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Bortezomib sensitizes thyroid cancer to BRAF inhibitor *in vitro* and *in vivo*

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Abstract

Although overall survival rate for patients with thyroid cancer (TC) is high, there is an alarming 10-year recurrence rate of up to 30% conferring a ~50% survival among these high-risk patients. The BRAF^{V600E} mutation is estimated to be present in over 50% of papillary thyroid cancer (PTC) cases besides being associated with carcinogenesis and poor prognosis. We assessed the status of NF-κB, Ki-67, cyclin D1 and BRAF^{V600E} in TC tissues and TC cell lines using immunohistochemistry and Western blot analysis. Concurrently, we evaluated the outcomes of combined targeting of the proteasome pathway in addition to selective BRAF inhibitors in cases of PTC. In this study, BRAF^{V600E}-bearing TC cells were treated with BRAF^{V600E} inhibitor, Vemurafenib alone or in combination with the proteasome inhibitor, Bortezomib. The combination of both drugs showed synergistic effects as evidenced by cell growth inhibition ($P < 0.05$), increased G2-phase cell cycle arrest and induced apoptosis ($P < 0.05$). In our TC xenograft model, the combination of Vemurafenib and Bortezomib significantly reduced tumor size ($P < 0.05$) and expression of the markers of cell growth and proliferation, Ki-67 and cyclin D1 ($P < 0.001$), when compared to monotherapy. Further analysis demonstrated that treatment with Bortezomib sensitized TC cells to Vemurafenib via mitochondrial dysregulation and apoptosis of TC cells, as evidenced by the increase in the expression of p53, Noxa protein, the loss of mitochondrial membrane potential, cytochrome c release and Poly (ADP-ribose) polymerase cleavage. Our results demonstrate a strong clinical potential for the combination of the Bortezomib and the BRAF inhibitor Vemurafenib as an efficient therapeutic approach for the treatment of TC.

Key Words

- ▶ BRAFV600E
- ▶ NF-κB
- ▶ thyroid cancer
- ▶ Vemurafenib
- ▶ Bortezomib

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Introduction

Thyroid cancer (TC) is the most common type of endocrine-related cancer (56,870 new cases in 2017) and represents approximately 3.4% of all new cancer cases (Howlader *et al.* 2017). Although over 90% of

differentiated thyroid cancers (DTC) are cured by surgery and have an excellent prognosis, recurrence is possible in patients with DTC, and a small percentage may have a fatal disease course. Anaplastic thyroid cancer (ATC) is

one of the most aggressive malignancies that can arise from a transformation of pre-existing DTC (Sanghvi 2013, Howlader *et al.* 2017). The survival rate of patients with ATC post-diagnosis is usually less than one year. Currently, effective standard therapies for patients with aggressive TC remain limited and need to be developed urgently (Smallridge 2012, Baumunk *et al.* 2013).

The mitogen-activated protein kinase (MAPK) signaling cascade regulates cell proliferation, differentiation, apoptosis and cell survival (Roberts & Der 2007, Li & Wu 2013). BRAF (v-ras murine sarcoma viral oncogene homolog B), a serine/threonine kinase, is one of the most well-characterized members of the MAPK pathway. The majority of somatic BRAF mutations occur as a substitution of valine with glutamic acid at amino acid residue 600 (V600E). The BRAF^{V600E} mutation constitutively activates its downstream pathway and results in oncogenic transformation of normal cells (Bennedbaek & Hegedüs 2003, Wan *et al.* 2004). Additionally, it also cooperates with PIK3CA mutations to promote anaplastic thyroid carcinogenesis (Bennedbaek & Hegedüs 1999, Charles *et al.* 2014). BRAF^{V600E} plays a critical tumorigenic role in several types of cancers, up to 60% of melanomas and 15% of colon cancers (Hegedüs 2009, Davies & Welch 2010, Rustgi *et al.* 2013, Salama & Flaherty 2013). The BRAF^{V600E} mutation is in up to 50% of papillary thyroid cancers (PTCs) and 25% of ATCs (Jeong *et al.* 2008, Nucera *et al.* 2010, Smallridge 2012, Baumunk *et al.* 2013). Selective BRAF^{V600E} inhibitors have shown promise in treatment of metastatic melanoma (Deandrea *et al.* 2008, Bollag *et al.* 2012). However, intrinsic and acquired resistance to selective BRAF inhibitors has proven to be a significant clinical obstacle in the treatment of patients with metastatic melanoma (Deandrea *et al.* 2008, Davies & Welch 2010, Bollag *et al.* 2012, Salama & Flaherty 2013).

