bortezomib (10, 20 nM) in serum starved medium for 24 and 48 hr, then harvested, fixed in ethanol, stained with propidium iodide (PI) (50 μ g/ml PI, 200 μ g/ml RNase) for 15 min and analyzed by flow cytometry (BD LSRII cytometer, BD Franklin Lakes, NJ). Percentages of cells in G1, S, and G2/M phase were determined using ModFit (Verity Software House, Topsham, ME).

Western Blot Analysis

Cells maintained in FGM were used for Western blot analysis of GRP78 and CHOP expression. For analyzing *in vitro* effects of proteasome inhibition cells were treated with increasing concentrations of bortezomib (0.1, 1, 10 nM) for 24 hr. Total cell lysates were prepared using RIPA (1% Nonidet P40, 0.5% Na-desoxy-cholat, 0.1% SDS) and protein content determined by BCA protein assay (Pierce). Samples adjusted to 20–50 μ g protein were separated on SDS-PAGE (10%; 12%) and transferred onto nitrocellulose (GRP78) or PVDF (CHOP) membranes (Amersham, Piscataway, NJ). Membranes were probed using anti-GRP78 (BD Bioscience, San Diego, CA, 1:4,000) and anti CHOP (anti-GADD153 (F-168), SantaCruz, Heidelberg, Germany, 1:1,000) as primary antibodies. Visualization was achieved using horseradish peroxidase conjugated secondary antibodies (Dako, Glostrup, Denmark, 1:2,000) and ECL Western blotting detection reagent (Amersham). Equal loading was verified by anti- β -Actin ((AC-74), Sigma, St. Louis, MA, 1:3,000) staining.

In Vivo Animal Experiment

Six-week-old athymic nude mice (nu/nu) were purchased (Harlan Winkelmann, Borcheln, Germany) and allowed to adapt to the laboratory environment for 1 week. Experiments were performed as described previously (21). Briefly, anaplastic Kat4 cells were immobilized in Matrigel[®] (300 mg/ml, BD Biosciences, San Jose, CA) and xenotransplanted subcutaneously into the right flank of the nude mice (3×10^6 cells/animal). After tumors reached 5 mm in diameter mice were randomized into two groups (n = 10) and treated with bortezomib (0.5 mg/kg, 2 days/week) by *i.p.* injections. Control groups were treated with vehicle or left untreated. Tumor size, animal weight, and side effects were monitored weekly. After 4 weeks mice were sacrificed and the tumor tissue removed and processed for

immunohistochemistry. Tumor volume (TV) was calculated as described elsewhere [21].

Immunohistochemical Studies

Three micrometer paraffin sections, prepared from the xenografts, were used for immunohistochemical analysis. Staining procedures were performed as detailed before [21]. The following antibodies were used: monoclonal mouse anti-Ki67 (Sigma; 1:800), goat anti-CD31 ((PECAM-1, M-20), Santa Cruz, 1:1,000), polyclonal rabbit anti-cleaved caspase-3 ((ASP175) Cell Signaling, Danvers, MA, 1:100) and rabbit polyclonal NF-κB p65 ((C20), Santa Cruz, 1:1,500). Further processing was carried out using ABC-Staining Kits (Vector Labs, Burlingame, CA) and hematoxylin according to Mayer.

Morphometrical analysis was performed using the Leica imaging system QWin (Leica, Wetzlar, Germany). Ki67 staining index was determined by counting the number of positive stained cells per high power field (×40) in 10 random areas within the maximal tumor cell load. Tumor angiogenesis was quantified following CD31 staining by assessment of the vascular surface density (VSD, 1 mm⁻¹) as described earlier [22,23]. Cleaved caspase-3 and NF-κB p65 staining was quantified by assessment of positive stained areas in 10 random areas (×40).

Statistical Analysis

Statistical analysis was performed using the Wilcoxon rank-sum test and an extension of the non-parametric Kruskal-Wallis test as indicated in figure legends. A *P*-value < 0.05 was considered to be significant.

