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Simultaneous suppression of the MAP kinase and NF- κ B pathways provides a robust therapeutic potential for thyroid cancer

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Abstract

The MAP kinase and NF- κ B signaling pathways play an important role in thyroid cancer tumorigenesis. We aimed to examine the therapeutic potential of dually targeting the two pathways using AZD6244 and Bortezomib in combination. We evaluated their effects on cell proliferation, cell-cycle progression, apoptosis, cell migration assay, and the activation of the MAPK pathway *in vitro* and the *in vivo* using tumor size and immunohistochemical changes of Ki67 and ppRB. We found inhibition of cell growth rate by 10%, 20%, and 56% ($p < 0.05$), migration to 55%, 61%, and 29% ($p < 0.05$), and induction of apoptosis to 10%, 15%, and 38% ($p < 0.05$) with AZD6244, Bortezomib, or combination, respectively. Induction of cell cycle arrest occurred only with drug combination. Dual drug treatment in the xenograft model caused a 94% reduction in tumor size ($p < 0.05$) versus 15% with AZD6244 and 34% with Bortezomib ($p < 0.05$) and also reduced proliferative marker Ki67, and increased pRb dephosphorylation. Our results demonstrate a robust therapeutic potential of combining AZD6244 and Bortezomib as an effective strategy to overcome drug resistance encountered in monotherapy in the treatment of thyroid cancer, strongly supporting clinical trials to further test this strategy.

Keywords

MAPK; NF- κ B; Thyroid cancer; AZD6244; Bortezomib

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Conflict of interest

The authors have nothing to disclose.

Introduction

In the last few decades, the incidence of thyroid cancer (TC) has rapidly increased, making it the 9th most common cancer among men and women in the United States [1]. Most patients are diagnosed with well-differentiated papillary TC (PTC) and respond well to treatment with surgical resection followed by radioactive iodine ablation. However, undifferentiated tumors with a more aggressive phenotype, such as anaplastic TC (ATC), have already shown local and distant metastases at the initial diagnosis and are associated with a high mortality [1]. Although the overall 10-year survival rate of PTC patients is about 90%, approximately 10–20% of patients with stage I or II have a disease recurrence [2].

Many standard chemotherapies for cancer have been developed but the dosage and treatment duration of these drugs are often limited due to their poor specificity and severe toxicity to noncancerous tissues. Some of the novel small-molecule drugs developed in recent years hold promise but intrinsic or secondary drug resistance is a common and significant challenge. Therefore, alternative therapeutic tools for the treatment of these tumors are in great demand. The resistance to combination therapies occurs much less frequently if monotherapy is used [3]. The drug combination approach using agents with different mechanisms of action which targeted aberrant pathways, dysregulated signaling molecules in tumors, and tumor-specific antigens has become a promising strategy to overcome side effects and drug resistance [3]. For example, Sorafenib for radioactive iodine-refractory, locally advanced or metastatic differentiated TC [4,5] has been approved but still has low efficacy and severe side effects [6]. The recently FDA-approved lenvatinib provides improved therapeutic profiles [7], but there is also room for further improvement.

BRAF, a member in the MAP kinase (MAPK) pathway, is frequently mutated (35–70%) of PTC in other TC types [8,9]. BRAF phosphorylates and activates MEK, which in turn activates downstream of MAPK and regulates transcription factors involved in cell differentiation, proliferation, and survival [8]. More than 95% of BRAF mutations detected in TCs are thymine-to-adenine transversions resulting in the substitution of valine by glutamate at residue 600 (V600E) [8,10]. There is an association between BRAF^{V600E} mutation and aggressive clinical outcomes such as invasion, metastasis, tumor recurrence and patient mortality [8,11,12]. In turn, BRAF^{V600E} mutation can cause constitutive activation of the MAPK signaling pathway. BRAF^{V600E} also plays a role in the cellular microenvironment of cancer [13,14]. Thus, the MAPK pathway and its mutated BRAF can be used as therapeutic targets of TC. Many MAPK inhibitors have been studied in clinical trials [15,16]. Targeting the mutant BRAF^{V600E} in TC harboring BRAF^{V600E} using anti-BRAF^{V600E} molecules as single agent has been studied [17]. Clinical trials targeting metastatic PTC harboring BRAF^{V600E} have been opened and are currently ongoing using Vemurafenib as adjuvant and neoadjuvant therapies. Although these studies have brought to light an exciting new area of translational TC research, the clinical effectiveness of these inhibitors was generally limited, raising questions on whether targeting the MAPK pathway alone is therapeutically sufficient.

