Preclinical therapeutics

Synergistic action of a RAF inhibitor and a dual PI-3 Kinase / mTOR inhibitor in thyroid cancer

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Abstract

Purpose:
In thyroid cancer clinical trials, agents targeting VEGF receptors 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, 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 ERK and PI3K pathways. In addition, RAF265 had significant RET inhibitory activity (IC50 25-50 nM for RET\textsuperscript{C634W}). The combination strongly inhibited proliferation of DTC and MTC lines with mutations in RAS, BRAF, PTEN, and RET. Synergy was demonstrated for B-CPAP (BRAF\textsuperscript{V600E}) and TT cells (RET\textsuperscript{C634W}). The combination of both drugs significantly inhibited growth of CAL62 (KRAS\textsuperscript{G12D/G12D}) 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 DTC and MTC.

Statement of Translational Relevance

The submitted manuscript is translational-relevant for several reasons.

First, the clinical problem -- advanced thyroid cancer results in approximately 1700 deaths per year in the U.S. There are no approved therapies for the majority of cases, although vandetanib recently was approved for advanced medullary thyroid cancer. Our preclinical development in thyroid cancer is aimed at this unmet need. Second, the signaling pathways that we examined are the two pathways most commonly activated by mutation in thyroid cancer. Third, the model systems that we chose are standard preclinical models for this field. Fourth, the drugs that we studied have clinical activity in other solid tumor types and are proceeding with development. Fifth, the preclinical activity that we observed justifies further study, and lays groundwork for a phase I combining these or similar agents.
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 1700 deaths are attributed to thyroid cancer annually in the U.S. (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 DTC (6). Taken together, the vast majority of DTC 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. MTC represents 3-5% 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 non-familial MTC tumors bear somatic RET mutations, which confer a powerful negative prognostic impact (7). The activated RET kinase is known to signal via ERK and PI3K, among other pathways, leading to enhanced proliferation and resistance to apoptosis.

Recent clinical trials for advanced radioiodine refractory DTC and for MTC have focused on semi-selective kinase inhibitors that target VEGF receptors, RET, additional RTK’s such as PDGFR and FGFR, and to a lesser extent, RAF. Among the strongest results reported to date are for pazopanib in DTC, with a 49% PR rate, and significant prolongation of progression-free survival (8). Among the strongest results reported for MTC are for vandetanib, associated with a 45% PR rate, and prolonged PFS in a randomized phase III trial (9). Other drugs with activity against DTC and/or MTC 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, et al. reported encouraging preclinical data for the RAF inhibitor PLX4720 (12). The related compound PLX4032 (Roche R05185426, Vemurafenib) resulted in PR or prolonged stable disease in the three DTC patients reported by Flaherty et al. in a Phase I trial focused primarily on melanoma (13). Based on poor responses to RAF inhibitors in RAS-mutant melanoma, a developing consensus is that thyroid cancer RAF inhibitor development 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 upsteam 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 two promising drugs in current development to simultaneously target the RAF-MEK-ERK and PI3K-AKT-mTOR pathways, using a combination with potential for further clinical development.

NVP-BEZ235 (Novartis) is an orally available, ATP-competitive class I PI3K inhibitor with IC$_{50}$ < 25 nM for p110 α, β, and δ. In addition, BEZ-235 potently inhibits Torc1 and Torc2 (IC$_{50}$ 20 nM). For a panel of 18 other kinases, the IC$_{50}$ is > 10,000 nM (16, 17). BEZ-235 has broad preclinical activity with a pro-
apoptotic effect in tumor models with PI3K pathway genetic alterations, and an anti-proliferative 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 WT and V600E mutant Braf as well as CRAF (IC$_{50}$ 3 to 60 nM 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 nM) (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).