RESULTS

Bortezomib (Velcade®) Inhibits Growth of Thyroid Cancer Cells

We first investigated the effect of bortezomib on various proliferating TC cell lines using the MTT assay for assessment of cell density. A time- and dose-dependent inhibition of cell proliferation was shown (Fig. 1). The antiproliferative effects were most pronounced

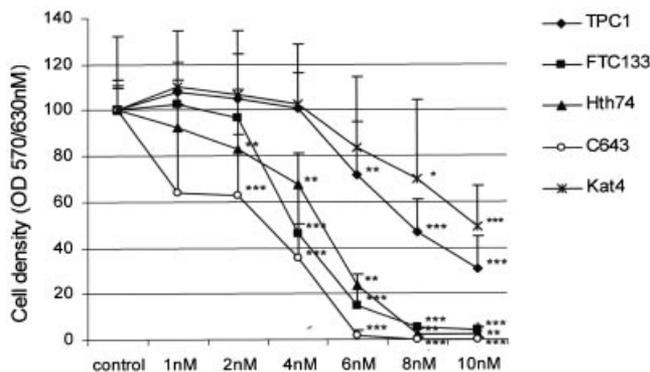


Fig. 1. Effects of bortezomib on proliferation of different TC cell lines: TPC1 papillary, FTC133 follicular, Hth74, C643, and Kat4 anaplastic. Cells were incubated with indicated concentrations of Bortezomib for 144 hr and cell density measured using the sulphorhodamine B assay. Data reported represent mean ± SD of three independent experiments with triplicates each. Control: cells grown under standard conditions. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 as revealed by the Wilcoxon rank-sum test.

in the anaplastic C643 and Hth74 cells and in follicular FTC133 cells. Here cell numbers were reduced by 100%, 98%, and 95% (10 nM bortezomib, 144 hr) compared to 70% in TPC1 (papillary) and about 50% in Kat4 (anaplastic) cells. IC₅₀ values at 144 hr were 2.6 ± 1.9 nM (C643), 3.6 ± 0.7 nM (FTC133), 4.6 ± 0.1 nM (Hth74), 8.1 ± 2.6 nM (TPC1) and 11.9 ± 2.1 nM (Kat4), revealing the C643 cells as the most sensitive to bortezomib treatment.

Bortezomib (Velcade®) Leads to Cell Cycle Arrest

After incubation with bortezomib (10 and 20 nM, 24 hr) and PI staining DNA profiles were created by flow cytometry and cells in G1, S, and G2/M phase were calculated in percentages. A dose-dependent accumulation of cells in G2/M, with a corresponding decrease of the number of cells in G1, was shown (Table I). The G2/M arrest induced was characterized by accumulation of up to 50% of cells in G2/M phase after treatment with 20 nM bortezomib for 24 hr, compared to values <5–20% in control cells. The effect was most pronounced in FTC133 (45.8% vs. 3.68% in untreated control), Hth74 (26% vs. 4.09%) and C643 (55.6% vs. 14.9%) and comparatively low in Kat4 cells (18.7% vs. 2.4%). In TPC1 cells DNA profile was not affected by bortezomib treatment.

Bortezomib (Velcade®) Elicits Pro- and Antiapoptotic Effects on Thyroid Cancer Cells

Bortezomib is described as inducing apoptotic cell death. We exposed TC cells to bortezomib (100 nM) for 24 hr to investigate the effects on apoptosis related proteins using an apoptosis protein array (Table II). As expected, caspase-3 was induced in all cell lines. Inducing or stabilizing effects were further exerted on TRAIL Receptors, p21/CIP1, p53 (three of five cell lines), and HSP60 (two of five cell lines) and p27/KIP1 (one cell line). Surprisingly, in FTC133 cells these pro-apoptotic proteins, except Caspase-3, were down-regulated. Stabilizing effects were also exerted on some anti-apoptotic proteins. Thus, Claspin expression was increased slightly in all cell lines, whereas HIF 1α and HO-1 proteins were increased in four of five and HSP70 and XIAP in two of five TC cell lines. From the anti-apoptotic proteins only X-linked inhibitor of apoptosis protein (XIAP), a member of the inhibitor of apoptosis protein family, was down-regulated in FTC133 cells. The anaplastic cell line Hth74 was the one most affected by bortezomib regarding pro-apoptotic proteins (Table II).