Despite the fact that Sorafenib (Nexavar), Lenvatinib and multiple kinase inhibitors including BRAF are currently approved for metastatic TC, the median progression-free survival is only approximately 1 year (Spiezia *et al.* 2009, Brose *et al.* 2014). The BRAF^{V600E} inhibitors Vemurafenib and Dabrafenib are currently FDA-approved for the treatment of BRAF^{V600E}-harboring melanoma and currently undergoing clinical trials for metastatic TC. Severe toxicities have been observed in these patients, and most patients, even those who responded initially, ultimately developed acquired resistance and rapid disease progression (Schlumberger & Torlantino 2000, Spiezia *et al.* 2009, Brose *et al.* 2014, Dadu *et al.* 2015). Therefore, new alternative therapies are needed for aggressive TCs.

NF- κ B has been shown to play a key role in TC by controlling the proliferative and anti-apoptotic signaling pathways of TC cells, including ATCs (Cooper *et al.* 2006, Hsu *et al.* 2014, Tsumagari *et al.* 2015). Paclitaxel induces cell survival through NF- κ B activation in ATCs, and the combination of paclitaxel and the NF- κ B inhibitor dehydroxymethylepoxyquinomicin has been suggested to compensate for paclitaxel resistance in ATCs (Durante *et al.* 2006, Polona *et al.* 2006).

Bortezomib, currently approved for the treatment of advanced multiple myeloma, is a proteasome inhibitor and can effectively control canonical and non-canonical NF- κ B signaling (Garrean *et al.* 2007, Hideshima *et al.* 2007). Although Bortezomib alone has a modest effect on advanced TC (Bailey *et al.* 2001, Cooper *et al.* 2006, Putzer *et al.* 2013, Tsumagari *et al.* 2015), a synergetic effect was suggested in combination with other chemotherapeutic agents, portending a promising role for Bortezomib in combination therapy regimens (Smith *et al.* 2001, Cooper *et al.* 2006, Mitsiades *et al.* 2006, Tsumagari *et al.* 2015). These observations demonstrate the potential for targeting NF- κ B for treatment in resistant and aggressive TC.

We therefore hypothesized that the combination of Vemurafenib and Bortezomib would provide synergetic effects in treating aggressive TC. Herein, we investigated the effect of the use of a combination of two FDA-approved drugs in *in vitro* and *in vivo* thyroid mouse xenograft models.

Materials and methods

Cell culture

The human anaplastic thyroid carcinoma-derived cell lines, SW1736 and KAT18 cells were provided by Dr Xing (University of Johns Hopkins, Baltimore, MD, USA). DRO and NPA cells were obtained from Dr Guy J F Julliard (University of California Los Angeles School of Medicine, Los Angeles, CA, USA). DRO, NPA and SW1736 cells carry mutant BRAF^{V600E} and KAT18 cells carry BRAF^{wt}. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 1% penicillin–streptomycin in a 37°C humidified incubator with 5% CO₂. Cells were treated with Vemurafenib (PLX4032, SelleckChem, Houston, TX, USA) and Bortezomib (LC Laboratories, Woburn, MA, USA) at various indicated concentrations and time points. The culture medium and drugs were replenished every 24 h during the treatment.

Human thyroid tissue specimens

Eleven PTC and three goiter tissue specimens were obtained from the Louisiana Cancer Research Center (LCRC) Biospecimen Core following the approval of the Institutional Review Board of Tulane University Health Sciences Center (TUHSC), USA. Real-Time PCR and Sanger sequencing for the detection of BRAF^{V600E} was utilized to detect mutation presence. Clinicopathologic characteristics were retrospectively retrieved from the patient records.