Nuclear factor kappa B (NF- κ B), a transcription factor, promotes genes involved in proliferation and inhibits apoptosis [18,19]. NF- κ B is one of the most important tumor-promoting factors and is therefore a promising therapeutic target in cancer. It is possible to interfere with NF- κ B activation at different levels, targeting components of its signaling cascade such as the IKK complex, the I κ B α inhibitory protein, the proteasome, and the RelA subunit of the transcriptionally active heterodimers. It has been reported that the anti-apoptotic behavior and invasiveness of TC cells transformed by oncogenic BRAF involved NF- κ B activation [20,21].

These results suggest that it may be a more effective therapeutic strategy to combine inhibitors, simultaneously targeting the MAPK and NF- κ B pathways in TC. Recently, the orally active MEK inhibitor AZD6244 has been studied in TC [22] after it had entered clinical trials on other human cancers [23–25]. Bortezomib, a proteasome inhibitor that suppresses the activity of NF- κ B, is currently approved for the treatment of multiple myeloma. In the present study, we investigated the therapeutic potential of combining AZD6244 and Bortezomib in the treatment of aggressive TC cells harboring BRAF^{V600E} mutation *in vitro* and in a mouse xenograft model.

Materials and methods

Cell cultures

K1 cells (PTC) were provided by Health Protection Agency Culture Collections (Salisbury, UK). SW1736 cells (ATC) were originally from Dr. N.E. Heldin (University of Uppsala, Uppsala, Sweden). NPA (PTC) and DRO (ATC) were from Dr. Guy J.F. Julliard (University California Los Angeles School of Medicine, Los Angeles, CA). All cells are carrying mutant BRAFV600E. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM Sodium pyruvate, and 1% penicillin–streptomycin in a 37 °C humidified incubator with 5% CO₂. Cells were treated with AZD6244 (SellekChem, Houston, TX) and Bortezomib (LC Laboratories, Woburn, MA) at various indicated concentrations and time points. The culture medium and drugs were replenished every 24 h during the treatment.

Cell proliferation assay

Cell proliferation assay was performed in triplicate and each experiment was repeated at least three times. Cells (800/well) 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 tetrazolium salt WST-8 (Cell Counting Kit-8, Dojindo Molecular Technologies Inc., MD) was added 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 AZD6244 and Bortezomib were calculated using the Reed–Muench method [26]. Trypan blue (Gibco, CA) exclusion assay was also performed for K1 and SW1736 cells.

Cell cycle assay

TC cells were harvested, spin down, and the resulting pellets were fixed in ice-cold 70% ethanol. Fixed cells were centrifuged, washed and re-suspended 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 (FACS, Calibur in the UAMS Flow Cytometry Core Facility, Tulane University, New Orleans, LA), followed by the determination of the percentage of cells in G1, S, and G2/M.

Apoptosis assay

TC cells were harvested, 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 a FACS as described. After treatment with either AZD6244 (1 μ M), Bortezomib (35 nM), or their combination for 48 h, K1 cells (4×10^6 cells) were washed with PBS and harvested for DNA fragmentation assay. Cell pellets were re-suspended in 600 μ l of lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), and 0.2% Triton X-100), and incubated in a cold room on a rotator for 30–45 minutes. Cell lysates were centrifuged at 12,000 g at 4 °C for 20 min, and supernatants containing low molecular-weight DNA were removed and digested with 0.5 mg/ml of proteinase K at 55 °C for 1 h. The DNA was extracted and precipitated in ethanol at –20 °C overnight. After rehydration in 30 μ l TE buffer (pH 8.0), the DNA sample was treated with RNase A (0.1 mg/ml) at 37 °C for 1 h. Eight μ g DNA was loaded and electrophoresed on 2% agarose gel and visualized with ethidium bromide fluorescence.