Bortezomib (Velcade®) Enhances Caspase-3 Activity

As presented in Table II expression of caspase-3 protein was induced by bortezomib treatment in the TC cells used in this study. Therefore, we further investigated the effect of bortezomib on caspase-3 activity using a luminescence-based caspase assay. Incubation with concentrations up to 100 nM bortezomib for 24 hr resulted in a significant dose-dependent increase of caspase-3 activity. Induction of caspase-3 activity differed depending on the cell line and ranged in magnitude from 8- to 20-fold compared to the vehicle treated control (Fig. 2A).

Bortezomib (Velcade®) Displays Different Effects on NF-κB Activity

The mechanism by which bortezomib acts includes the inhibition of inhibitory-κB (IκB)-degradation by the proteasome which in turn leads to decreased NF-κB activity. Using an ELISA based assay, baseline NF-κB DNA binding activity was demonstrated for all cell lines tested (Fig. 2B). Bortezomib treatment (as indicated) resulted either in increased (TPC1, Hth74) or suppressed (C643) NF-κB DNA binding activity, depending on the cell line. In FTC133

TABLE I. G2/M-Arrest Induced by Bortezomib in Different TC Cell Lines

Cell line	TPC1		FTC133		Hth74		C643		Kat4.1	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
Untreated	31.68	63.92	79.64	3.68	89.72	4.09	59.13	14.88	83.38	1.27
10 nM	30.75	65.81	63.48	17.55	86.30	13.70	27.63	50.58	84.96	5.21
20 nM	30.64	63.94	36.98	45.78	54.75	26.04	16.03	55.61	69.94	18.68

Note: TC cells incubated with and without bortezomib at indicated concentrations and analyzed by FACS after PI-staining. Numbers represent values of one experiment.

(follicular) and Kat4 cells NF- κ B DNA binding activity was hardly affected (Fig. 2C).

GRP78 Expression Shows no Correlation to Bortezomib-Sensitivity and is not Affected by Bortezomib Treatment

GRP78, a protein belonging to the class of ER-stress chaperones, prevents cells from apoptosis and the level of GRP78 protein expression has been supposed to predict sensitivity to proteasome inhibition by different drugs (Wang et al. 2007). In the cell lines used in this study GRP78 protein was found to be constitutively expressed at nearly the same level (Fig. 3A). Treatment with bortezomib with concentrations up to 10 nM for 24 hr failed to display distinct effects on GRP78 level (Fig. 3B).

CHOP is Expressed in Thyroid Cancer Cell Lines and Differently Affected by Bortezomib Treatment

CHOP belongs as well as GRP78 to the ER-stress chaperones and is involved in the unfolded protein response (UPR). Over-expression of CHOP promotes apoptosis and was suggested to predict sensitivity to proteasome inhibition [15]. In this study CHOP protein was found to be expressed in all TC cell lines but at different levels. It was highly expressed in Kat4 cells, followed by TPC1 and C643 cells, and only minorly in FTC133 and Hth74 cells (Fig. 3C).

TABLE II. Summary of the Effect of Bortezomib on Apoptosis-Related Proteins In Vitro

Protein	Cell line				
	TPC1	FTC133	Hth74	C643	Kat4
Cleaved Caspase-3	(+)	++	++	++	++
Claspin	+	+	+	+	++
TRAIL R1/DR4	++	-	+	(-)	++
TRAIL R2/DR5	++		++	+	(+)
HIF-1 α	+	+		++	+
HO-1/HMOX1/HSP32	++	+		++	++
HSP 60		+	(+)	+	+
HSP 70	+		++		(+)
p21/CIP/CDNK1A	++	-	++		+
p27/KIP1		-	++		
phospho p53 (S15)	++	-	++		+
phospho p53 (S46)	++	--	++		+
phospho p53 (S392)	++	--	++		+
XIAP		-	++	(+)	+

Note: Proteins induced: (+) weak, +middle, ++strong or down-regulated: (-) weak, -middle, --strong) as revealed by apoptosis protein array performed as described in Material and Methods section.

