

# Combined inhibition of cellular pathways as a future therapeutic option in fatal anaplastic thyroid cancer

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**Abstract** Conventional treatment by surgery, radioiodine, and thyroxin-suppressive therapy often fails to cure anaplastic thyroid cancer (ATC). Therefore several attempts have been made to evaluate new therapy options by use of “small molecule inhibitors”. ATC was shown to respond to monotherapeutic proteasome and Aurora kinase inhibition *in vitro* as well as in xenotransplanted tumor cells. Aim of this study was to evaluate the effect of combined treatment targeting the ubiquitin–proteasome system by bortezomib and Aurora kinases by use of MLN8054. Three ATC cell lines (Hth74, C643, and Kat4.1) were used. The antiproliferative effect of combined treatment with bortezomib and MLN8054 was assessed by MTT-assay and cell cycle analysis (FACS). Proapoptotic effects were evaluated by measurement of Caspase-3 activity, and effects on VEGF secretion were analyzed by

ELISA. Compared to mono-application combined treatment with bortezomib and MLN8054 resulted in a further decrease of cell density, whereas antagonizing effects were found regarding cell cycle progression. Caspase-3 activity was increased up to 2.7- and 14-fold by mono-application of MLN8054 and bortezomib, respectively. When the two drugs were used in combination, a further enhancement of Caspase-3 activity was achieved, depending on the cell line. VEGF secretion was decreased following bortezomib treatment and remained unchanged by MLN8054. Only in C643 cells, the bortezomib-induced down-regulation was enhanced when MLN8054 was applied simultaneously. In conclusion, our data demonstrate that targeting the proteasome and Aurora kinases simultaneously results in additional antitumoral effects *in vitro*, especially regarding cell growth and induction of apoptosis. The efficacy of this therapeutic approach remains to be revised by *in vivo* and clinical application.

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## Introduction

Anaplastic thyroid carcinoma (ATC) is a malignancy with fatal prognosis—less than 20 % of affected patients survive 1 year from diagnosis, and median survival is only 3–8 months—and frequently resistant to conventional treatment by surgery and radioiodine. Despite the various attempts to improve the outcome, the clinical course of this disease did not change within the last decades [1]. ATC is a rare tumor that makes up less than 2 % of thyroid carcinomas (TC); however, it accounts for up to 40 % of the

tumor-related deaths of TC. Since TC incidence has been reported to rise in several countries [2], it is unclear, whether the incidence of ATC that may appear de novo or from a preexistent differentiated carcinoma will rise too accordingly. Hence patients suffering from ATC are in desperate need for innovative and effective treatment options.

New insights in development and biological behavior of ATC based on preclinical and clinical research focussing on genetic alterations and dysfunction of signaling pathways offer the possibility of targeted therapies [3]. Apart from the “classical” chemotherapeutics, a large number of drugs targeting new pathways had been developed in the last years. Some of them were shown to successfully inhibit tumor-promoting pathways even in ATC cells in preclinical models [3, 4]. However, only few of them entered clinical trials, generally restricted to advanced differentiated or medullary thyroid cancer. The rare phase II trials that included ATC patients partially displayed clinical responses, but in summarize only showed few effectivity of monotherapy. The application of imatinib, fosbretabulin, or combretastatin resulted in a rate of 6-month overall survival between 23 and 45 % [5–7]. Therefore, rising evidence advocates targeted combination therapy to improve the clinical effect of innovative compounds.

Among the various new drugs proteasome and Aurora kinases inhibitors were demonstrated to be effective in preclinical investigations. Bortezomib, a well evaluated inhibitor of the proteasome, displayed severe antitumoral activity in multiple myeloma (MM) [8], but partially also in solid tumors [9] and was therefore approved for the treatment of MM by the US Food and Drug Administration in 2003. So far very few data focusing the role of proteasome inhibitors in ATC are yet available and the clinical impact remains somewhat confused. Bortezomib was shown to reduce tumor cell proliferation and increase apoptosis of ATC cells in vitro [10, 11] and a case report of second-line treatment with bortezomib refers to bortezomib induced necrosis in the relapsed tumor mass in a patient suffering from ATC [11].

So far, three members of Aurora kinase family were identified: Aurora A, B, and C, known to be key regulators of mitosis. Aurora A is required for correct function of the centrosomes, Aurora B for attachment of the mitotic spindle to the centromere, and Aurora C, mainly detected in germ-line cells, was suggested to be a co-factor of Aurora B in tumor cells [15].

Inhibiting effects targeting Aurora kinases were demonstrated preclinically by in vivo siRNA experiments and by blocking Aurora kinase activity in ATC cell lines using VX-680 as inhibitor [13, 14]. By using MLN8054, our group showed profound antitumor activity to ATC cells in vitro and in vivo [15]. Aurora kinase inhibitors entered clinical Phase I and II studies, targeting various tumor

entities—unfortunately not thyroid cancer—demonstrating some encouraging results. Since MLN8054 and bortezomib appear to have different antitumoral activity—MLN8054 by mainly influencing cell proliferation by reduced mitosis and bortezomib by mainly promoting tumor apoptosis and reducing tumor vascularity, the combination of antitumor activity by influencing both—cell cycle and cell survival—seemed meaningful to us. Therefore, we conducted this study to evaluate the effect of combined anti-proteasome and anti-Aurora kinase action on ATC.