Cell proliferation assay

The cell proliferation assay was performed in triplicate and each experiment was repeated at least thrice. TC cells were seeded into 96-well plates and treated with either drug at the indicated concentrations. After 1, 3 and 5 days of treatment, 10 μ L of tetrazolium salt WST-8 (Cell Counting Kit-8, Dojindo Molecular Technologies Inc., MD, USA) was added to cells and incubated for 4 h at 37°C. The plates were read at 450 nm using a microplate reader. For each cell line, the 50% inhibition concentration (IC₅₀) of Vemurafenib and Bortezomib was calculated using GraphPad Prism (version 5.0). Trypan blue (ThermoFisher) exclusion assay was also performed for each cell line. The effectiveness of Vemurafenib and Bortezomib combination therapy was evaluated by a combination index using isobologram analysis which quantifies drug–drug interactions between Vemurafenib and Bortezomib using the described equation (Kanotra *et al.* 2008, Abd Elmageed *et al.* 2017).

Colonogenic assay

Cells at a concentration of 500 cells per well were seeded in 6-well plates with 2 mL of media in each well. After overnight incubation (37°C, 5% CO₂), each well was treated with Vemurafenib, Bortezomib or a combination of both drugs. After 14 days of treatment, the media were aspirated from each well and a solution of 1% methylene blue and 50% methanol was added. The cells were incubated in this solution for 30 min to fix and stain them. The colony number in each well was counted in duplicate plates.

Cell cycle assay

TC cells were harvested and centrifuged, and the resulting pellets were fixed in ice-cold 70% ethanol. Fixed cells

were recentrifuged, washed and resuspended in PBS containing RNase A (1 mg/mL) and propidium iodide (PI) was added (1.0 mg/mL). PI-stained cells were analyzed by a fluorescence-activated cell sorter (FACSCalibur in the UAMS Flow Cytometry Core Facility, Tulane University, New Orleans, LA, USA), followed by determination of the percentages of cells in G₁-, S- and G₂/M-phases of the cell cycle.

Apoptosis assay

After treatment with Vemurafenib (1 μ M), Bortezomib (20 nM) or both for 48 h, TC cells (2 $\times$ 10⁵ cells) were washed with PBS and harvested for the apoptosis assay. The cells were washed and resuspended in cold PBS. PI and Annexin V were added (1.0 mg/mL) following the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit, Sigma). Cells stained by PI and Annexin V were analyzed by fluorescence-activated cell sorting (FACS) as described previously.

Measurement of mitochondrial membrane potential ($\Delta\psi$ m) using JC-1

DRO and SW1736 cells were stained with 10 μ M JC-1 for 30 min at room temperature in the dark. The intensities of green (520–530 nm) and red fluorescence (>550 nm) of 50,000 individual cells were analyzed by flow cytometry as described previously (Esnault *et al.* 2010, El Jamal *et al.* 2016).

Detection of reactive oxygen species (ROS)

The detection of ROS in Bortezomib, Vemurafenib-treated DRO and SW1736 cells was performed by staining with DHR 123 (Sigma) and analyzed by FACS as described previously (Selimovic *et al.* 2011).

Western blot analysis

Cells treated with inhibitors at the indicated concentrations were lysed in PhosphoSafe Extraction Reagent (EMD Biosciences, Inc., Madison, WI, USA), and protein concentrations were determined using the BCA method (ThermoFisher) as described previously (Cooper *et al.* 2006, Tsumagari *et al.* 2015). Briefly, protein samples were boiled in an equal volume of sample loading buffer for 5 min, with equal amounts of protein electrophoresed on a 4–20% Tris–HCL polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with

5% skim milk in TBST buffer, membranes were hybridized with the indicated antibodies. The following antibodies were used at the indicated dilution: anti-I κ B α (Sc-7182, Santa Cruz, CA, USA), 1:1000; anti-p-I κ B α (AF4809, R&D system), 1:1000; anti-PARP (#9542, Cell Signaling Technology Inc.), 1:500; anti-GAPDH (Santa Cruz, CA, USA). Protein expression signals developed by ECL (Pierce, ThermoFisher Scientific) were determined by a gel documentation system (Bio-Rad, Model 700) equipped with Quantity One software.