However no correlation was found between constitutive CHOP expression and sensitivity to proteasome inhibition by bortezomib. In response to bortezomib CHOP protein expression increased in FTC133 and Hth74 cells, to a lesser extent in TPC1 cells, and decreased in C643 cells. In Kat 4 cells the level of CHOP expression remained unchanged (Fig. 3D). The mode of action did not correlate with the sensitivity of the cell lines used, neither regarding the anti-proliferative nor the pro-apoptotic effects.

Bortezomib Inhibits Growth of Xenotransplanted Anaplastic Thyroid Cancer Cells

Since TC cells were shown to be highly sensitive to bortezomib treatment in vitro we further evaluated the potential of bortezomib in suppressing tumor growth in vivo. The effect was explored on anaplastic Kat4 cells, xenotransplanted to nude mice. Application of bortezomib (0.5 mg/kg, 2 day/week) by i.p. injections for 4 weeks resulted in marked tumor growth inhibition (Fig. 4A). Average TV was reduced by 63% compared to the vehicle treated control group. However the differences between the bortezomib and vehicle treated group were not significant ($P = 0.1462$).

Bortezomib Affects Proliferation, Angiogenesis, Apoptosis, and NF- κ B Activity In Vivo Ki 67

The effect of bortezomib on tumor cell proliferation in vivo was analyzed by Ki67 immunohistochemistry in ATC xenografts. Tumor growth inhibition was found to be accompanied by decreased tumor cell proliferation. In the treatment group, the number of Ki67 stained cells was significantly reduced to 11.1 ± 5.16 compared to 35.2 ± 5.56 in the vehicle treated group ($P < 0.05$; Fig. 4B). Based on this data bortezomib was calculated to reduce the proliferative activity by about 69% in xenografted ATC tumors.

VSD

As revealed by CD31 staining and morphometrical analysis bortezomib also exhibited potent antiangiogenic activity in vivo. VSD was significantly reduced ($P < 0.05$) in the treatment group (0.009 ± 0.002 (1/mm)) compared to control tumors (0.021 ± 0.005 (1/mm)) as shown in Fig. 4C. Expressed as a percentage VSD was reduced by about $\approx 57\%$.

Caspase-3

To investigate the effects of bortezomib on apoptosis, tumor sections were stained with an antibody to cleaved caspase-3. Caspase-3 reactivity, quantified as percentages of positively stained areas in 10 random fields of the tumor mass, differed greatly between the animals treated with bortezomib ($3.82 \pm 1.91\%$) and the vehicle treated animals ($0.89 \pm 0.514\%$; Fig. 4D). Thus, in vivo caspase-3 activity was increased about fourfold by bortezomib.