## Methods

### Reagents

MLN8054 (4-[(9-chloro-7-[2,6-difluorophenyl]-5H-pyrimido[5,4-d][2]benzazepin-2-yl)amino]-benzoic acid) and bortezomib (Velcade®) were kindly provided by Millenium Pharmaceuticals (Cambridge, MA). For in vitro use stock solutions of 10 mM MLN8054 and 2.5 mM bortezomib were prepared in dimethylsulfoxide (DMSO).

### Cell lines and culture conditions

Three ATC cell lines were used: Hth74, C643, and Kat4.1. The Hth74 and C643 cells were obtained from Dr. Nils-Erik Heldin (University of Uppsala, Sweden) [16] and the Kat4.1 cells from Dr. K.B. Ain (University of Kentucky, USA) [17]. Cells were grown in full growth medium (FGM) as described previously [18] and for use in experiments FGM was changed to serum-starved conditions (2 % FCS = DMEM-h21/Ham's F12 1:1 (v/v) supplemented with 2 % FCS). Trypan blue exclusion was used to assess cell viability.

### Cell proliferation studies

ATC cells were plated in 96-well plates at a density of  $1 \times 10^4$  cells/well in FGM. After 24 h, FGM was changed to 2 % FCS, and cells were treated with MLN8054 (1 and 10  $\mu$ M) or bortezomib (5 and 10 nM) alone or in combination for up to 144 h. After 24, 72, and 144 h, cell density was determined by SRB-assay (OD 490 nm). Experiments were repeated three times with triplicates each.

### Cell cycle analysis

Cells ( $1 \times 10^6$  each) were seeded in 75 ml flasks and grown at about 80 % confluence. Then FGM was changed to 2 % FCS, and cells were incubated for 24 h with final concentrations of MLN8054 (1 and 10  $\mu$ M) and bortezomib (5 and 10 nM) alone or in combination. After that cells

were harvested, stained with propidium iodide (PI) (50 µg/ml PI, 200 µg/ml RNase) for 15 min, and analyzed by flow cytometry (FACS) (BD LSRII cytometer, BD Franklin Lakes, NJ). Data analysis was carried out using FlowJo software (TreeStar Inc. Ashland, OR, USA). Experiments were performed thrice.

#### Caspase-3 activity

Cells were plated into triplicate wells of 96-well plates ( $1 \times 10^4$  cells/well) in FGM, switched to 2 % FCS after 24 h, and incubated with MLN8054 (1 and 10 µM) and bortezomib (5 and 8 nM) alone or in combination for 24 h. Then Caspase-3 activity was assessed using a luminescence-based Caspase assay (Caspase GloTM 3/7 Assay, GloMax Multimode Reader, Promega, Mannheim, Germany). Experiments were repeated three times.

#### VEGF secretion

Cells were plated in 12-well plates ( $1 \times 10^5$  cells/well) in FGM. After 4 h, medium was changed to serum-starved conditions overnight before the drugs were added in following concentrations: MLN8054 1 and 10 µM and bortezomib 5 and 10 nM alone and in combination. Incubations were continued for 48 h. Then conditioned medium was harvested, centrifuged ( $15,000 \times g$ , 15 min, 4 °C), and stored at -80 °C until analyzed. VEGF protein concentrations were quantified using a commercially available VEGF ELISA (VEGF DuoSet, RnDSystems, Minneapolis, MA, USA) as described elsewhere [18]. Experiments were repeated three times. The VEGF content per sample was calculated as cell number adjusted (pg VEGF/ml) according to the standards used. The Hth74 cells were not included in this experiment because lack of VEGF secretion under standard culture conditions. As demonstrated elsewhere Hth74 cells secrete VEGF only when stimulated with EGF [18].

#### Determination of drug–drug interactions

To determine the impact of combined treatment of MLN8054 (1 and 10 µM) and bortezomib (5 and 10 nM) on cell viability, Caspase-3 activity and VEGF secretion the Bliss additivity model of drug–drug interactions [19] was used. According to this model, the combined response C for the effect of two single compounds A and B is  $C = A + B - (A \times B)$ , where each effect is expressed as a fractional inhibition between 0 and 1. Then the fractional product R (response of combined treatment as measured)/C was calculated and according to the results interaction was defined as follows: fractional product  $R/C > 1$  means Bliss synergism,  $R/C = 1$  Bliss additivity, and  $R/C < 1$  Bliss antagonism [cf. Supplementary Material (SM)].

#### Statistical analysis

A linear model was used to test for a downward trend in the response to increase concentrations of MLN8054 and bortezomib in the individual cell lines. For combinations of various concentrations of MLN8054 and bortezomib, analysis of variance (ANOVA) was used to detect effects from each of the substances and to investigate the possibility of an antagonistic/synergistic interaction in each cell line in addition to the Bliss model.