Materials and Methods

Cell lines. B-CPAP, 8505C, and CAL62 were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). C643 was obtained from NE Heldin, Uppsala University. FTC133 was obtained from Matthew Ringel, Ohio State University. Cell culture conditions were as follows: B-CPAP, 8505C and CAL62 - RPMI 1640 with 10% FBS. C643 - RPMI 1640 with 10% FBS, 1mM sodium pyruvate, and non-essential amino acids (Irvine Scientific, Santa Ana, CA). FTC133 - DMEM:Ham’s F12 (1:1) (Gibco) with 10% FBS. TT cells were cultured in RPMI 1640 with 16% FBS. All media were supplemented with penicillin-streptomycin. All cell lines not obtained directly from the DSMZ repository were analyzed for polymorphisms in 8 highly polymorphic markers using the PowerPlex DNA
Fingerprinting system (Johns Hopkins Genetic Core Facility) and confirmed versus published data.

Inhibitor treatments. RAF265 and BEZ-235 (Novartis, E. Hanover, NJ) were prepared as 10 mM stock solutions in DMSO, following the manufacturer’s instructions. For analysis of ERK and PI3K pathway inhibition, cultured cells were treated with indicated doses of inhibitors for 4 hours. For growth inhibition assays, cells were treated at day 0, with medium change and fresh drug as indicated in figure legends.

Western blotting. Cells were treated for 4 h or 5 d as described above, then washed with PBS and harvested by scraping with 1x sodium dodecyl sulfate lysis buffer (2% sodium dodecyl sulfate and 62.5 mM Tris (pH 6.8)). Lysates were electrophoresed on 4–20% gradient polyacrylamide gels and transferred onto PVDF membranes. Blots were probed at 4° C overnight with primary antibody to pERK (CST, Beverly, MA, #9101) diluted 1:1000 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 GAPDH (Trevigen). Anti-rabbit secondary antibodies (SantaCruz) were diluted 1:2000. Blots were visualized using Supersignal Pico Chemiluminescence (Pierce Chemical Co., Rockford, IL).

Growth analyses. Growth assays were performed in triplicate using the 3,4,5-dimethylthiazol2,5-diphenyltetrazolium (MTT) assay (M2128, Sigma-Aldrich) following 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 the mean absorbance ± SEM, based on 3-6 independent experiments, normalized to control cells. GI_{50} was determined as the X-intercept of Log_{10}(FA/FU) plotted versus Log_{10}(concentration), determined by linear regression (23). GI_{50} values were reported as the mean +/- standard deviation of 3-6 independent experiments.
Flow cytometry cell cycle analyses. Thyroid cancer cells were treated for 48 hours with drug combinations or vehicle control, 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, Franklin Lakes, NJ) gated for single nuclei. Cell cycle profiles were determined using 10,000 gated nuclei with ModFit LT 2.0 software (Verity, Topsham, MA).

Animal studies. Animal studies were approved by the Johns Hopkins Animal Care and Use Committee and performed in accordance with NIH guidelines. CAL62 or TT cells suspended in Matrigel (5 x 10^6 cells/200 µl) were inoculated s.c. into the right flank of 4 to 6-week-old female athymic nude (nu/nu) mice (Harlan Laboratories, Indianapolis, IN). Once palpable, tumor volumes were calculated with calipers using the formula: length x width x height x (0.5236). After tumors reached ~0.1 cm^3 in average 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 cm^3. Kaplan-Meier analysis for tumor progression was performed 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, and tumors 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, Burlingame, CA) with DAB as chromogen, and a hematoxylin counterstain. Anti-CD31 staining (Abcam, Cambridge, MA ab28364, 1:50) followed a pressure cooker antigen retrieval method according to the manufacturer’s protocol. Microvessel density was
estimated as the mean of CD31-reactive foci in 10 high power fields in the tumor periphery, using a two-tailed paired T test for significance. Light microscopy was performed 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 BRAF<sup>V600E</sup> mutation. The estimated IC<sub>50</sub> in cell culture was 100-200 nM (Figure 1, panel A). Using the CAL62 DTC line with a KRAS<sup>G12R</sup> mutation, we observed that BEZ-235 potently inhibited AKT activation, with an IC<sub>50</sub> of approximately 10 nM (Figure 1, panel B). We initially performed MTT proliferation assays at these doses across a panel of thyroid cancer lines with a range of genotypes including BRAF<sup>V600E</sup>, KRAS<sup>G12R</sup>, HRAS<sup>G12A</sup>, and PTEN<sup>-/-</sup> (Figure 1, panel C). BEZ-235 at 10 nM was strongly inhibitory for PTEN<sup>-/-</sup> FTC133 cells, with additional activity against the RAS mutant and BRAF mutant lines. Somewhat surprisingly, RAF265 at 200 nM also had activity across the panel, including non-BRAF mutant lines. In combination, the two drugs were highly effective, with MTT values reduced to approximately 10% of control, even at these modest doses, roughly at IC<sub>50</sub> values for their respective targets. Target inhibition persisted in vitro for at least 72h, using fully inhibitory doses (Figure 1, panel D).