Western blotting analysis

Cells treated with inhibitors at the indicated concentrations were lysed in PhosphoSafe™ Extraction Reagent (EMD Biosciences, Inc, Madison, WI) and protein concentrations were determined using the BCA method (Thermo Scientific, Rockford, IL) as described previously [22]. Briefly, protein samples were boiled in an equal volume of sample loading buffer for 5 min. Equal amounts of proteins were 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 blots were incubated with either anti- β -actin from Sigma (St. Louis, MO, USA) or primary antibodies against phospho-p44/42 MAPK (ERK1/2) and total-p44/42 MAPK (ERK1/2) from Cell Signaling (Beverly, MA, USA). Blots were then incubated with the secondary antibody, goat anti-mouse IgG (LI-COR Biotechnology, Lincoln, Nebraska, USA). The antigen-antibody complexes were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA). Protein expression was determined by a gel documentation system (Bio-Rad, Model 700) equipped with Quantity One software.

Cell migration assay

About 2×10^5 cells in 1% FBS were seeded on the top of a Boyden chamber, followed by drug treatment with AZD6244 (1 μ M), Bortezomib (35 nM), or both for 24 h. Cells were fixed and stained with H&E and counted under a light microscope. At least five fields were counted and the average number of cells was obtained for each treatment conditions.

***In vivo* studies**

Six-week-old inbred homozygous athymic BALB/C nude (nu/nu) male mice (Charles River, Wilmington, MA, USA) 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 K1 cells with Matrigel™ basement membrane matrix (BD Bioscience, 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 AZD6244 (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, or a combination of AZD6244 and Bortezomib, or vehicles (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 fractional inhibition of tumor growth was calculated on the basis of 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 in each group were resected on Day 28 of the efficacy study, fixed in formalin, embedded, cut, and mounted. The expression of Ki-67 was assessed by IHC according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, USA). After antigen retrieval, embedded tumors were treated by permeabilization with 0.1% Sodium Citrate plus 0.1% Triton X-100. The embedded tumors were then subjected to IF staining with ppRb (1:200, GeneTex, Irvine, CA, USA) antibody at 4 °C overnight followed by washing with cold PBS and incubated with Alexa Fluor 488-labeled anti-rabbit secondary antibody (1:500) (Invitrogen). The cells were examined by fluorescence microscopy (Olympus America Inc, Center Valley, PA). Fluorescent intensities from images of six randomly selected microscopic fields of cells were semi-quantitatively analyzed by densitometry (ImageJ software, NIH Image).

Statistical analysis

Differences between experimental and control groups were analyzed by analysis of variance (ANOVA) using GraphPad software (La Jolla, CA, USA). A *p* value of <0.05 was considered to be significant.

Results

Treatment of TC cells with AZD6244 and Bortezomib enhanced the inhibition of cell growth

We evaluated the *in vitro* effects of Bortezomib, which targets the NF-κB pathway, on cell proliferation and survival using two four TC cell lines. TC cell lines harboring the BRAF^{V600E} mutation, PTC (K1 and NPA) and ATC (DRO and SW1736), were exposed to Bortezomib (0~100 nM) for 120 hours and cell viability was determined by cell proliferation assay (Fig. 1A). The IC₅₀ of Bortezomib and AZD6244 are shown in Table 1. To determine whether simultaneous suppression of MAPK and NF-κB pathways can exert synergistic