## Results

#### Tumor cell proliferation

As stated previously, mono-application of MLN8054 or bortezomib leads to a significant dose-dependent decrease of cell number depending on the sensitivity of the particular cell lines towards the two drugs [15, 19]. Concerning MLN8054, Hth74 cells were found most sensitive followed by Kat4.1 and C643 cells (IC<sub>50</sub> MLN8054: 0.1, 1, and >10 µM, respectively [15]), whereas C643 cells were shown to be most sensitive to bortezomib followed by Hth74 and Kat4.1 cells (IC<sub>50</sub> bortezomib about 3, 5, and 12 nM respectively [20]).

Combined treatment with MLN8054 (1 and 10 µM) and bortezomib (5 and 10 nM) resulted in a significant decrease of cell numbers when compared to mono-application in the majority of the experimental conditions (Table 1; Fig. 1). To define synergies, the data were analyzed using the Bliss additivity model. The results are described for each cell line below and summarized in Table S1 (SM).

In the C643 cell line, combined treatment with MLN8054 at 10 µM and bortezomib 5 nM decreased cell numbers significantly ( $P \leq 0.001$ ) to  $7.3 \pm 3.6$  % compared to  $62.8 \pm 12.9$  and  $36.8 \pm 19.5$  % when MLN8054 respectively bortezomib were used alone (Table 1; Fig. 1). Analyzing these data by the Bliss additivity model, a synergistic effect [ $R/C = 1.200$  (Table S1, SM)] can be stated, whereas additive effects were proved for the other drug combinations.

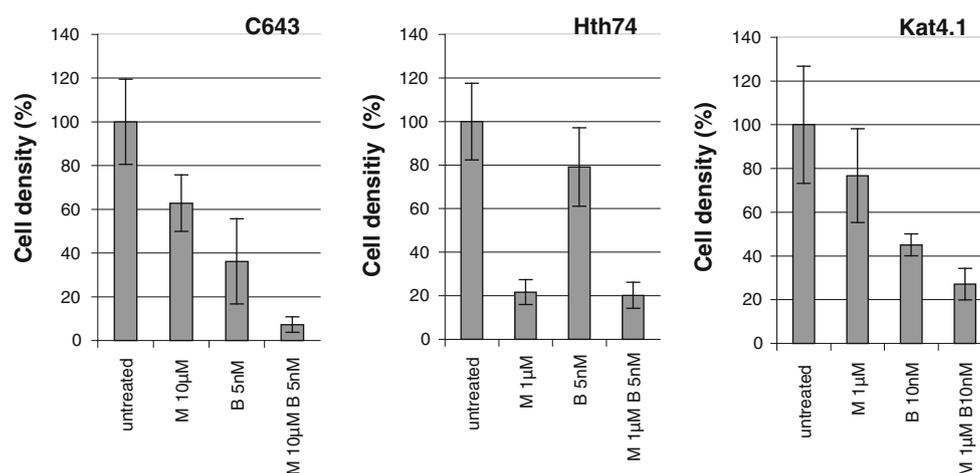
When the two drugs were used in final concentrations near the IC<sub>50</sub> values in Kat4.1 cells (MLN8054 1 µM and bortezomib 10 nM), cell numbers decreased to  $76.7 \pm 21.4$  % (MLN8054 1 µM, alone),  $45.1 \pm 5$  % (bortezomib 10 nM, alone), and  $27.1 \pm 7.2$  % (combined) (Table 1; Fig. 1), which implies synergistic effects [ $R/C = 1.114$ , Table S1 (SM)]. Synergistic effects were also found for the other drug combinations, most pronounced at 1 µM MLN8054 and 5 nM bortezomib [ $R/C = 1.423$ , Table S1 (SM)], which might be due to the higher sensitivity of Kat4.1 cell to MLN8054 compared to bortezomib.

**Table 1** Effect of combined treatment of MLN8054 and bortezomib on proliferation of different ATC cell lines

	C643	Hth74	Kat4.1
DMSO	100 ± 19	100 ± 18	100 ± 27
MLN8054 1 µM	63 ± 10	22 ± 6	77 ± 21
Bortezomib 5 nM	36 ± 19	79 ± 18	101 ± 29
MLN8054 1 µM + Bortezomib 5 nM	20 ± 8	20 ± 6	68 ± 17
MLN8054 1 µM	63 ± 10	22 ± 6	77 ± 21
Bortezomib 10 nM	6 ± 3	3 ± 3	45 ± 5
MLN8054 1 µM + Bortezomib 10 nM	8 ± 2	2 ± 0	27 ± 7
MLN8054 10 µM	63 ± 13	20 ± 7	44 ± 10
Bortezomib 5 nM	36 ± 19	79 ± 18	101 ± 29
MLN8054 10 µM + Bortezomib 5 nM	7 ± 4	22 ± 9	40 ± 14
MLN8054 10 µM	63 ± 13	20 ± 7	44 ± 10
Bortezomib 10 nM	6 ± 3	3 ± 3	45 ± 5
MLN8054 10 µM + Bortezomib 10 nM	9 ± 3	1 ± 2	17 ± 5