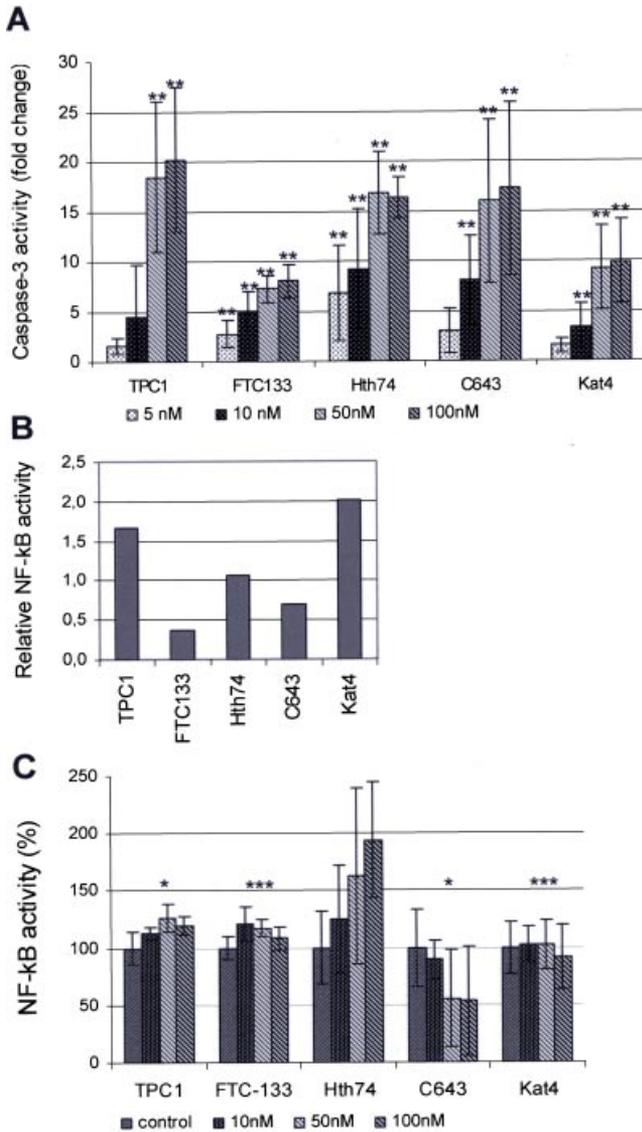


Fig. 2. In vitro effects of bortezomib on caspase-3 activity (A), baseline level of NF-κB activity (B) and in vitro effects of bortezomib on NF-κB DNA-binding activity (C). A: Caspase-3 activity of TC cells incubated with indicated concentrations of bortezomib for 24 hr was determined and the fold change compared to vehicle treated samples was calculated. Values represent mean ± SD of three independent experiments with duplicates each. ***P* < 0.01 as revealed by the Wilcoxon rank-sum test. B: Baseline DNA-binding activity of NF-κB in TC cells included in this study as quantified by an ELISA based assay. C: DNA-binding activity of NF-κB in TC cells incubated with indicated concentrations of bortezomib for 24 hr was quantified by ELISA as described in Materials and Methods section. Results presented as percent of the value of untreated control samples of two independent experiments with duplicates each. Control: DMSO-treated cells. For statistical analysis a linear model adjusting for the repetition of experiments in separate well plates was used to test for an effect of bortezomib on NF-κB activity in each cell line. **P* < 0.05, ****P* < 0.001.

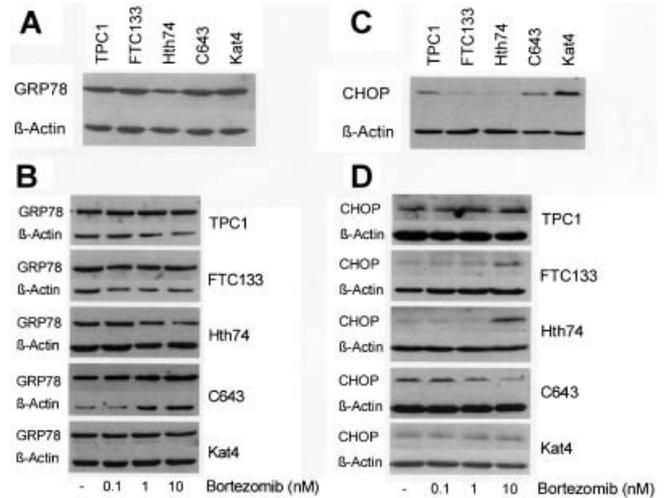


Fig. 3. GRP78 (A) and CHOP (C) expression in TC and bortezomib effects on expression of GRP78 and CHOP protein (B,D). A: whole cell lysate (50 μg/lane) separated on SDS-Page and analyzed for GRP expression by immunoblotting showing nearly the same expressions level for all cell lines. C: Western blot of whole cell lysates showing different expression of CHOP protein in the cell lines used in this study. B,D: whole cell lysates prepared from TC cells treated with indicated concentrations of bortezomib for 24 hr were analyzed by Western blotting (50 μg/lane) using anti-GRP78 and anti-CHOP antibodies. B: no bortezomib-effect on expression of GRP78 was found. D: expression of CHOP protein is differently affected by bortezomib in the TC cells used. β-Actin was used to ensure equal gel loading.