GI<sub>50</sub> 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 six tested thyroid cancer cell lines with their diverse genotypes. The lowest values were recorded for FTC133 (PTEN<sup>-/-</sup>, 1.7 nM) and CAL62 (KRAS<sup>G12R</sup>, 3.8 nM). However, BEZ-235 also caused powerful growth inhibition of BRAF and RET mutant lines, perhaps reflecting the dual inhibitory capacity for mTOR as well as PI3K. RAF265 was most potent for the BRAF mutant line B-CPAP (GI<sub>50</sub> 91.6 nM) and for FTC133 (27.4 nM). In contrast, 8505C was relatively resistant to this single agent despite a BRAF mutant genotype (368 nM). In general, GI<sub>50</sub> values for the two drugs corresponded to their IC<sub>50</sub> values for the tested kinase targets. No clearcut relationship was seen for GI<sub>50</sub> and mutation type 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 nM) (Figure 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 immunoblot for pAKT and pS6. BEZ-235 (500 nM), as expected, completely blocked these activities, with no significant effect on pERK. BEZ235 also partially reduced total S6 abundance, the significance of which is unclear. The combination of the two 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 (11, 14) (Supplemental Figure 1).

We hypothesized, based on structural similarity to sorafenib, that RAF265 could have RET inhibitory activity, similar to sorafenib (24). Analyzing pRET\textsuperscript{Y905}, we indeed found that RAF265 potently inhibited RET auto-phosphorylation in TT cells, with an estimated IC\textsubscript{50} of approximately 25 nM (Figure 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 employed together at a fixed ratio of 1:4, in order 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 five day endpoint, in a modification of the method of Brachmann, et al (18).
In TT cells (panel B), and more weakly in B-CPAP cells (panel A), we observed a reduction in MTT value below baseline at combined doses of 200 nM (BEZ-235) and 800 nM (RAF-265). 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 <1.0 indicate synergy (see insets, both panels).

Flow cytometry cell cycle analyses were performed for cultured BRAF and RAS mutant lines (Figure 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 three 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 based on internal data from the pharmaceutical supplier (Novartis) optimizing for tolerability and to achieve plasma levels comparable to 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 (Figure 5, panel A). Kaplan-Meier analysis indicated significant inhibition of progression compared to 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, Figure 5, Panel B). Interestingly RAF265 also appeared to have some single agent activity, although the majority of MTC xenografts progressed on this drug alone.

We performed IHC for pERK\textsuperscript{T202/Y204} in order to track pharmacodynamic activity of RAF265, and for pAKT\textsuperscript{S473} and pS6\textsuperscript{S235/S236} to track action of BEZ-235, using the chosen \textit{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 drug exposure. The combination of the two drugs strongly reduced reactivity for all three kinase targets. Very similar data were obtained for TT MTC xenografts (supplemental figure 2).