Effect of combined treatment of anaplastic thyroid cancer cells (C643, Hth74, and Kat4.1) with MLN8054 (1 and 10 µM) and bortezomib (5 and 10 nM) for 144 h

Data represent cell density as determined by SRB-assay and calculated as percentage compared to the untreated control (DMSO). Mean value ± SD of two independent experiments with triplicates each are shown



**Fig. 1** Anaplastic thyroid cancer cells treated with MLN8045 (M) and bortezomib (B) at final concentration around the IC50 values for each individual cell line (C643 IC50: MLN8054 ~ 10 µM, bortezomib ~ 3nM); Hth74 IC50: MLN8054 0.1 µM, bortezomib ~ 5 nM; Ka4.1 IC50: MLN8054 ~ 1 µM,

In Hth74 cells, MLN8054 at a final concentration of 1 µM already resulted in a significantly decreased cell number (to about 21.7 ± 5.7 %), and no further decrease was seen when MLN8054 at this concentration was used together with bortezomib at 5 nM bortezomib (20.2 ± 6.0 %) (Table 1; Fig. 1). For this drug combination as well as for the others, barely additive effects were calculated [Table S1 (SM)]. This might be due to the high sensitivity of Hth74 cells towards Aurora kinase inhibition by MLN8054 (IC50 0.1 µM).

Taken together, it can be stated that combined treatment with MLN8054 and bortezomib resulted in synergistic

bortezomib 12 nM). After 144 h, cells were analyzed for cell density by use of SRB-assay and cell density calculated as percentage compared to the untreated control (DMSO). Data represent mean value ± SD of two experiments with triplicates each

effects on cell viability depending on the cell line (C643 and Kat4.1) and were most pronounced when concentrations near the IC50 value were employed.

Effects of combined treatment with MLN8054 and bortezomib on cell cycle progression

When cells were analyzed for DNA content by flow cytometry, treatment with MLN8054 (1 µM) alone led to an accumulation of about 43–46 % cells in G2/M compared to 13–15 % in control samples that was only slightly enhanced by increased drug concentrations (Table 2).

Cell cycle progression was less affected by bortezomib. Here, final concentrations of 5 and 10 nM resulted in accumulation of 16–21 %, respectively, 24–34 % cells in G2/M (Table 2). No further enhancement of G2/M accumulation was observed after combined treatment (Table 2). In contrast, the two drugs act partially antagonistic, especially at concentrations of bortezomib as high as 10 nM. The increase of cells in G2/M was generally accompanied by decreased cell numbers in G1-phase and to a minor extent in S-phase of the cell cycle. The SubG1 fraction was smaller than 1 % in C643 and Hth74 cell and about 2–2.5 % in Kat4.1 cell independently from the absence or presence of the two drugs and no distinct a SubG1 peaks were found.

#### Effects of combined treatment with MLN8054 and bortezomib on Caspase-3 activity

To evaluate the effect of combined treatment on apoptosis in ATC cells, Caspase-3 activity was determined as described in “Methods”. Following MLN8054 treatment (1 and 10  $\mu$ M), Caspase-3 activity increased up to 2.7- and 2-fold in Hht74 and Kat4.1 cells, whereas in C643 cells only minor changes were observed (Table 3). Mono-application of bortezomib (5 and 10 nM) however increased Caspase-3 activity significantly up to 14-fold (Table 3). The effects achieved by co-incubation differed depending on the cell line (Table 3). In C643 cells, MLN8054 and bortezomib act synergistically when final concentrations of 10  $\mu$ M, respectively, 5 nM were used, whereas other combinations resulted in additive or antagonizing effects (Table 3). In Hth74 cells, co-application of bortezomib at 5 nM and MLN8054 at 1  $\mu$ M resulted in a

synergistic effect, and additive or antagonizing effects were seen at other combinations (Fig. 2; Table 3). In Kat4.1 cells, Caspase-3 activity was affected synergistically when bortezomib at 5 nM was applied together with MLN8054 (1 and 10  $\mu$ M), and antagonizing effects were found when bortezomib at 10 nM was combined with MLN8054.

In conclusion, our data demonstrate that in ATC cells proteasome inhibition by bortezomib is more effective in inducing proapoptotic effects than Aurora kinase inhibition by MLN8054 and combined treatment by the two drugs rarely displayed synergistic interaction.

#### Effects of combined treatment with MLN8054 and bortezomib on VEGF secretion

Cell density adjusted VEGF secretion into the medium was only minor affected by mono-application of MLN8054 (Fig. 3a). Bortezomib, however, caused a dose-dependent decrease in C643 cells, whereas VEGF secretion remained nearly constant in Kat4.1 (Fig. 3b). Surprisingly C643 cells treated with 12.5 nM bortezomib seem to secrete more VEGF than those treated with lower concentrations. This might be caused by the release of intracellular stored VEGF from cells affected severely by bortezomib. The failure of bortezomib to affect VEGF secretion in Kat4.1 cells in the actual experimental set-up might be due to comparatively less sensitivity toward proteasome inhibition by bortezomib (Kat4.1 IC<sub>50</sub> 12 nM vs. IC<sub>50</sub> 2.6 nM for C643 cells).

