Synergistic Action of a RAF Inhibitor and a Dual PI3K/mTOR Inhibitor in Thyroid Cancer

Ning Jin1, Tianyun Jiang1, David M. Rosen1, Barry D. Nelkin1, and Douglas W. Ball1,2

Abstract

Purpose: In thyroid cancer clinical trials, agents targeting VEGF receptors (VEGFR) and RET, among other kinases, have led to partial responses but few complete or durable responses. The RAF–MEK–ERK and PI3K–AKT–mTOR signaling pathways are frequently activated in differentiated and medullary thyroid cancer (DTC and MTC) and may provide therapeutic targets for these diseases. We tested a novel drug combination targeting RAF, phosphoinositide 3-kinase (PI3K), and mTOR, plus VEGFR2 and RET, in thyroid cancer preclinical models with defined genetic backgrounds.

Experimental Design: RAF265, an ATP-competitive pan-RAF inhibitor active against VEGFR2, and BEZ-235, a PI3K inhibitor also active against Torc1 and Torc2, were tested alone and in combination in a panel of thyroid cancer lines. We tested RAF265 and BEZ-235 for kinase inhibition, growth inhibition and cell-cycle alterations, and inhibition of signaling targets and tumor growth in xenograft models.

Results: Both drugs potently inhibited their kinase targets in the extracellular signal-regulated kinase (ERK) and PI3K pathways. In addition, RAF265 had significant RET inhibitory activity (IC50 = 25–50 nmol/L for RETC634W). The combination strongly inhibited proliferation of DTC and MTC cell lines with mutations in RAS, BRAF, PTEN, and RET. Synergy was shown for B-CPAP (BRAFV600E) and TT cells (RETC634W). The combination of both drugs significantly inhibited growth of CAL62 (KRASG12R/G12R) and TT xenografts, thoroughly inhibiting ERK and PI3K pathway signaling.

Conclusions: Combined blockade of ERK and PI3K signaling potently inhibits growth in preclinical models representing the key genotypes seen in refractory thyroid cancer. These targets and therapies are promising for further development in both differentiated and medullary thyroid cancers.

Introduction

Thyroid cancer, with an estimated incidence of 44,670 cases in the United States in 2010, represents nearly 95% of endocrine malignancies (1). Approximately 1,700 deaths are attributed to thyroid cancer annually in the United States (1). Roughly 95% of thyroid cancer cases, including papillary, follicular, and Hürthle cell cancers are classified as differentiated thyroid cancer (DTC), sharing the potential for adjuvant treatment with radioactive iodine. Prevalent mutations in DTC tumors include BRAF and RAS mutations, activation of RET via rearrangement, and, less commonly, PTEN loss, AKT1 activating mutation, and alterations in PI3K genes (2–5). AKT activation is associated with increased invasiveness in differentiated thyroid cancer (6). Taken together, the vast majority of differentiated thyroid cancer patients have a detectable genetic alteration affecting the RAF–MEK–ERK, PI3K–AKT–mTOR pathways, or both.

Medullary thyroid cancer (MTC) has a distinct cell of origin, the calcitonin-producing parafollicular C cell, and a major hereditary component—the MEN 2 syndrome. Medullary thyroid cancer represents 3% to 5% of thyroid cancer cases and a disproportionate number of thyroid cancer deaths. Germline point mutations in RET are seen in virtually all MEN 2 patients. In addition, 40% of nonfamilial MTC tumors bear somatic RET mutations, which confer a powerful negative prognostic impact (7). The activated RET kinase is known to signal via extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K), among other pathways, leading to enhanced proliferation and resistance to apoptosis.

Recent clinical trials both for advanced radioiodine-refractory differentiated thyroid cancer and for medullary thyroid cancer have focused on semiselective kinase
Inhibitors that target VEGF receptors (VEGFR), RET, additional RTKs such as platelet-derived growth factor receptor and fibroblast growth factor receptor, and, to a lesser extent, RAF. Among the strongest results reported to date are for pazopanib in differentiated thyroid cancer, with a 49% partial response rate, and significant prolongation of progression-free survival (8). Among the strongest results reported for medullary thyroid cancer are for vandetanib, associated with a 45% partial response rate and prolonged progression-free survival in a randomized phase III trial (9). Other drugs with activity against differentiated thyroid cancer and/or medullary thyroid cancer include sunitinib, sorafenib, axitinib, and cabozantinib, sharing a major common target of VEGFR2 with additional receptor tyrosine kinases including RET in most cases (10, 11).