Based on the potential of RAF265 to inhibit VEGFR2 signaling (20), we examined microvessel density (MVD), using immunohistochemistry for CD31. In CAL62 xenografts, RAF265 resulted in a significant decline in MVD (16.1 CD31-reactive vessel foci/hpf ± 2.5 vs. 30.9 ± 4.3 seen for vehicle, p=.015, see supplemental figure 3). No significant change in MVD was observed in TT xenografts treated with RAF265. Based on these analyses, the drug combination at the chosen doses appeared to be highly effective in inhibiting the ERK and PI-3K 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 combination treatment with agents inhibiting RAF, VEGFR, plus PI3K and mTOR was strongly growth inhibitory, both \textit{in vitro} and \textit{in vivo}. 


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 RET<sub>C634W</sub>, a constitutively active form of the kinase seen in MEN 2A. Oncogenic RET mutations (both germline and somatic) predominate in aggressive cases of MTC and somatic chromosomal rearrangements of RET occur in DTC. 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 demonstrated 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 pro-survival signaling (29) and modulation of expression of pro-apoptotic and anti-apoptotic BH3 proteins (30). Some of these preclinical studies have employed 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 et al 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. Multi-targeted 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 anti-tumor 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.
Acknowledgement

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Figure Legends

Figure 1

Figure 1. Target kinase and growth inhibition of thyroid cancer cell lines by RAF265 and BEZ-235. A. Dose-response effect for RAF inhibitor RAF265 in two BRAF mutant lines, treated 4 h. Estimated IC₅₀ for pERK ~100-200 nM. B. Dose-response effect for combined PI-3K/mTOR inhibitor BEZ-235 in KRAS mutant line CAL62. Estimated IC₅₀ for pAKT ~10 nM. C. MTT assays for cell lines treated with RAF265 200 nM or BEZ-235 10 nM or the combination at day 0 and at day 3, and tested at day 5, compared to vehicle control. Values are expressed as mean and S.E.M. of 3 experiments performed in triplicate. Significant growth inhibition was seen in all five of the treated lines, with indicated genotype. D. Persistence of target inhibition over 72h in 8505C cells treated once with BEZ-235100 nM or RAF265 1600 nM.

Figure 2

Downstream kinase inhibition by BEZ-235 and RAF265 or combination in thyroid cancer cell lines. Cells were treated 4 hours with indicated doses. Total kinase as well as active kinase levels are indicated by immunoblot, with GAPDH loading control. The two BRAF and the KRAS mutant lines had complete inhibition of pAKT and pS6 with BEZ-235 or the combination. Both BRAF but not the KRAS mutant line had complete inhibition of pERK with RAF265 or the combination. RAF265 also inhibited RET activity, assayed by pRET⁴⁹⁰⁵ in TT cells (right panels).
**Figure 3** Synergistic activity of RAF265 and BEZ-235 in thyroid cancer cell lines. A B-CPAP cells or B TT cells treated with RAF265 (black squares), BEZ-235 (black diamonds) or combination (black triangles) were treated at day 0 with indicated doses. MTT analyses performed at day 5 are represented as mean values (± S.D.) normalized to control = 1.0 and starting value = 0. (Negative values represent 5 day MTT scores below the starting value). Combination indices (insets) calculated using method of Chou and Talalay (23).

**Figure 4**

Combined RAF265 and BEZ-235 induces profound G1 arrest in cultured thyroid cancer. Cell lines were treated at day 0 with vehicle, RAF265 1.6 μM, BEZ-235 100 nM or combination, and analyzed by FACS at day 2. Calculated S phase fractions are indicated.