The results of a total of five experiments were summarized to determine the effect of combined treatment with MLN8054 and bortezomib on VEGF secretion. In C643 cells, VEGF secretion decreased significantly ( $P \leq 0.5$ ) to

**Table 2** Effect of combined treatment with MLN804 and bortezomib on cell cycle progression in ATC cell lines

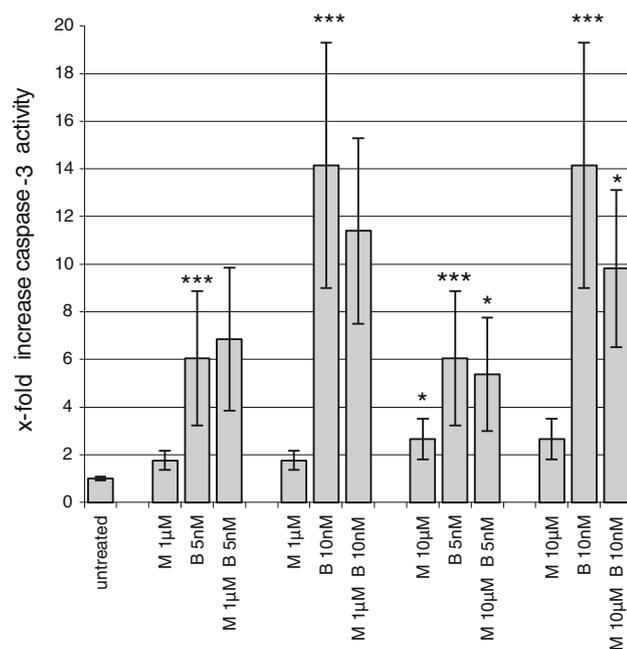
	DMSO	M 1 $\mu$ M	M 10 $\mu$ M	B 5 nM	B 10 nM	M 1 $\mu$ M B 5 nM	M 1 $\mu$ M B 10 nM	M 10 $\mu$ M B 5 nM	M 10 $\mu$ M B 10 nM
<b>C643</b>									
G1	75 $\pm$ 0.6	42 $\pm$ 1.3	45 $\pm$ 1.3	61 $\pm$ 16	56.8 $\pm$ 16	49 $\pm$ 3.8	73 $\pm$ 3.7	49 $\pm$ 2.5	52 $\pm$ 4.3
G2	14 $\pm$ 1.5	43 $\pm$ 1.4	45 $\pm$ 1.6	21 $\pm$ 3.4	34.1 $\pm$ 2.3	42 $\pm$ 3.4	13 $\pm$ 2.9	43 $\pm$ 2.5	36 $\pm$ 0.5
S	10 $\pm$ 0.8	13 $\pm$ 0.7	9 $\pm$ 0.4	16 $\pm$ 4.6	7.8 $\pm$ 0.9	8 $\pm$ 0.6	12 $\pm$ 0.6	7 $\pm$ 0.6	10 $\pm$ 2.7
<b>Hth74</b>									
G1	79 $\pm$ 2.5	47 $\pm$ 8.6	45 $\pm$ 10	78 $\pm$ 2.6	67.4 $\pm$ 4.9	55 $\pm$ 17.6	67 $\pm$ 11.2	61 $\pm$ 11.9	67 $\pm$ 9.1
G2	13 $\pm$ 3.5	46 $\pm$ 9.5	44 $\pm$ 10	17 $\pm$ 2.9	25.0 $\pm$ 9.0	38 $\pm$ 13.7	30 $\pm$ 12.4	35 $\pm$ 12.1	27 $\pm$ 8.6
S	8 $\pm$ 1.2	6 $\pm$ 1.2	5 $\pm$ 0.7	4 $\pm$ 2.1	4.4 $\pm$ 0.5	5 $\pm$ 3.2	3 $\pm$ 0.7	3 $\pm$ 1.4	5 $\pm$ 0.5
<b>Kat4.1</b>									
G1	46 $\pm$ 0.7	7 $\pm$ 0.0	10 $\pm$ 1.5	41 $\pm$ 6.0	43 $\pm$ 3.9	17 $\pm$ 5.8	20 $\pm$ 1.1	14 $\pm$ 2.4	20 $\pm$ 1.7
G2	15 $\pm$ 2.6	43 $\pm$ 6.9	47 $\pm$ 1.8	16 $\pm$ 1.9	24 $\pm$ 4.5	40 $\pm$ 4.0	41 $\pm$ 1.1	44 $\pm$ 0.4	44 $\pm$ 1.1
S	24 $\pm$ 2.3	22 $\pm$ 3.2	15 $\pm$ 2.1	32 $\pm$ 8.6	16 $\pm$ 1.0	20 $\pm$ 2.1	15 $\pm$ 0.9	16 $\pm$ 2.2	11 $\pm$ 0.8