NF-κB

Tumor growth inhibition caused by bortezomib treatment seems further to be accompanied by decreased NFκB activity as revealed by immunohistochemistry using an antibody recognizing the NF-κB p65 subunit, which translocates into the nucleus after activation of NF-κB by phosphorylation. Staining was predominately cytoplasmic and was decreased about threefold in the bortezomib treated tumors (1.89 ± 0.97%) compared to those in the vehicle treated group (5.29 ± 4.63%; Fig. 4E). However, this decrease was not statistically significant.

DISCUSSION

In addition to the antitumoral action of proteasome inhibitors like bortezomib on hematologic malignancies, such as MM, increasing evidence raised new hope of proving this class of drugs to be effective in solid tumors as well. However, the results of the first clinical studies that proved effectiveness in advanced prostate and non-small cell lung cancer failed to display antitumoral effects in other types of solid tumors [9]. Therefore, further preclinical and clinical studies are needed to clarify the potential for possible clinical application. In TC bortezomib was shown to act anti-proliferatively and pro-apoptotically when applied to ATC tumor cells in vitro [11,16], but data regarding its effect in vivo or in clinical investigations are lacking. The present study of bortezomib in ATC showed it to exert effective antitumor action in vitro and in vivo, displayed by a decrease in proliferation and neoangiogenesis and an increase in apoptosis.

In this study bortezomib was shown to affect cell viability in all histological types of TC cells with IC₅₀-values in the range of about 2–12 nM. These values are in line with those reported by Mitsiades

et al. [11] for TC. Higher IC₅₀ values have been reported for other solid tumors, up to 1,000 nM for example in breast cancer cell lines [24,25]. Thus, TC cells can be said to be sensitive to bortezomib treatment. In this context it is important to notice that clinically achievable doses are limited to values of 10 nmol/ml bortezomib [26].

Bortezomib treatment resulted in a dose-dependent G₂/M-arrest in all cell lines except TPC1 cells (papillary), an outcome that has been shown in prostate, hepatocellular, breast cancer, and mesothelioma cell lines before [8,24,25]. However, incubation of TC cells with concentrations as high as 25 nM was reported to induce accumulation of cells in the Sub-G₁ region, indicating induction of apoptosis [11]. Since bortezomib concentrations higher than 20 nM severely affect viability of TC cells (own observation), this might be due to the experimental conditions used.

Prominent pro-apoptotic activity of bortezomib in TC cells as shown by an up to twenty-fold increase of caspase-3 activity can be asserted, an observation that was documented for TC cells before

[15]. However, the effect on caspase-3 activity in ATC cells was not always correlated with inhibition of NF- κ B activity, as already noted earlier [15]. Bortezomib-caused induction of apoptosis was further indicated by stabilization of caspase-3 protein shown using an apoptosis protein array. In addition to caspase-3 TRAIL-Receptors, XIAP, p53, HIF1 α , Claspin as well as p21/Cip1, and p27/Kip1 were also found to be affected by bortezomib treatment. Bortezomib-induced accumulation of p53 protein, a known effect of proteasome inhibition, was found in three of five TC cell lines in the present study. According to Mitsiades et al. [27] this effect is independent of NF- κ B and was thought to induce apoptosis via a p53-dependent pathway [28]. Stabilizing effects on p21/CIP1 and p27/KIP1 were shown for different types of cancer like mesothelioma and hepatocellular carcinomas [24,29,30], as well as for the TC cells used in the present study. In this context p21/CIP1 was shown to be induced transcriptionally by p53 [31]. The accumulation of p21/CIP1 and p27/KIP1, both of which are important cell cycle regulators, is of great interest, since their expression is down-regulated in various types of cancers [9] and especially the accumulation of p27/KIP1 was recently suggested to possibly account for most of the biological effects induced by proteasome inhibition [32].