In vivo studies

Six-week-old inbred homozygous athymic BALB/C nude (nu/nu) male mice (Charles River) were housed in a pathogen-free barrier facility. All animal work was performed at the Tulane University School of Medicine in accordance with federal, local and institutional guidelines and with approved IACUC protocol from Tulane University. Mice were subcutaneously inoculated with 2×10^6 SW1736 cells with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) in the right flank, and tumor growth was monitored with calipers. After the tumors became palpable, tumor-bearing mice were randomly divided into four groups and administered Vemurafenib (dissolved in 0.5% hydroxypropyl methyl cellulose, 0.1% Polysorbate 80, 50 mg/kg, oral gavage) once per day, Bortezomib (dissolved in 1% DMSO, 0.5 mg/kg, i.p.) twice per week, a combination of Vemurafenib and Bortezomib or vehicle (0.5% hydroxypropyl methyl cellulose, 0.1% Polysorbate 80 once per day and 1% DMSO) once per week. Tumor volume was measured every other day and calculated according to the following formula: $\pi \times a^2 \times b/6$, where a is the short axis of the tumor, b is the long axis of the tumor and $\pi \approx 3.14159$. Fractional inhibition of tumor growth was calculated based on the tumor volume. Mice were sacrificed by euthanasia and tumors were harvested on the last day of treatment.

Immunohistochemical (IHC) and immunofluorescence (IF) analyses

Fresh tumors from each group were resected on Day 28 of the efficacy study, fixed in formalin, embedded, cut and mounted. The expressions of BRAF^{V600E} (VE1) (Spring Bioscience, Pleasanton, CA, USA), anti-NF- κ B p65 (4-2H22L23, Thermo Scientific), Ki-67 (Thermo Scientific) and cyclin D1 (Novus Biologicals, Littleton, CO, USA) were assessed by IHC and IF according as described previously (Kano *et al.* 2008, Abd Elmageed *et al.* 2017).

Statistical analysis

All data relating to the study were summarized using descriptive statistics, such as the mean, proportion and standard deviation. The repeated measure analysis of variance method and correlation analysis were applied to find associations and to compare mean differences among different cell lines, tumor samples and concentration levels. The violation of the assumption of sphericity (i.e., the variances of the differences between all groupings of related sets (levels) are equal) for using ANOVAs with repeated measures (within-subject factors) was performed using Mauchly's sphericity test. Bonferroni's multiple comparison method was used for a post hoc analysis. A two-sided, 5% significance level was used throughout the analyses. All analyses, summaries and listings were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC, USA).

Results

Analysis of BRAF^{V600E} and NF- κ B in human TC tissue and cell lines by IHC and Western blot, respectively

We started first with IHC analysis of BRAF^{V600E} and NF- κ B in human TC tissues as well as in goiter specimens. Eleven PTC tissues (Stage III/IV) harboring BRAF^{V600E} (confirmed by real-time PCR, data not shown) and three goiter tissues were stained with BRAF^{V600E} and NF- κ B-specific antibodies. All cases of PTC showed overexpression of VE1 and NF- κ B as compared to goiter tissues (Fig. 1A and B). The analysis of both BRAF^{V600E} and NF- κ B in the TC-derived cell lines NPA, DRO and SW1736 (BRAF^{V600E}), and in KAT18 (BRAF^{wt}) was performed. Data from the Western blot (Fig. 1C) revealed the expression of BRAF^{V600E} in NPA, DRO and SW1736, but not in KAT18, the BRAF^{wt}-expressing cell line. Similarly, the Western blot analysis of the NF- κ B pathway of the cytoplasmic and nuclear protein of mutated and wild-type BRAF TC cells (Fig. 1C) demonstrated that the basal activation of NF- κ B in BRAF^{V600E}-expressing cells was higher than those noted in BRAF^{wt}-expressing cells, suggesting that acquired resistance could be attributed to activation of NF- κ B pathway.