inhibitory effects on TC cell lines, cells were exposed to sub-IC₅₀ concentrations of AZD6244 (1.0 μ M) as we previously described [22] and Bortezomib (35 nM). We selected to utilize K1 and SW1736 cells in the following experiments because they have shown relatively higher IC₅₀ compared to other tested cells. Viability of K1 and SW1736 cells was assessed at 72 hours post exposure to these compounds (Fig. 1B). In K1 and SW1736 cells treated with each drug individually, cell viability was decreased by 10% and a 37% after treating the cells with AZD6244 or Bortezomib, relative to non-treated control cells. However, the exposure of these cells to both compounds simultaneously showed a markedly enhanced inhibition of cell viability, by 54% and 67% ($p < 0.001$) after 72 hours of treatment in K1 and SW1736, respectively. These results demonstrate that the combination of AZD6244 and Bortezomib can be synergistically suppressed by the combination of AZD6244 and Bortezomib.

Combination of AZD6244 and Bortezomib significantly induced apoptosis and cell cycle arrest in TC cells

Next, we examined the apoptotic effects of AZD6244 and Bortezomib using flow cytometric analysis and DNA fragmentation assay after 48 hours of exposure to the two compounds individually or simultaneously. Individual exposure of K1 cells to AZD6244 or Bortezomib for 48 hours caused a slight apoptotic change (10–15%); however, treatment of cells with combined drugs significantly increased cell apoptosis (~40%, $p < 0.01$) (Fig. 2A). Using DNA fragmentation assay, AZD6244 or Bortezomib alone caused a slight DNA fragmentation, while the exposure of K1 cells to the two compounds resulted in a remarkable increase of the laddering effect even at lower concentrations of Bortezomib (10 nM) with AZD6244 (0.5 μ M), indicating that a drug combination significantly triggered cell apoptosis in K1 cells harboring the BRAF^{V600E} mutation (Fig. 2B).

Next, we sought to examine the effects of AZD6244 and Bortezomib on cell cycle progression after 24 hours of exposure to these compounds individually or simultaneously. Flowcytometric analysis was performed to determine the distribution of cells in G1-, G2- and S-phases of the cell cycle. In K1 cells, exposure to either compound alone did not show any changes in cell cycle compared to untreated cells, which was 37% in the G1 phase, 3% in the G2-phase and 60% in the S-phase. Intriguingly, the drug combination showed a significant increase in the G2-phase to 59.2% and a decrease of cells entering the S-phase to 9.1% (Fig. 2C). The exposure to both compounds resulted in a drastic drop in the cellular proliferative responses. These results showed that cell cycle arrest occurred at the G2-phase after exposure of AZD6244, Bortezomib alone or in combination.

We used Western blotting to confirm that AZD6244 could specifically suppress the MAPK pathway. K1 cells were treated with AZD6244 or Bortezomib individually or simultaneously for 0.5, 2.0 and 4.0 hours. As expected, cells treated with AZD6244 showed a significant reduction in phosphorylation of ERK1/2 after 0.5 hour. In contrast, no reduction of phosphorylation of ERK1/2 was seen in the cells treated with Bortezomib alone (Fig. 3). However, the combination of AZD6244 and Bortezomib induced prolonged inhibition of ERK1/2 phosphorylation up to 4 hours.

Combination of AZD6244 and Bortezomib suppressed the migration of K1 cells

As cancer cell migration is mediated by signaling pathways, such as the RAS/RAF/MEK/ERK pathway, we evaluated the effects of AZD6244, Bortezomib alone and in combination on cell migration using a modified Boyden chamber assay. The combination of AZD6244 and Bortezomib significantly reduced cell migration compared to untreated cells while the use of each compound alone exhibited a smaller effect on cell migration (Fig. 4).