Percentages of cells in G1, G2, and S were determined after exposure to MLN8054 (M) and bortezomib (B) at indicated concentrations for 24 h as detailed in “Methods”. Data represent mean value  $\pm$  SD of three independent experiments

**Table 3** Effect of combined treatment with MLN8054 and bortezomib on Caspase-3 activity of different ATC cell lines

	C643	Hth74	Kat4.1
Untreated control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
1 μM MLN8054	1.0 ± 0.1	1.8 ± 0.4	1.5 ± 0.2***
5 nM Bortezomib	2.6 ± 0.2***	6.0 ± 2.8***	1.8 ± 0.5***
1 μM MLN8054 + 5 nM Bortezomib	2.6 ± 0.2	6.8 ± 3.0	2.6 ± 0.9
1 μM MLN8054	1.0 ± 0.1	1.8 ± 0.4	1.5 ± 0.2**
10 nM Bortezomib	5.5 ± 1.4***	14.1 ± 5.2***	5.0 ± 2.***
1 μM MLN8054 + 10 nM Bortezomib	4.9 ± 1.1	11.4 ± 3.9	5.2 ± 1.8
10 μM MLN8054	1.1 ± 0.1	2.7 ± 0.8*	2.0 ± 0.4***
5 nM Bortezomib	2.6 ± 0.2***	6.0 ± 2.8***	1.8 ± 0.5**
10 μM MLN8054 + 5 nM Bortezomib	3.0 ± 0.2	5.4 ± 2.4*	2.7 ± 0.4
10 μM MLN8054	1.1 ± 0.1	2.7 ± 0.8	2.0 ± 0.4***
10 nM Bortezomib	5.5 ± 1.4***	14.1 ± 5.2***	5.0 ± 2.1***
10 μM MLN8054 + 10 nM Bortezomib	5.0 ± 1.2	9.8 ± 3.3*	5.1 ± 1.4

Increase of Caspase-3 activity following MLN8054 and bortezomib treatment at indicated concentrations and as calculated by means of the raw data, which were determined as detailed in “Methods”. Documented data (*x*-fold change) represent mean value ± SD of three experiments with triplicates each. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$



**Fig. 2** Effect of combined treatment with MLN8054 (M) (1 and 10 μM) and bortezomib (B) (5 and 10 nM) on Caspase-3 activity exemplary documented for one ATC cell line (Hth74). Caspase-3 activity was determined after 24 h using a luminescence-based assay as detailed in “Methods”. Raw data were used to calculate the *x*-fold increase of Caspase-3 activity compared to untreated controls. Data represent mean ± SD of three independent experiments with triplicates each. \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$

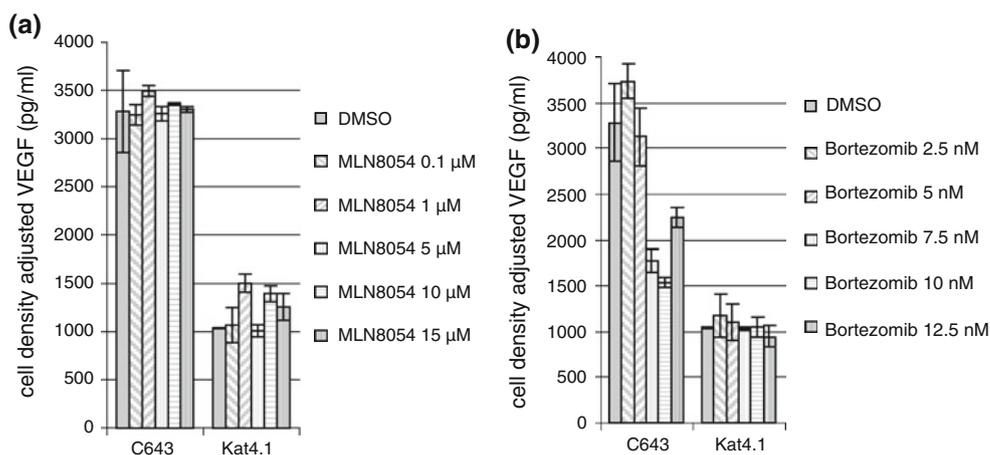
about  $64.5 \pm 24.7\%$  when MLN8054 at 10 μM was applied together with bortezomib at 5 nM, suggesting synergistic effects [ $R/C = 3.431$ , Table S2 (SM)] (Fig. 4a). In contrast, antagonizing effects [ $R/C = 0.620$ ,

Table S2 (SM)] were found using the two drugs in concentrations near the IC<sub>50</sub> value (MLN8054 1 μM; bortezomib 10 nM) in the Kat4.1 cell line. Here VEGF secretion into the medium was reduced to  $82.7 \pm 41.9\%$  compared to  $91.1 \pm 38.9$  and  $78.9 \pm 40.5\%$  using MLN5084 respectively bortezomib alone (Fig. 4b). Comparable results were found when MLN8054 (1 μM) was used in combination with 20 nM bortezomib (data not shown).