The exposure of TC cells to Vemurafenib or Bortezomib is associated with the inhibition of cell growth

To assess the inhibitory effects of Vemurafenib and Bortezomib on TC cells, we analyzed the viability of TC cell lines using a cell viability assay. First, we estimated the IC₅₀ values of the TC cell lines harboring BRAF^{V600E}

Figure 1

(A) Immunohistochemistry of PTC-bearing BRAF^{V600E} using NF-κB-, Ki67- and Cyclin-D1-specific antibodies. Advanced PTC and goiter were stained with anti-NF-κB, Ki67 or Cyclin D1 as indicated in PTC (right lane) and goiter (left lane). Magnification was 40×. (B) Immunofluorescence staining of PTC with VE1 and/or NF-κB. (C) Western blot analysis of the thyroid cancer cell lines (NPA, DRO, SW1736) expressing mutated BRAF^{V600E} and the thyroid cancer cell line (KAT18) expressing BRAF^{wt} up on the expression of BRAF^{V600E} and activation of NF-κB. GAPDH was used as internal control for loading and transfer.

mutation (NPA, DRO and SW1736) or BRAF^{wt} (KAT18) for the treatment with Vemurafenib (10 μM) and Bortezomib (100 nM) for 120 h and cell viability was determined (Fig. 2A). Then, the cell lines NPA, DRO, SW1736 or KAT18 were exposed to the estimated concentrations of Vemurafenib and Bortezomib. Upon treatment of NPA, DRO and SW1736 cells with Vemurafenib and Bortezomib, we noted significant inhibition of cell viability by 9–22% and 17–29%, respectively, when compared to control cells (Fig. 2B). In contrast, exposure of KAT18, the BRAF^{wt}-expressing cells, to Vemurafenib and Bortezomib showed a cell growth inhibition of 2% and 15%, respectively. Interestingly, the exposure of these cells to both compounds simultaneously for 72 h (Fig. 2B) showed a synergistic effect as evidenced by the level of the growth inhibition of NPA, DRO and SW1736 cells of 44, 58 and 64% ($P < 0.01$), respectively. Although the effect of Vemurafenib or Bortezomib was not significant in KAT18, the combination significantly inhibited the growth of the BRAF^{wt} KAT18 cells (Fig. 2B). The cell

viability data were further confirmed by the application of a colonogenic assay. Accordingly, the SW1736, BRAF^{V600E}-expressing cells were subjected to a colonogenic assay following the treatment with Vemurafenib, Bortezomib or combination of the two. As expected, a reduction of 25 or 39% of colonies was noted in SW1736 cells in response to the exposure to Vemurafenib or Bortezomib, respectively (Fig. 2C). More interestingly, the combination of Vemurafenib and Bortezomib showed a reduction of 93% in colony formation when compared to control cells ($P < 0.01$).

Apoptosis induced by the combination of Vemurafenib and Bortezomib in TC cells is associated with mitochondrial dysregulation and accumulation of ROS

Next, we investigated whether Vemurafenib- and Bortezomib-induced growth inhibition of DRO and SW1736 is mediated by an apoptotic mechanism. Both cells were exposed for 48 h to the recommended

Figure 2

Cell viability assay in TC cells. (A) DRO, SW1736 and KAT18 cells were treated with Vemurafenib (0–10 µM) and Bortezomib (0–100 nM) for 120 h, and cell viability was assayed by WST-8 assay. (B) NPA, DRO, SW1736 or KAT18 cells were exposed to sub-IC₅₀ concentrations of Vemurafenib (0.1, 0.1, 1.0 or 10 µM) and Bortezomib (15, 15, 20 or 35 nM), respectively, for 72 h, and cell viability was tested by Trypan blue nuclear exclusion assay. B; Bortezomib; V; Vemurafenib. (C) SW1736 cells were treated with Vemurafenib (1.0 µM) and Bortezomib (20 nM) for 14 days and assessed by colonogenic assay.

concentration of the anticancer agents Vemurafenib and Bortezomib, both individually or in combination. The cells were then subjected to a flow cytometry analysis using Annexin V/PI staining. Data obtained from flow cytometry (Fig. 3A) demonstrated the induction of apoptosis in treated cells. The exposure to Vemurafenib was found to trigger apoptosis up to 15% in both cell lines, whereas the exposure of the same cell lines to Bortezomib demonstrated up to 18% apoptosis rates; however, treatment of cells with a combination of both drugs showed up to a 61% apoptosis rate ($P < 0.01$) in DRO and SW1736 cells, respectively (Fig. 3A).