Bortezomib has been shown to sensitize different tumor cells to TRAIL-induced apoptosis [reviewed in Sayers and Murphy [33]]. Among other things this effect was suggested to be mediated by increased expression of TRAIL-Receptors [34–36]. Therefore, bortezomib/TRAIL cotreatment has become a promising approach for cancers resistant to induction of apoptosis [37]. In our study TRAIL-Receptors were increased by bortezomib in three of five (TRAIL-R1) and four of five (TRAIL-R2) TC cell lines. This is consistent with a study from Conticello et al. [12], where not only up-regulation of TRAIL-receptors, but also synergistic effects of combined treatment with bortezomib and TRAIL were documented for ATC in vitro. Various studies demonstrated that proteasome inhibition by bortezomib leads to decreased NF- κ B activity due to the proteasome dependent regulation of I κ B, even for TC cells [11,38]. In the cell lines used in the present study NF- κ B activity was diversely affected by bortezomib, but these effects did not correlate with the bortezomib-induced effects on cell survival. This is consistent with findings on TC cells using MG132 as inhibitor [15] and was also reported for endometrium cancer and MM [39–41]. Therefore, proteasome-dependent pathways apart from NF- κ B signaling might be involved

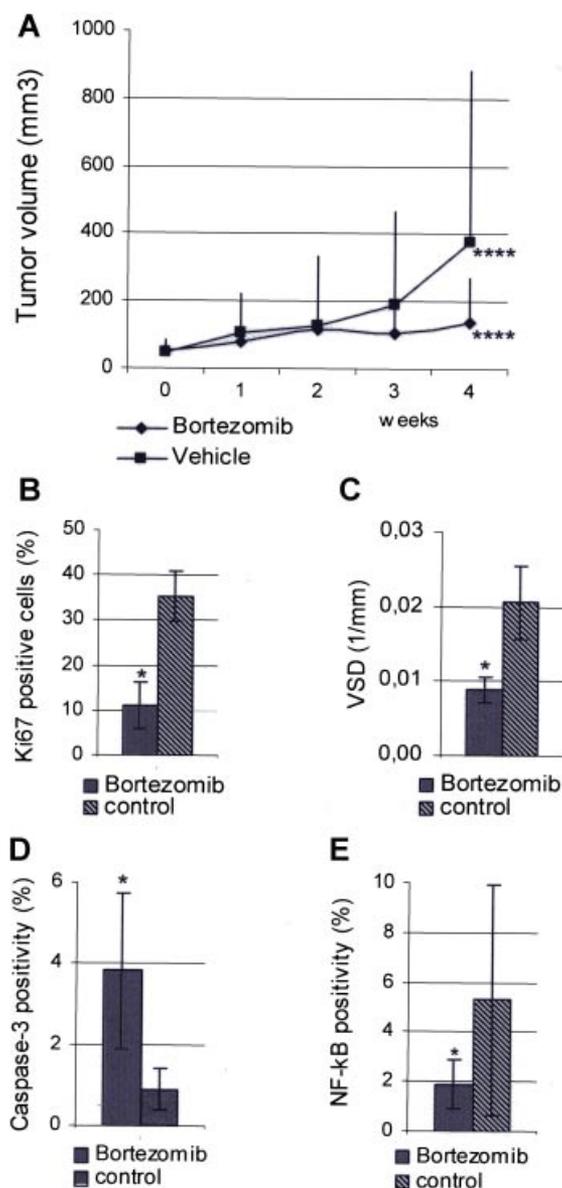


Fig. 4. Bortezomib efficacy in vivo. **A:** tumor growth inhibition induced by bortezomib. TC cell (Kat4) tumor bearing mice were treated with bortezomib (0.5 mg/kg, i.p. application, twice a week) for 4 weeks and TV calculated as $TV = (l \times b^2) \times 0.5$. Graph depicts average TV for vehicle and bortezomib treated animals \pm SD for each group ($n = 10$). For statistical analysis an extension of the non-parametric Kruskal–Wallis test to time-series was used to calculate the P -value for differences in the TV growth pattern between the bortezomib and vehicle treated group. **** $P = 0.1462$. **B–E:** Immunohistochemical analysis of the tumor samples regarding proliferation (B), angiogenesis (C), apoptosis (D), and NF- κ B activity (E) as detail in Materials and Methods section. **B:** rate of proliferation as revealed by Ki67 immunostaining and counting Ki67 positive cells in 10 fields ($\times 40$) per slide. Means \pm SD of Ki67 positive cells depicted for treated and control group ($n = 10$ each). **C:** In vivo effect of bortezomib on angiogenesis as revealed by measurement of VSD following CD31 immunostaining. **D:** Means \pm SD of caspase-3 positivity (%) for the bortezomib treated and control group ($n = 10$) depicted, 10 fields ($\times 40$) per slide analyzed. **E:** Means \pm SD of NF- κ B positive area (%) depicted for the bortezomib treated and control group ($n = 10$). Ten fields ($\times 40$) per slide analyzed. The Wilcoxon rank-sum test was used to compare the percentage of Ki67 positive cells, VSD, Caspase-3 positive and NF κ B positive area in percent respectively between the bortezomib and control groups. * $P < 0.05$.

in the response of TC cells to bortezomib as it was suggested for MM and gastric cancer [35,42].