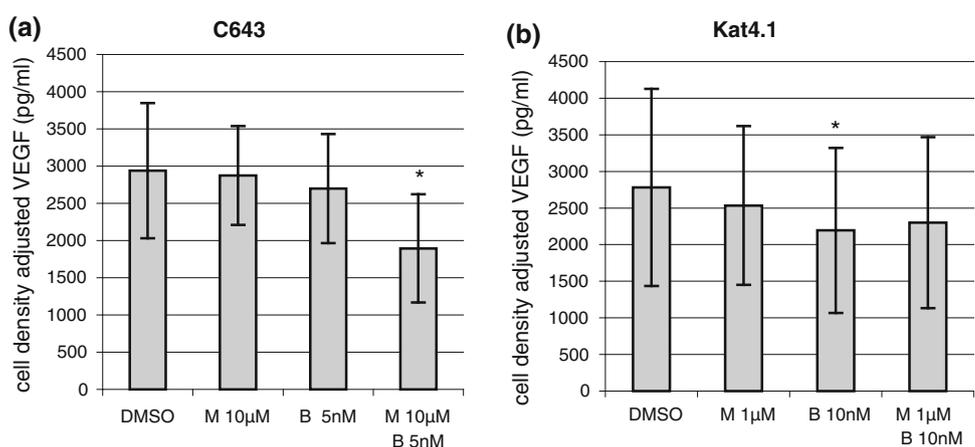
## Discussion

ATC, which are generally unable to trap iodine and do not respond to thyrotropin-suppressive therapy, exhibit an extremely aggressive behavior and represent some of the most lethal malignant diseases. This common failure of established therapeutic strategies in ATC urgently mandates new options of treatment. Some of them, based on new developed drugs, have successfully been evaluated in preclinical settings [21]. Since only few of them entered clinical trials including ATC patients, the results of monotherapeutic drug application displayed somewhat disappointing results [22]. Some studies, including our own reports, demonstrated effective preclinical in vitro and in vivo results for proteasome and Aurora kinase inhibitors depending on the individual tumor biology of ATC cell lines [12, 14, 15, 23]. However—until today—monotherapeutic dosage of new drugs with acceptable toxicity generally failed to improve the clinical outcome of ATC. Therefore, combined application of a proteasome and an Aurora kinase inhibitor to ATC cells was evaluated to possibly increase the individual antitumor effects, since

**Fig. 3** Effect of MLN8054 (a) and bortezomib (b) on VEGF secretion of ATC cell lines C643 and Kat4.1. Cells were incubated for 48 h with MLN8054 (M) respectively bortezomib (B) at indicated concentrations. VEGF content of the medium was determined by ELISA and adjusted to cell density as determined by SRB-assay. Data represent mean  $\pm$  SD of one experiment with triplicates each



**Fig. 4** Effect of combined treatment with MLN8054 (a) and bortezomib (b) on VEGF secretion of C643 and Kat4.1 cells. Cells were incubated for 48 h with MLN8054 (M) respectively bortezomib (B) at indicated concentrations. VEGF content of the medium was determined by ELISA and adjusted to cell density as determined by SRB-assay. Data represent mean  $\pm$  SD of five experiments. \*  $P \leq 0.05$



targeting the mitotic process on one hand and supporting the programmed cell death on the other to achieve a synergistic antitumorigenic result seemed promising to us.

The results of mono-application of the drugs confirmed our own and other previous reports [10, 15, 20, 24]. Combined therapy resulted in a further decrease of cell viability as demonstrated for the three ATC cell lines used in this study (Fig. 1; Table 1). Depending on the cell line and drug-combination synergistic and/or antagonizing effects were calculated. The synergistic effects were pronounced when the drugs were used at final concentrations appropriate to the individual IC50 values of each drug, which were reported elsewhere [15, 20].

So far, no other reports exist concerning combined application of MLN8054 and bortezomib to ATC cell lines. However, for a human plasma cell leukemia cell line (OPM1), a synergistic antiproliferative effect was demonstrated by combining an Aurora kinase inhibitor (MLN8237) and bortezomib [25]. A phase I/II study has recently been initiated to investigate the combined action of Aurora kinase inhibition and bortezomib in relapsed or refractory MM. Synergistic effects on cell viability were also shown for MM cells using the pan-Aurora kinase

inhibitor VE465 and bortezomib [26], but to the best of our knowledge, no reports exist concerning the combined treatment with MLN8054 or MLN8237 and bortezomib in solid tumors.