Combination of AZD6244 and Bortezomib synergistically reduced tumor growth in vivo

Our *in vitro* results showed that the combination of AZD6244 and Bortezomib was robustly effective in suppressing the growth of K1 cells with the BRAF^{V600E} mutation. To further evaluate the efficacy in inducing the cell toxicity of AZD6244 in combination with Bortezomib as a potential treatment option for TC, we used a mouse xenograft tumor model derived from K1 cells. Six-week-old inbred homozygous athymic BALB/C nude (nu/nu) male mice were implanted with 2.0×10^6 K1 cells. After the tumor size reached 50–100 mm³, AZD6244 and Bortezomib were administered to mice for 4 weeks. The effect of drug combination using AZD6244 and Bortezomib on tumor size was determined with calipers (Fig. 5A). Tumor growth curves for these studies are depicted in Fig. 5B. Tumor size was reduced by either AZD6244 (15%) or Bortezomib (21%) alone, while the combination of the two inhibitors resulted in a more pronounced tumor growth inhibition by 94% compared with the control mice receiving vehicle alone ($p < 0.05$).

To characterize the mechanism of tumor growth inhibition observed in the tumor xenograft models by AZD6244 and Bortezomib, the mitosis of tumor tissues was assessed by Ki-67 expression using immunohistochemical analysis. Active cell proliferation was observed in tumor tissue sections with a 56% relative proliferation rate (Fig. 5B). Monotherapy with AZD6244 or Bortezomib slightly decreased the percentage of Ki-67-positive proliferating tumor cells, with relative proliferation rates of 42% and 39%, respectively (Fig. 5B). Combined treatment with AZD6244 and Bortezomib markedly decreased the percentage of Ki-67-positive proliferating tumor cells to 10% ($p < 0.01$), which was consistent with the marked *in vitro* inhibition of ERK1/2 phosphorylation. We next determined whether the combination of AZD6244 and Bortezomib affected cell cycle progression *in vivo*.

Retinoblastoma gene (Rb), a tumor suppressor similar to p53, is known to be required for the maintenance of G2-phase arrest; the prolongation of G2-phase arrest is correlated with a gradual accumulation of hypophosphorylated Rb protein (pRb) [27]. In the light of our *in vitro* results of cell cycle analysis, we thought to examine the phosphorylation of pRb. IHC staining results indicated that the use of AZD6244 or Bortezomib alone decreased the level of phosphorylation of pRb to relatively modest levels (AZD6244: 30.7% and Bortezomib: 19.9%), while the combination decreased the phosphorylation of pRb (7.0%) to an extremely low level in TC cells ($p < 0.05$) (Fig. 5C).

Discussion

Severe side effects and drug resistance are two major obstacles limiting the use of recently developed molecularly targeted agents and classical systemic chemotherapies. Genetic

alterations including RET/PTC rearrangements, Ras and BRAF mutations can cause aberrant activation of the MAPK pathway in TC [28,29]. Single agent-based monotherapy targeting a limited pathway often fails to overcome aggressive cancer, although initial response is sometimes promising. Finding novel strategies to overcome drug resistance and sensitize the tumor cells to existing antitumor drugs is an urgent and arduous challenge in managing patients with aggressive TC; success in tackling this challenge would have a significant clinical impact.

The NF- κ B signaling pathway plays an important role in thyroid tumorigenesis and maintenance of malignant phenotype in TC cell lines [10]. Activation of NF- κ B appears to require the phosphorylation by IKK α and subsequent degradation of phosphorylated I κ B protein by 26S proteasomes, which allow rapid translocation of NF- κ B from the cytoplasm to the nucleus [30]. NF- κ B regulates the expression of genes involved in cellular proliferation and apoptosis [31]. BRAF^{V600E} mutation also activates I κ B α and causes activation of NF- κ B [20]. The MAPK pathway plays a regulatory role in NF- κ B activation, and the MEK inhibitor (PD98059) has been reported to induce NF- κ B activation by augmenting I κ B degradation [32] or increasing I κ B phosphorylation [33]. Prior studies showed that the combination of MEK inhibitor (PD0325901) and NF- κ B pathway inhibitor (PS1145) was associated with a synergistic inhibition in TC cells [34], although these inhibitors are not currently applicable in the clinical setting. It was also shown that the combination of an ERK inhibitor and proteasome inhibitor (Bortezomib) synergized the anticancer effect in human medulloblastoma [35]. Therefore these findings are supportive of our hypothesis and results that suppression of the NF- κ B pathway is important to increase the inhibitory effects of the MAPK pathway in TC and may help reduce drug resistance of the MAPK pathway inhibitors.