We next examined whether there were effects on cell cycle progression after exposure to Vemurafenib and Bortezomib individually and in combination. In SW1736 cells, individual exposure to either compound did not result in any cell cycle changes compared to untreated cells, which were 60–63% in G1-phase, 3–5% in G2-phase and 33–36% in S-phase. Intriguingly, the two drugs in combination resulted in a significant increase in the proportion of cells in the G2-phase, up to 30%, and a decrease of cells entering G1-phase, down to 32% (Fig. 3B). Consequently, exposure to Vemurafenib and Bortezomib simultaneously resulted in a drastic drop in the cellular proliferative responses.

We next analyzed whether the induced apoptosis of TC cells is associated with the inhibition of NF-κB, mitochondrial dysregulation and/or an accumulation of ROS. Apoptosis was confirmed at the molecular level by analysis of the apoptotic marker Poly ADP-ribose polymerase protein (PARP) using Western blot. Although the exposure of TC cells to either Vemurafenib or Bortezomib does not show PARP cleavage, their combination was found to trigger PARP cleavage in both cell lines (Fig. 3C). As a proteasome inhibitor, we speculated that Bortezomib may inhibit the ubiquitination of the inhibitor of NF-κB kinase (IKBα) and thereby contributes in the modulation of Vemurafenib-induced apoptosis of TC cells. As expected, the Western blot analysis of the IKBα in control and treated cells (Fig. 3C) revealed the degradation of IKBα in control and Vemurafenib-treated cells, but not in TC cells treated with either Bortezomib or Vemurafenib combined with Bortezomib (Fig. 3C). Accordingly, the phosphorylation of IKBα was elevated in control and Vemurafenib-treated cells and inhibited in Bortezomib or in Vemurafenib- and Bortezomib-treated cells (Fig. 3C). This suggests the involvement of Bortezomib-induced inhibition of NF-κB pathway in the modulation of Vemurafenib-induced apoptosis of TC cells.

Figure 3

Drug combination induces apoptosis and cell cycle arrest in TC cells. (A) DRO and SW1736 cells were treated with Vemurafenib (0.1 or 1.0 μ M) and Bortezomib (10 or 20 nM) for 48 h. TC cells were stained with propidium iodide and anti-Annexin V. The percentage of apoptotic cells is indicated (* P <0.05, ** P <0.01). (B) SW1736 cells were treated with Vemurafenib and/or Bortezomib, and cell cycle progression was assessed by flow cytometric assay. (C) Western blot analysis demonstrates the phosphorylation and the corresponding degradation of I κ B α and cleavage of PARP in control and treated SW1736 and DRO cells. GAPDH is used as an internal control for loading and transfer. Data are representative of three independent experiments. (D) Flow cytometry analysis of treated and control cells following the JC-1-staining shows the loss of mitochondrial membrane potential in response to the exposure of TC cells to the combination therapy. (E) Flow cytometry analysis of the reactive oxygen species (ROS) in control and treated TC cell following the incubation with DHR123 substrates. Data are representative of three independent experiments performed separately. B, Bortezomib; V, Vemurafenib.

To show whether the induced apoptosis of TC cells is mediated via mitochondrial dysregulation-dependent mechanisms, the control and treated cells were subjected to flow cytometry analysis following the staining with JC-1. Data from the flow cytometry analysis (Fig. 3D) demonstrated the loss of mitochondrial potential ($\Delta\Psi$ m) only in cells treated with the combination of both Vemurafenib and Bortezomib when compared to control or single drug-treated cells. This represents evidence for the involvement of mitochondrial dysregulation-dependent mechanisms in the modulation of TC cell apoptosis in response to the combination of both Vemurafenib and Bortezomib. We also investigated whether the induced apoptosis of TC cells is associated with the accumulation of ROS. TC control and treated cells were prepared for flow cytometry analysis following the incubation with DHR123 substrates for 1 h under normal tissue culture conditions. Data obtained from flow cytometry (Fig. 3E) showed the accumulation of ROS in Vemurafenib-treated SW1736 (2.11%) and DRO (2.50%) cells, and in Bortezomib-treated SW1736 (3.33%) and DRO (7.52%)

cells, and when compared to corresponding control cells. The exposure SW1736 and DRO cells to the combined therapy (Vemurafenib and Bortezomib) increased the level of ROS accumulation to 17.18% and 28.18%, respectively, when compared to control cells (Fig. 3E). This suggests a possible role for ROS accumulation in the modulation of induced apoptosis in TC cells treated with this drug combination therapy.