When effects exerted on cell cycle progression were analyzed by FACS, an accumulation of cells in the G2/M phase caused by Aurora kinase inhibition was seen (Table 2). This has already been reported for thyroid cancer cell lines [15] and other solid tumors like prostate and colon carcinoma [24]. Although bortezomib itself was reported to induce G2/M arrest in myeloma as well as solid tumor cells including thyroid cancer [20, 27, 28], combined Aurora kinase and proteasome inhibition did not result in additional cell accumulation in the G2/M phase. In contrast, it exerts antagonistic effects especially when bortezomib was used in concentrations of 10 nM. Similar results were obtained when bortezomib was used in combination with docetaxel or taxol in breast and prostate respective breast cancer cells [29, 30]. Here the antagonizing effect of bortezomib was accompanied by down-regulation of Cdk1, which was reported to be a distinct feature of bortezomib action earlier [31]. Cdk1 itself promotes G2/M transition and enables cells entry into mitosis.

Therefore, the bortezomib-mediated Cdk1 inhibition was supposed to explain the antagonistic effect on G2/M accumulation exerted by bortezomib [29]. Aurora kinases, involved either in the spindle checkpoint control (Aurora A) or acting as chromosomal passenger proteins (Aurora B), act downstream of Cdk1. Inhibition of these mitotic kinases interferes with cell cycle progression and results in mitotic delay, polyploidy and multinucleation as well as accumulation in a pseudo G1 state depending on the drug used [32]. MLN8054, known to inhibit Aurora A selectively over Aurora B, was documented to induce defects in the mitotic spindle assembly followed by a transient spindle checkpoint-dependent mitotic arrest, apparent as G2/M arrest [33]. Since this happens also as downstream of Cdk1, it might be possible that bortezomib counteracts MLN8054 action similarly to the studies mentioned above.

In this study, mono-application of MLN8054 and bortezomib to ATC cell lines confirmed our previous investigations concerning proapoptotic effects [15, 20], and bortezomib seems to be more potent in induction of apoptosis than MLN8054. Combined treatment, however, enhanced the proapoptotic effects as shown by measurement of Caspase-3 activity. The effects varied depending on the experimental condition and cell line and were found to be barely additive or slightly synergistic (Table 3). This is in line with recently published data using an Aurora kinase inhibitor (MLN8237) and bortezomib in combination for the treatment of MM cells [25]. In this study, dephosphorylation of Chk1, leading to mitotic progression (G2/M transition) mediated by Cdk1 activation, and further induction of p53, p27, and p21, indicating changes in the p53 pathway, following MLN8237 treatment, were supposed to be responsible for the enhancement of apoptosis caused by combined treatment with bortezomib. MLN8054, a precursor-molecule of MLN8237, was reported to affect apoptosis by regulating different proteins including p53, p21, and Bax [33, 34]. Therefore, similar mechanisms may account for the observations made in our study, but again this has to be clarified in further studies.

VEGF secretion is a common feature of thyroid cancer cells and several approaches have been made to influence the growth of thyroid cancers using anti-VEGF strategies, primarily by use of monoclonal antibodies (Bevacizumab, Avastin<sup>®</sup>) and tyrosine kinase inhibitors (TKI) like ZD6474 (Zactima<sup>®</sup>) and Sorafenib (Nexavar<sup>®</sup>) for example. Various extracellular receptors and intracellular pathways are involved in the regulation of the VEGF, which promotes angiogenesis. Recently, it has been shown that VEGF expression is also stimulated by NF $\kappa$ B-activity [35, 36]. Moreover, it has been shown that bortezomib is competent to inhibit VEGF gene expression and VEGF secretion in endothelial and cancer cells [31, 37–40]. Antiangiogenic effects of bortezomib were further confirmed in vivo

[31, 41–43]. In our study, bortezomib-mediated VEGF-suppressive effects were documented for the first time for two ATC cell lines. This effect differed relevant according to the overall sensitivity of the cell lines towards bortezomib (Fig. 3b). Aurora kinase inhibition by MLN8054 on the other hand failed to affect VEGF secretion, confirming the results of a study on the cervical cancer cell line SiHa in which ZM447439 was used as inhibitor [44]. Since we demonstrated a decrease in tumor vascularity by Aurora kinase inhibition in ATC xenotransplants before, this must be due to other mechanisms of antiangiogenesis that have to be elucidated by additional analysis [15]. Surprisingly in C643 cells, shown to be very sensitive to bortezomib application elsewhere [20], combined treatment with bortezomib and MLN8054 seems to act synergistically on VEGF secretion, implicating additional mechanisms of VEGF regulation by combined drug action.

In summary, combined therapy of ATC cells by use of two drugs targeting different central cellular functions, namely, cell cycle and cell survival, resulted in additional antitumoral effects in most of the experimental conditions. However, depending on the individual cell line, even antagonistic effects—for example regarding cell accumulation in the G2/M phase—were observed. Nevertheless, an additional tumor-cell targeting action by combined proteasome and Aurora kinase inhibition can be confirmed for ATC cells in vitro that has to be approved in more complex systems like tumor xenotransplantation models to gain suitable data before a possible clinical application. Based on these results, it can be hypothesized, that the effectivity of combined targeting of the proteasome and Aurora kinases strongly depends on the individual sensitivity of tumor cells.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical standards** The authors declare that the experiments comply with the current laws of the country in which they were performed.

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