There has been increasing interest in targeting RAF, focusing on the BRAF mutant subset of DTC. Salerno and colleagues reported encouraging results on preclinical data for the RAF inhibitor PLX4720 (12). The related compound PLX4032 (Roche R05185426; vemurafenib) resulted in partial response or prolonged stable disease in the 3 differentiated thyroid cancer patients reported by Flaherty and colleagues in a phase I trial focused primarily on melanoma (13). On the basis of poor responses to RAF inhibitors in RAS-mutant melanoma, a developing consensus is that development of thyroid cancer RAF inhibitor probably should be restricted to the BRAF mutant subset of DTC and ATC (14). Selective RAF inhibitors such as PLX4032 can lead to paradoxically increased ERK signaling, especially in settings where there is upstream pathway activation, such as with RAS mutations and low to moderate RAF inhibitor doses (15).

In the current thyroid cancer preclinical study, we combined RAF-MEK-ERK inhibition with inhibition of PI3K-AKT-mTOR and VEGFR2. We hypothesize that both PI3K-AKT-mTOR and RAF-MEK-ERK signaling are important for thyroid cancer resistance to apoptosis and tumor progression. In these settings, targeted anti-VEGFR therapy or RAF inhibition could be ineffective if PI3K survival signaling remains intact. We selected a combination of 2 promising drugs in current development to simultaneously target the RAF-MEK-ERK and PI3K-AKT-mTOR pathways.

NVP-BEZ235 (Novartis) is an orally available, ATP-competitive class I PI3K inhibitor with IC_{50} less than 25 nmol/L for p110-α, -β, and -δ. In addition, BEZ-235 potently inhibits Torc1 and Torc2 (IC_{50} = 20 nmol/L). For a panel of 18 other kinases, the IC_{50} is more than 10,000 nmol/L (16, 17). BEZ-235 has broad preclinical activity with a proapoptotic effect in tumor models with PI3K pathway genetic alterations and an antiproliferative effect across several other tumor types (18). In a phase I clinical trial in advanced solid tumors, BEZ-235 was well tolerated, with significant activity in breast cancer patients with PI3K pathway oncogenic activation (19).

RAF265/CHIR-265 (Novartis) is an ATP-competitive pan-RAF inhibitor with activity against wild-type and V600E mutant BRAF as well as CRAF (IC_{50} = 3–60 nmol/L in a cell-free system). RAF inhibition in BRAF mutant melanoma cells is associated with apoptosis and cell-cycle arrest. RAF265 also inhibits VEGFR2 and proliferation of human endothelial cells (IC_{50} = 30 nmol/L; refs. 20, 21). RAF265 had significant clinical activity in a phase I melanoma expansion cohort (22). In a recent preclinical study, the combination of everolimus and RAF265 was more effective than RAF265 alone in culture and xenograft models with RAS and PI3K pathway activation (21).
Western blotting

Cells were treated for 4 hours or 5 days as described earlier, washed with PBS, and then harvested by scraping with 1× sodium dodecyl sulfate lysis buffer [2% sodium dodecyl sulfate and 62.5 mmol/L Tris (pH 6.8)]. Lysates were electrophoresed on 4% to 20% gradient polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Blots were probed at 4°C overnight with primary antibody to pERK (CST 9101) diluted 1:1,000 in 5% milk, total ERK (CST 9102), pAKT (CST 4058), total AKT (CST 9272), pS6 (CST 2215), total S6 (CST 2217), pRET (CST 3221), total caspase 3 (CST 9665), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen). Anti-rabbit secondary antibodies (Santa Cruz) were diluted 1:2,000. Blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.).

Growth analyses

Growth assays were done in triplicate using the MTT assay (M2128; Sigma-Aldrich) following the manufacturer’s instructions. Cells were seeded in 24-well plates using phenol red-free media. MTT absorbance was determined 5 days after exposure to drugs or DMSO alone. Data are represented as mean ± SEM absorbance values, based on 3 to 6 independent experiments, normalized to control cells. GI50 was determined as the X-intercept of log10(Fx/Ft1), where Fx is the fraction affected and Ft1 is the fraction unaffected, plotted versus log10(concentration), determined by linear regression (23). GI50 values were reported as the mean ± SD of 3 to 6 independent experiments.

Flow cytometric cell-cycle analyses

Thyroid cancer cells were treated for 48 hours with drug combinations or vehicle control and then trypsinized and lysed in Hoechst 33258 staining solution (0.56% Nonidet P-40, 3.7% formaldehyde, and 11 μg/mL Hoechst 33258 in PBS) for flow cytometry. Nuclei were analyzed using a LSR flow cytometer (BD Biosciences) gated for single nucleus. Cell-cycle profiles were determined using 10,000 gated nuclei with ModFit LT 2.0 software (Verity).