The combination of Vemurafenib and Bortezomib synergistically reduced tumor growth *in vivo*

We assessed the clinical reliability of the Bortezomib and Vemurafenib combination as a potential treatment option for aggressive TC cells bearing BRAF^{V600E} mutation using a mouse xenograft tumor model derived from SW1736 cells. Six-week-old inbred homozygous athymic BALB/C nude (nu/nu) male mice were subcutaneously implanted with 2.0×10^6 SW1736 cells. After the tumor size reached 50–100 mm³, Vemurafenib and/or Bortezomib were administered to the mice for 4 weeks. The effect of

mono- or combination therapy on tumor size was monitored at regulated time intervals until the end of the experiment. Tumors were stained by H&E and apoptotic changes were seen in the group treated with Vemurafenib, Bortezomib or combination. Apoptotic changes were most prominent in the group treated with combination therapy (Fig. 4A). The tumor growth rates of control and treated mice are calculated and depicted in Fig. 4B. Robust tumor growth was seen in the control group without treatment (760% compared to initial time point). In contrast, a significant tumor growth reduction of 35% and 26% was noted in Vemurafenib- and Bortezomib-treated mice, respectively. The combination of the two inhibitors resulted in a significant tumor size reduction of 64% when compared with the control mice ($P < 0.05$).

To characterize the mechanism of Vemurafenib- and Bortezomib-induced tumor growth inhibition that was observed in the thyroid xenograft model, the mitotic index

of tumor tissues was assessed by the Ki-67 expression using IHC analysis. Active cell proliferation was observed in tumor tissue sections with a 69% relative proliferation rate (Fig. 4B). Monotherapy with Vemurafenib or Bortezomib slightly decreased the percentage of Ki-67-positive proliferating tumor cells, with relative proliferation rates of 74% and 47%, respectively (Fig. 4B). The combination of Vemurafenib and Bortezomib markedly decreased the percentage of Ki-67-positive proliferating tumor cells to 11% ($P < 0.01$). We next determined whether the combination of Vemurafenib and Bortezomib affected cell cycle progression *in vivo* by analyzing cyclin D1 expression (Fig. 4C). Cyclin D1, a key regulator for cell cycle progression, is required for maintenance of the G2-phase; the prolongation of G2 and phase arrest is correlated with the expression of cyclin D1 (Stacey 2003, Jensen *et al.* 2012). The statistical analysis of IHC staining results indicated that the use of Vemurafenib or

Figure 4

Effect of drug combination on xenograft thyroid tumors in mice. Mice were engrafted with 2×10^6 SW1736 cells and treated with Vemurafenib and/or Bortezomib. (A) At the end point after treating with the drug Vemurafenib, Bortezomib or combination (V+B), tumors resected and stained with H&E. (B) Treatment of mice with the drug combination (V+B) produced a synergistic effect (64% tumor size reduction compared to initial tumor size), while Vemurafenib (35%) or Bortezomib (26%) alone slightly increased tumor size compared to initial tumor. Data are expressed as the arithmetic mean $\pm$ the standard deviation and considered significant at $P < 0.05$. (C) Statistical analysis of IHC of Ki-67 in control and treated mice with Vemurafenib, Bortezomib or combination for 4 weeks. Nuclear staining was counted from at least five fields and expressed as percentage, * and ** represent significance at $P < 0.05$ and $P < 0.01$, respectively, compared with control (no drug). (D) Statistical analysis of IHC of Cyclin D1 in control and treated mice with Vemurafenib, Bortezomib or combination for 4 weeks.

Bortezomib alone decreased the expression level of cyclin D1 to relatively modest levels (Vemurafenib: 89.8% and Bortezomib: 79.2%), while the combination decreased the expression level of cyclin D1 to 9.0%, an extremely low level in TC cells ($P < 0.05$) (Fig. 4C).