**Figure 5**

Combinatorial activity of RAF265 and BEZ-235 in thyroid cancer in vivo. CAL62 (A) and TT (B) xenografts were implanted in 10-12 athymic nu/nu mice, grown to 0.1 cm³, then treated with vehicle alone, RAF265 (10 mg/kg thrice weekly), BEZ-235 (20 mg/kg daily), or a combination of both drugs, by oral gavage. Tumor volumes are expressed relative to starting volumes (± s.e.m.). In both panels, diamonds indicate vehicle, squares RAF265, triangles BEZ-235, and circles a combination of both drugs. * Combination p=0.047 vs. Vehicle. ** Combination p<0.001 vs. Vehicle.
Figure 6

*Inhibition of target kinases in CAL62 xenografts treated with BEZ-235, RAF265, or combination.* Tumors were harvested 24 hours after RAF265 or 4 hours after BEZ-235 or combination treatment. Formalin-fixed, paraffin-embedded sections were tested for reactivity to pAKT, pERK, and pS6 antibodies (Immunoperoxidase, 200x).
References

5. Xing M. Genetic alterations in the phosphatidylinositol-3 kinase/Akt pathway in thyroid cancer. Thyroid 2010;20:697-706.


Table 1

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<th>Genotype</th>
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<tr>
<td>CAL62</td>
<td>KRAS&lt;sup&gt;G12R/G12R&lt;/sup&gt;</td>
<td>3.8 ±0.66</td>
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<td>8505C</td>
<td>BRAF&lt;sup&gt;V600E/V600E&lt;/sup&gt;</td>
<td>5.5 ±1.0</td>
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<td>B-CPAP</td>
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<td>FTC133</td>
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<td>C643</td>
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$G_{I50}$, nM

Table 1. Calculated $G_{I50}$ values for BEZ-235 and RAF265 (±S.D.) across a panel of thyroid cancer cell lines. See Materials and methods for calculation method. Known mutations in each cell line are indicated (11, 33, 34).
Figure 1

A

8505C BRAF<sup>V600E</sup>

RAF265 nM:

0 50 100 200 400 800

B-CPAP BRAF<sup>V600E</sup>

0 50 100 200 400 800

pERK<sup>T202/Y204</sup>

ERK

GAPDH

B

CAL62 KRAS<sup>G12R</sup>

BEZ-235 nM:

0 2 10 50 250 1000

pAKT<sup>S473</sup>

AKT

GAPDH
Figure 2

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- **pAKT<sup>S473</sup>**
- **AKT**
- **pS6<sup>S235/S236</sup>**
- **S6**
- **pERK<sup>T202/Y204</sup>**
- **ERK**
- **GAPDH**

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Figure 3
**Figure 4**

- **B-CPAP BRAF**
  - **Control**: 16% (G1/S, G2/M)
  - **RAF265**: 17% (G1/S, G2/M)
  - **BEZ235**: 16% (G1/S, G2/M)
  - **Combined**: 8% (G1/S, G2/M)

- **8505C BRAF**
  - **Control**: 25% (G1/S, G2/M)
  - **RAF265**: 21% (G1/S, G2/M)
  - **BEZ235**: 20% (G1/S, G2/M)
  - **Combined**: 3% (G1/S, G2/M)

- **CAL62 KRAS**
  - **Control**: 33% (G1/S, G2/M)
  - **RAF265**: 25% (G1/S, G2/M)
  - **BEZ235**: 12% (G1/S, G2/M)
  - **Combined**: 12% (G1/S, G2/M)
Figure 5

(A) Relative Tumor Volume of CAL62-KRAS<sup>mut</sup>

(B) Relative Tumor Volume of TT RET<sup>mut</sup>

Days

Relative Tumor Volume

0 5 10 15 20 25 30

0 1 2 3 4 5 6
Figure 6

<table>
<thead>
<tr>
<th></th>
<th>pAKT&lt;sup&gt;S473&lt;/sup&gt;</th>
<th>pERK&lt;sup&gt;T202/Y204&lt;/sup&gt;</th>
<th>pS6&lt;sup&gt;S235/S236&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Vehicle</td>
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<td>BEZ-235</td>
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<tr>
<td>RAF265</td>
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<tr>
<td>Combination</td>
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Scale bar: 10 μm
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Ning Jin, Tianyun Jiang, David M. Rosen, et al.

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