Animal studies

Animal studies were approved by the Johns Hopkins Animal Care and Use Committee and conducted in accordance with NIH guidelines. CAL62 or TT cells suspended in Matrigel (5 × 10⁵ cells/200 μL) were inoculated subcutaneously into the right flank of 4- to 6-week-old female athymic nude (nu/nu) mice (Harlan Laboratories). Once palpable, tumor volumes were calculated with calipers using the following formula: length × width × height × 0.5236. After tumors reached approximately 0.1 cm³ in size, animals were sorted into groups of 13 to achieve equal distribution of tumor size in all treatment groups. Animals were treated with DMSO vehicle alone, RAF265 (10 mg/kg thrice weekly), BEZ-235 (20 mg/kg daily), or a combination of both drugs, by oral gavage. Animals were euthanized by CO₂ asphyxiation when tumors reached approximately 1 cm³ in size. Kaplan–Meier analysis for tumor progression was done with GraphPad Prism software, using a 4-fold increase in tumor volume from onset of treatment as a threshold for tumor progression.

Immunohistochemistry

Four hours after treatment with drugs or DMSO control, mice were sacrificed, tumors were harvested in 10% paraformaldehyde overnight, followed by incubation in 70% ethanol. Paraffin sections were incubated with anti-pERK (CST 4376; 1:100), pS6 (CST 2215; 1:100), pAKT (CST 3787; 1:50) or using a Vectastain ABC kit (Vector Labs) with 3,3′-diaminobenzidine as a chromogen and hematoxylin as a counterstain. Anti-CD31 staining (Abcam ab28364; 1:50) followed a pressure cooker antigen retrieval method according to the manufacturer’s protocol. Microvessel density (MVD) was estimated as the mean of CD31-reactive foci in 10 hpf in the tumor periphery, using a 2-tailed paired t test for significance. Light microscopy was carried out with an Olympus Vanox and Nikon DMX1200 CCD camera, using Nikon ACT-1 image capture software.

Results

Growth inhibition of thyroid cancer cell lines by RAF265 and BEZ-235

In dose–response studies, we found that RAF265 inhibited ERK activation in 8505C and B-CPAP, two DTC lines with a BRAFV600E mutation. The estimated IC50 in cell culture was 100 to 200 nmol/L (Fig. 1A). Using the CAL62 DTC line with a KRASG12R mutation, we observed that BEZ-235 potently inhibited AKT activation, with an IC50 of approximately 10 nmol/L (Fig. 1B). We initially carried out MTT proliferation assays at these doses across a panel of thyroid cancer lines with a range of genotypes including BRAFV600E, KRASG12R, HRASG12A, and PTENnull (Fig. 1C). BEZ-235 at a concentration of 10 nmol/L was strongly inhibitory for PTENnull FTC133 cells, with additional activity against the RAS mutant and BRAF mutant lines. Somewhat surprisingly, RAF265 at a concentration of 200 nmol/L also had activity across the panel, including non-BRAF mutant lines. In combination, the 2 drugs were highly effective, with MTT values reduced to approximately 10% of control, even at these modest doses, roughly at IC50 values for their respective targets. Target inhibition persisted in vitro for at least 72 hours, using fully inhibitory doses (Fig. 1D).

GI50 concentrations for BEZ-235 and RAF265 as single agents are indicated in Table 1. The PI3K/mTOR inhibitor BEZ-235 was strikingly potent for all 6 tested thyroid cancer cell lines with their diverse genotypes. The lowest values were recorded for FTC133 (PTENnull, 1.7 nmol/L) and CAL62 (KRASG12R, 3.8 nmol/L). However, BEZ-235 also caused powerful growth inhibition of BRAF and RET mutant lines, perhaps reflecting the dual inhibitory capacity for mTOR and PI3K. RAF265 was most potent for the BRAF mutant line B-CPAP (GI50 = 91.6 nmol/L) and for
FTC133 (27.4 nmol/L). In contrast, 8505C was relatively resistant to this single agent despite a BRAF mutant genotype (368 nmol/L). In general, GI50 values for the 2 drugs corresponded to their IC50 values for the tested kinase targets. No clear-cut relationship was seen for GI50 and mutation types across the cell panel.