GRP78 and CHOP expression were suggested to predict sensitivity to proteasome inhibitors in ATC [15]. In the present study GRP78 was expressed at similar levels in all cell lines and no changes were observed after bortezomib treatment. This is in line with the high sensitivity to bortezomib as displayed by IC₅₀ values ≤10 nM and the positive effects on induction of apoptosis documented. CHOP expression varied depending on the cell line and was affected differently by bortezomib. In contrast to Wang et al. [15] no correlation between bortezomib sensitivity and CHOP expression was found. Since changes in CHOP expression induced by bortezomib seem to be time-dependent this might be due to experimental conditions and demands further clarification.

When xenotransplanted ATC cells were treated by bortezomib a strong reduction in tumor growth of about 70% in a 4 week period of drug application was seen, however it failed to reach a significant level. To our best knowledge this is the first time that a relevant decrease in tumor growth was demonstrated in an in vivo ATC tumor model. This effect consisted of reduced tumor cell proliferation, diminished VSD within the tumor mass and an increase in tumor cell apoptosis (Fig. 4). Although bortezomib was demonstrated to inhibit tumor growth in hematological malignancies, it is worthwhile to notice that solid tumors in general, with the exception of prostate cancer and non-small cell lung cancer, do not respond too well to bortezomib [9]. The reasons for this phenomenon remain to be further elucidated and have prompted various additional studies, including pharmacodynamic investigations intending to clarify the mechanism of bioavailability in responder and non-responder tumors in vivo [43]. Although the complex principle of proteasome inhibition, influencing several pathways, is not yet understood, we found significant changes within the experimental tumors that can explain the reduced tumor growth by bortezomib treatment. The suppression of tumor cell proliferation, induction of apoptosis (caspase-3) and reduced vascularity have been shown in xenotransplanted prostate cancer cells before [44]. The decreased vascularity could be due to inhibiting proangiogenic factors [44–46] or due to direct suppression of vascular endothelial cells by bortezomib [47], resulting in a significantly lower VSD within the experimental tumors (Fig. 4C). Moreover, the bortezomib-dependent decrease in in vivo expression of NF-κB, shown for ATC cells to decrease proliferation by selective NF-κB inhibition in vitro [48], completed the range of possible anti-tumor effects. In summary, proteasome inhibitors represent a powerful novel possibility of anti-tumor therapy that obviously displays an extremely wide range of effectiveness depending on the individual tumor biology. Therefore successful clinical application, possibly as a combined therapy, requires a careful preclinical evaluation of the histotype-associated mode of anti-tumor action. Based on the data of the present study, clinical evaluation of bortezomib treatment, either alone or in combination, for ATC is warranted.

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