In the present study, we utilized *in vitro* and *in vivo* models of TC to evaluate the efficacy of AZD6244 in combination with Bortezomib as a potential therapeutic strategy for the treatment of TC patients expressing mutant BRAF^{V600E}. We have shown that single use of either AZD6244 or Bortezomib moderately decreased cell proliferation, which was profoundly augmented with combined therapy. Mechanistically, our results suggest that the combination of AZD6244 and Bortezomib resulted in a synergistic anti-proliferative effect in TC cell lines, which are involved in the induction of cell apoptosis. It is important to note that p53 is wild type in K1 [36] while it is null in SW1736 [37]. Bortezomib induces p53-independent apoptosis [38]. The effect of the combination of drugs on apoptosis may be independent of any changes in the p53 gene since the K1 cells harbor a wild-type p53. This anti-proliferative effect of combined drugs was also demonstrated in the animal model by showing a significant tumor growth inhibition compared with untreated controls or monotherapy. The combination drug strategy significantly reduced Ki67 expression. In addition, we were able to reduce the dose of the two drugs to minimize the chance of unwanted side effects while preserving the synergistic anti-tumor effects of the combination use of AZD6244 and Bortezomib. It has been previously reported that Bortezomib induced G₂/M cell-cycle arrest in colorectal cancer cell lines [39], whereas AZD6244 induced G₁ cell-cycle arrest and favored apoptosis in a TC cell line [22]. In our study, the drug combination synergistically induced a cell-cycle arrest in the K1 cells *in vitro* and *in vivo*,

with a reduction in the percentage of cells in the S-phase (60.9%–9.1%) and a concomitant accumulation of cells in the G2-phase (2.5%–53.1%). In parallel, animal studies showed that monotherapy exerted a modest effect on cell-cycle distribution with the indicated doses. NF- κ B has been shown to promote phosphorylation of pRB via cyclinD1 expression and subsequent activation of CDK [40,41]. Bortezomib inhibits both NF- κ B and phosphorylation of pRb eventually leading to apoptosis [42]. The cell cycle arrest observed in the xenograft model was also corroborated by hypo-phosphorylation of pRb, suggesting its role in the inhibitory effects of the combination use of the two drugs we tested here on tumor growth.

Because cell proliferation is a phenotypic endpoint of malignant neoplasm, we utilized other *in vitro* models including a modified Boyden chamber and found that the drug combination resulted in significant inhibition of TC cell migration. This suggests that the combined drug treatment not only inhibits tumor growth but also may play a role in the metastatic process [43]. It is well known that MEK is a downstream effector of the EGFR, VEGFR, and IGF-1R signaling pathways, and previous data have shown that VEGFR induced cell migration in TC [44]. Therefore MEK activation could play a role in cell migration. In addition, the NF- κ B pathway induces migratory activity [30]. Thus, our results suggest that the combination of AZD6244 and Bortezomib may act together on the downstream signaling pathway to block the phenotypic effects of IGF-induced TC migration.

A prominent strength of our study testing the combined effects of AZD6244 and Bortezomib is their high potential for clinical applicability in TC patients, based on their excellent safety profiles reported in clinical trials in other human cancers [25,45–47]. Our current findings suggest that targeting the NF- κ B pathway may help overcome the resistance of TC to MEK inhibitors, and the combination of inhibitors targeting the NF- κ B and MAPK pathways provides an attractive novel therapeutic strategy for aggressive TC.

In summary, the present study demonstrates that the combination of AZD6244 and Bortezomib can synergize their abilities to induce cell cycle arrest and apoptosis and inhibit the proliferation of TC cells harboring BRAF^{V600E} mutation. This was also corroborated by our *in vivo* studies which confirmed the synergistic antitumor effects of both drugs used in combination. Thus, the combination of AZD6244 and Bortezomib is a promising therapeutic strategy for TC, which is worth further testing in future clinical trials.