**Downstream kinase inhibition by combined BEZ-235 and RAF265**

We further explored the capacity of these drugs to inhibit downstream kinases, singly and in combination. In BRAF mutant cell lines, we observed complete inhibition of pERK by a higher dose of RAF265 (800 nmol/L; Fig. 2). Inhibition was less complete in RAS mutant CAL62 cells. In the tested lines, RAF265 did not lead to significant alterations in PI3K, AKT, or mTOR activity, as assessed by immunoblotting for pAKT and pS6. BEZ-235 (500 nmol/L), as expected, completely blocked these activities, with no significant effect on pERK. BEZ-235 also partially reduced total S6 abundance, the significance of which is unclear. The combination of the 2 drugs at these higher doses completely abrogated AKT, mTOR, and ERK activity in the BRAF mutant lines and was associated with low-level residual ERK activity in the RAS mutant cells. We observed a minimal up-regulation of pERK with the lowest tested doses of RAF265 in KRAS mutant CAL62 cells, similar to results previously reported for allosteric RAF inhibitors such as PLX4032 (refs. 11, 14; Supplementary Fig. S1).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genotype</th>
<th>GI50, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BEZ-235</td>
</tr>
<tr>
<td>CAL62</td>
<td>KRASG12R/G12R</td>
<td>3.8 ± 0.66</td>
</tr>
<tr>
<td>8505C</td>
<td>BRAFV600E/V600E</td>
<td>5.5 ± 1.0</td>
</tr>
<tr>
<td>B-CPAP</td>
<td>BRAFV600E/wt</td>
<td>22.8 ± 3.3</td>
</tr>
<tr>
<td>FTC133</td>
<td>PTENat</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>C643</td>
<td>HRASG12A/wt</td>
<td>6.4 ± 3.4</td>
</tr>
<tr>
<td>TT</td>
<td>RETC634W</td>
<td>25.4 ± 5.9</td>
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**Table 1. Calculated GI50 ± SD values for BEZ-235 and RAF265 across a panel of thyroid cancer cell lines**

NOTE: See Materials and Methods for calculation method. Known mutations in each cell line are indicated.
We hypothesized, based on structural similarity to sorafenib, that RAF265 could have RET inhibitory activity, similar to sorafenib (24). Analyzing pRETY905, we indeed found that RAF265 potently inhibited RET autophosphorylation in TT cells, with an estimated IC50 of approximately 25 nmol/L (Fig. 2).

Synergistic activity of RAF265 and BEZ-235 in thyroid cancer cell lines

Figure 3 illustrates combination index experiments in which BEZ-235 and RAF265 were used together at a fixed ratio of 1:4 to test for potential synergy (25). In this plot, the relative MTT values are expressed on a scale where zero is assigned to untreated cells at the start of the experiment and 1 to untreated cells at the 5-day endpoint, in a modification of the method of Brachmann and colleagues (18). In TT cells (Fig. 3B), and more weakly in B-CPAP cells (Fig. 3A), we observed a reduction in MTT value below baseline at combined doses of 200 nmol/L (BEZ-235) and 800 nmol/L (RAF265). The analysis for synergy, using the method of Chou and Talalay (23), was positive in both lines across a range of doses with median combination indices of 0.2 for CAL62 and 0.78 for TT, where values less than 1.0 indicate synergy (see insets, Fig. 3A and B).

Flow cytometric cell-cycle analyses were carried out for cultured BRAF and RAS mutant lines (Fig. 4). In all tested lines, the combination of both drugs was highly effective in inducing a G1 arrest, with the S-phase fraction dropping from 16% to 8% (B-CPAP), 25% to 3% (8505C), and 33% to 12% (CAL62). We did not detect any significant changes in the sub-G0 population in all treated groups. In a further test for apoptosis, immunoblot analysis for cleaved caspase 3 was negative after 3-day treatment in these 3 cell lines (data not shown).

Inhibition of DTC and MTC xenografts by RAF265 and BEZ-235

The synergistic effect of the combination of RAF265 and BEZ-235 in vitro suggested that the combination would likely be effective in tumor xenografts in vivo. To test the in vivo activity of this drug combination, athymic nu/nu mice bearing palpable DTC (CAL62) or MTC (TT) tumors were randomized to receive RAF265, BEZ-235, a combination of both drugs, or vehicle alone. Dose levels were selected on the basis of internal data from the pharmaceutical supplier (Novartis) optimizing for tolerability and for achieving plasma levels comparable with human phase I clinically achievable levels. For CAL62 cells bearing a KRASG12R mutation, minimal growth was observed over the course of the experiment for the tumors treated with both drugs (Fig. 5 A). Kaplan–Meier analysis indicated significant inhibition of progression compared with control (P < 0.05). Neither BEZ-235 nor RAF265 alone resulted in significant inhibition of progression. No significant weight loss was seen with the drug combination. For MTC xenografts, we again observed a strongly significant inhibition of progression (P < 0.001 vs. control; Fig. 5 B). Interestingly, RAF265 also appeared to have some single-agent activity, although the majority of MTC xenografts progressed on this drug alone.