Discussion

Targeted inactivation of tumor growth-driving signals is an attractive strategy for anti-tumor therapy. Monotargeting therapy does not provide significant benefit for patients, particularly in those with advanced disease. Monotherapy over longer periods of treatment time is also associated with the eventual development of acquired resistance.

In the present study, we demonstrated the potential clinical viability of a combination therapy for the treatment of aggressive TC bearing a BRAF^{V600E} mutation (Stride & Coussios 2010, Ho *et al.* 2017). We found that the combination of Vemurafenib with Bortezomib has synergetic therapeutic advantages over the treatment with Vemurafenib or Bortezomib individually. We confirmed the clinical relevance of these findings both *in vitro* and *in vivo*. In addition, we provided insight into the possible cellular and molecular mechanisms involved in combination-therapy-induced tumor reduction. Our study shows that this reduction can be attributed to mitochondrial dysregulation leading to the initiation of apoptotic processes.

BRAF inhibitors have been FDA-approved and are promising agents for the treatment of cancer types with BRAF^{V600E} mutation (Okita *et al.* 2013, Griewank & Schilling 2017). These cancer types include TC, melanoma and colorectal carcinoma (Holt & Roy 2001, Cohen *et al.* 2017). Although monotherapies with mutant BRAF kinase inhibitors such as Vemurafenib have shown promising clinical outcomes in melanoma treatment and in clinical trials for TC, these successes have been hampered by the development of acquired resistance once the primary treatment has started (Stride & Coussios 2010, Ho *et al.* 2017). Consequently, some small molecule inhibitors such as MEK inhibitors have been developed based on their excellent anti-tumorigenic effects (Gyöngy & Coussios 2010, Welsh & Corrie 2015). Although the combination of MEK inhibitors and Bortezomib has showed a synergistic effect (Cooper *et al.* 2006, Kandil *et al.* 2013, Tsumagari *et al.* 2015), the side effects of MEK inhibitors have limited their widespread clinical utilization. These side effects include acute cardiac events, ocular issues, interstitial lung disease and pneumonitis (seen in patients

treated in the METRIC trial) (Gyöngy & Coussios 2010, Welsh & Corrie 2015). Vemurafenib, a BRAF^{V600E} inhibitor, might be an attractive drug to combine with Bortezomib, which targets the NF- κ B pathway, and in contrast to MEK inhibitors, Vemurafenib has advantages regarding side effect profiles. Thus, it can be anticipated that the combination of Vemurafenib with Bortezomib in the treatment of aggressive TC may reduce both the side effect burden and the development of acquired resistance to BRAF inhibitors.

Our results suggest the overexpression of BRAF^{V600E} is correlated with the activation of NF- κ B in advanced TC and also the induced death of aggressive TC, in response to the combination of Vemurafenib and Bortezomib, is regulated by the inhibition of the survival pathway NF- κ B, while the other mechanism is mediated by mitochondrial dysregulation leading to apoptosis.

This anti-proliferative effect of Vemurafenib and Bortezomib combination therapy was also demonstrated in a mouse model. The combination of Vemurafenib with Bortezomib showed significant tumor regression when compared to monotherapy or to untreated controls. The reduction of tumor volume was correlated with the reduction of Ki-67 expression. The therapeutic advantage of the drug combination is attributed to the fact that the administrated dose is less than the dose that would be required to trigger the side effects of the drugs.

The clinical relevance of this study relies on the potential use of this synergistic effect of currently FDA-approved anticancer agents Vemurafenib and Bortezomib. Our current findings provide evidence that the combination of Vemurafenib and Bortezomib at lower doses can overcome tumor resistance to monotherapy and subsequently minimize the adverse effects of monotherapy. The combination strategy may help prevent the development of acquired resistance of TC to BRAF^{V600E} inhibitor-based therapy. The acquired resistance to BRAF inhibitors is common in TC shortly after initial treatment with monotherapy.

In summary, our results demonstrate that the inhibition of the BRAF^{V600E}-mediated pathway together with activation of Bortezomib-mediated pathways of apoptosis may provide a clinically relevant therapeutic approach for the treatment of aggressive TC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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