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Highlights

- We examined simultaneous targeting of two pathways, the MAP kinase and NF- κ B pathways.
- We used two FDA approved drugs, AZD6244 and Bortezomib.
- Dual drug treatment caused significant apoptosis *in vitro*.
- Dual drug treatment caused the inhibition of cell growth and migration *in vitro*.
- Dual drug treatment in the xenograft model caused a significant reduction in tumor size.

Fig. 1.

Cell viability assay in TC cells. A. K1, NPA, DRO, and SW1736 cells were treated with Bortezomib (0–100 nM) for 120 h and cell viability was assayed by MTT assay. B. K1 and SW1736 cells were treated with AZD6244 (1.0 μ M) and Bortezomib (35 nM) for 72 h and cell viability was tested by Trypan blue nuclear exclusion assay (* p < 0.01, ** p < 0.001).

Fig. 2.

Apoptosis assay in K1 cells. A. K1 cells were treated with AZD6244 (1.0 μM) and Bortezomib (35 nM) for 48 h. K1 cells were stained with propidium-iodide and anti-Annexin V. The percentage of apoptotic cells is indicated (* $p < 0.05$, ** $p < 0.01$). B. K1 cells were treated with AZD6244 and/or Bortezomib and apoptosis was assessed by DNA fragmentation assay. C. K1 cells were treated with AZD6244 (1 μM) and/or Bortezomib (35 nM) and cell cycle was assessed by flow cytometry assay.

Fig. 3.

K1 cells were treated with AZD6244 (1 μ M) or/and Bortezomib (35 nM) for 0.5, 2.0 and 4.0 h. Cells were harvested and proteins were extracted, immunoblotted and hybridized with pERK, tERK and β -actin in Western blotting assay. The combination of AZD6244 and Bortezomib prolonged the inhibition of phosphorylation of ERK. AZD6244 + Bortezomib (A+B).

Fig. 4. Combination of AZD6244 and Bortezomib suppresses migration of K1 cells. About 2×10^5 cells in 1% FBS were seeded on the top of Boyden chamber, followed by drug treatment with AZD6244 (B), Bortezomib (C) or both (D) or control vehicle (no drug) (A) for 24 h. Cells were fixed and stained with H&E and counted under a light microscope. At least five fields were counted and the average of cell numbers was obtained (E). *, ** represents significance at $p < 0.05$ and $p < 0.01$, respectively, compared with DMSO. AZD6244, Bortezomib or combination (A+B).

Fig. 5. Effect of drug combination using AZD6244 and Bortezomib on xenograft thyroid tumor in mice. Mice were engrafted with 2×10^6 K1 cells and treated with AZD6244 and/or Bortezomib. A. H&E staining is shown ($\times 40$). Treatment of mice with the drug combination (A+B) produced a synergistic effect (93.1% tumor size reduction) compared to AZD6244 (15.0%) or Bortezomib (27.5%) alone. Data are expressed as mean $\pm$ SEM and considered significant at $p < 0.05$. B. Untreated (a), treated with AZD6244 (b), Bortezomib (c) or combination (d) for 4 weeks. Paraffin-embedded tissue sections were incubated with Ki-67

antibody. Nuclear staining was counted from at least five fields and expressed as percentage (e), * and ** represent significance at $p < 0.05$ and $p < 0.01$, respectively, compared with control (no drug). C. Tumor sections were immunoreactive with anti-ppRb and nuclei were stained with PI.

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Table 1Genotypes and their IC₅₀ of AZD6244/Bortezomib.

Cell line	Derived from	BRFA status	IC ₅₀	
			AZD6244	Bortezomib
K1	PTC	V600E	6.1 μM	45 nM
NPA	PTC	V600E	0.45 μM	28 nM
SW1736	ATC	V600E	1.5 μM	38 nM
DRO	ATC	V600E	0.28 μM	22 nM