We carried out immunohistochemistry for pERK<sub>Y202/Y204</sub> to track pharmacodynamic activity of RAF265 and for pAKT<sub>S473</sub> and pS6<sub>S235/24</sub> to track action of BEZ-235, using the chosen in vivo doses. As shown in Figure 6, vehicle-treated CAL62 xenograft tumors had dense, predominantly nuclear reactivity for pERK and pAKT and intense predominantly cytoplasmic reactivity for pS6 in a high fraction of cells. Four hours after BEZ-235 treatment, we observed virtually complete abrogation of pAKT and pS6 reactivity, with no effect on pERK. Conversely, RAF265 strongly reduced pERK, with little effect on pAKT and pS6 24 hours after dosing.
after drug exposure. The combination of the 2 drugs strongly reduced reactivity for all 3 kinase targets. Very similar data were obtained for TT MTC xenografts (Supplementary Fig. S2).

On the basis of the potential of RAF265 to inhibit VEGFR2 signaling (20), we examined MVD, using immunohistochemistry for CD31. In CAL62 xenografts, RAF265 resulted in a significant decline in MVD (16.1 CD31-reactive vessel foci/hpf vs. 30.9 ± 4.3 seen for vehicle, P = 0.015; Supplementary Fig. S3). No significant change in MVD was observed in TT xenografts treated with RAF265. On the basis of these analyses, the drug combination at the chosen doses seemed to be highly effective in inhibiting the ERK and PI3K pathways in DTC and MTC xenograft models and caused significant delays in tumor progression.

Discussion

In summary, we showed in DTC and MTC preclinical models that the combination treatment with agents...
inhibiting RAF, VEGFR, plus PI3K and mTOR, was strongly growth inhibitory, both in vitro and in vivo. The tested drug combination resulted in profound G1–G0 arrest and was associated with consistent inhibition of target kinases. In addition, we showed for the first time that RAF265 potently inhibits RETC634W, a constitutively active form of the kinase seen in MEN 2A. Oncogenic RET mutations (both germline and somatic) predominate in aggressive cases of medullary thyroid cancer and somatic chromosomal rearrangements of RET occur in differentiated thyroid cancer. It is currently unknown whether drugs targeting this range of kinases will be adequately tolerated in human phase I studies. If the toxicity proves acceptable, then the synergistic action we observed could be beneficial in patients with advanced thyroid cancer.

The improved efficacy of combination treatment targeting both RAF and PI3K pathways is supported by several recent clinical and preclinical studies. Synergy between a MEK inhibitor and either a PI3K, AKT, or mTOR inhibitor has been shown both in cell culture and in xenografts in several tumor types (25–30). The mechanisms reported to underlie this synergy include downstream convergence of translational control of prosurvival signaling (29) and modulation of expression of proapoptotic and antiapoptotic BH3 proteins (30). Some of these preclinical studies have used either BEZ-235 or RAF265. For example, a recent preclinical study in neuroendocrine tumor cell lines reported that RAF265 was growth inhibitory but strongly induced AKT activation. Combined treatment with BEZ-235 led to AKT inhibition and enhanced growth inhibition (31). Engelman and colleagues showed that BEZ-235 caused regression in a PIK3CA-inducible murine lung cancer model but not in a KRAS-inducible model (26). Combination treatment with the MEK inhibitor AZD6244 caused marked synergy in shrinking the KRAS-induced cancers as well (26). This observation parallels the phase I experience with single-agent BEZ-235 in which activity was reported in PIK3CA-dependent breast cancer (19). These findings and our current study using a RAF inhibitor in addition to BEZ-235 suggest a promising role for dual pathway inhibition in the setting of RAS and other mutant genotypes.

The effective targeting of BRAF in melanoma, with rapidly emerging resistance mechanisms involving the PI3K and MAPK pathways, suggests that single targeted therapies are unlikely to achieve stable durable responses in other BRAF mutant cancers including thyroid cancer. Multitargeted kinase inhibition of both the PI3K and MAPK arms may be more successful. Our study targeted both of these signaling arms as well as VEGFR2 and RET, resulting in antitumor synergy. Strategies that target additional survival mechanisms in thyroid cancer, in combination with a subset of the kinases targeted here, may offer future directions for therapeutic development.
Disclosure of Potential Conflicts of Interest

D.W. Ball is a consultant/advisory board member of Exelixis, Inc., and Eisai, Inc. No potential conflicts of interest were disclosed.

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