Concomitant BRAF and PI3K/mTOR Blockade Is Required for Effective Treatment of BRAFV600E Colorectal Cancer

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Abstract

Purpose: BRAFV600E mutations are associated with poor clinical prognosis in colorectal cancer (CRC). Although selective BRAF inhibitors are effective for treatment of melanoma, comparable efforts in CRC have been disappointing. Here, we investigated potential mechanisms underlying this resistance to BRAF inhibitors in BRAFV600E CRC.

Experimental Design: We examined phosphoinositide 3-kinase (PI3K)/mTOR signaling in BRAFV600E CRC cell lines after BRAF inhibition and cell viability and apoptosis after combined BRAF and PI3K/mTOR inhibition. We assessed the efficacy of in vivo combination treatment using a novel genetically engineered mouse model (GEMM) for BRAFV600E CRC.

Results: Western blot analysis revealed sustained PI3K/mTOR signaling upon BRAF inhibition. Our BRAFV600E GEMM presented with sessile serrated adenomas/polyps, as seen in humans. Combination treatment in vivo resulted in induction of apoptosis and tumor regression.

Conclusions: We have established a novel GEMM to interrogate BRAFV600E CRC biology and identify more efficacious treatment strategies. Combination BRAF and PI3K/mTOR inhibitor treatment should be explored in clinical trials. Clin Cancer Res; 19(10); 2688–98. ©2013 AACR.

Introduction

BRAF is a critical component of the mitogen-activated protein kinase (MAPK) signaling pathway (1). BRAFV600E mutations have been identified in melanoma, papillary thyroid carcinoma, and colorectal cancer (CRC), and result in constitutive MAPK signaling that promotes carcinogenesis (2). BRAFV600E mutations are seen in 15% of CRC and are associated with decreased survival (3). In particular, microsatellite stable (MSS) BRAFV600E CRC is associated with these poor clinical outcomes (4). With respect to CRC with high microsatellite instability (MSI-H) in the adjuvant setting (mainly stage II and III CRC), patients have better outcomes and the presence of BRAF mutation does not affect outcomes (5). However, in the setting of metastasis, BRAFV600E/MSI-H tumors have poorer outcomes than BRAF wild-type/MSI-H tumors (6, 7).

As more than 600,000 people worldwide die from CRC each year, robust therapies targeting MSS BRAFV600E CRC is a critical unmet clinical need (8). The development of selective BRAF inhibitors is a compelling goal for drug discovery (9). For instance, a recent phase III melanoma clinical trial resulted in a 48% response following treatment with the BRAF inhibitor PLX4032 (vemurafenib) versus 5% for standard of care dacarbazine chemotherapy (10). However, acquired resistance to vemurafenib quickly emerges (11, 12). Potential resistance mechanisms include: (i) secondary mutations in RAS, HRAS, NRAS, or MEK1; (ii) activation of COT-1 kinase; and (iii) induction of phosphoinositide 3-kinase (PI3K)/mTOR signaling through recruitment of platelet-derived growth factor receptor β (PDGFRβ) or insulin-like growth factor 1R (IGF-1R; refs. 13–16).

CRC treatment with BRAF inhibitors has been disappointing. For example, vemurafenib treatment in 19 patients with BRAFV600E CRC yielded only 1 partial and 4 minor responses. Furthermore, 5 patients presented with a mixed response pattern of both regressing and progressing lesions (17). Although these results highlight the therapeutic potential for such BRAF inhibitors, they underscore the
Translational Relevance

**BRAF**V600E mutations are associated with poor clinical prognosis in colorectal cancer (CRC). Although selective BRAF inhibitors are effective for melanoma treatment, comparable efforts in CRC have been disappointing. Further understanding of potential resistance mechanisms to treatment is an unmet clinical need in CRC. Using colon-restricted delivery of adenovirus-expressing cre recombinase to floxed mice, we developed a novel genetically engineered mouse model (GEMM) for **BRAF**V600E CRC that recapitulates the sessile serrated adenoma–carcinoma pathway in humans. In conjunction with *in vitro* cell line studies, we used this GEMM to identify combined phosphoinositide 3-kinase (PI3K)/mTOR signaling as a potential resistance mechanism and to show the efficacy of concomitant BRAF and PI3K/mTOR blockade for therapy. Taken together, this GEMM provides a robust preclinical platform to interrogate **BRAF**V600E CRC biology and to refine further combinatorial therapeutic regimens and suggests that combined treatment with BRAF and PI3K/mTOR inhibitors should be explored in future clinical CRC trials.

Although PI3K/mTOR signaling has been implicated in resistance to BRAF inhibitors in melanoma, it also plays a dominant role in CRC carcinogenesis, being constitutively activated in approximately 30% of all patients (21). However, 60% of **BRAF**V600E CRCs have been reported to have increased p-AKT expression, suggesting that the PI3K/mTOR axis is activated in a significant proportion of **BRAF**V600E CRC (22). Taken together, these findings suggest that PI3K/mTOR signaling may represent a significant resistance mechanism to BRAF inhibitor treatment in **BRAF**V600E CRC.

We have described multiple novel genetically engineered mouse models (GEMM) for sporadic CRC (23, 24). These GEMMs are ideally suited for preclinical trials, using optical colonoscopy to assess dynamic tumor responses to treatment. In our studies, we used *in vitro* and *in vivo* experiments incorporating human CRC cell lines and a new GEMM for **BRAF**V600E CRC that recapitulates the sessile serrated adenoma/polyp (SSA/P) pathway seen in humans, respectively. Our findings provide compelling preclinical evidence that combination BRAF and PI3K/mTOR inhibition should be explored in clinical trials. Furthermore, our results establish a novel GEMM for investigation of the SSA/P pathway, interrogation of **BRAF**V600E CRC biology, and identification of more efficacious treatment strategies.

**Materials and Methods**

**In vitro** treatment of human CRC cell lines

The following cell lines were used in this study: VACO432, RKO, VT1, T29, HCT-116, and DLD-1. VACO432, RKO, VT1, and T29 cell lines (a kind gift from B. Vogelstein, Johns Hopkins Medical Institutions, Baltimore, MD) were maintained in McCoy’s (Invitrogen) with 10% FBS (Invitrogen) and 1 × penicillin/streptomycin (Invitrogen). HCT-116 and DLD-1 (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), as described earlier. VACO432/VT1 and RKO/T29 are isogenic pairs (25).

VACO432 and RKO are homozygous for **BRAF**V600E, whereas both VT1 and T29 have the **BRAF**V600E allele knocked out. VACO432 and VT1 are heterozygous for a PIK3CA**H1047R** mutation (this mutation is uncharacterized). RKO and T29 are homozygous for the PIK3CA**H1047R**-activating mutation. RKO is also heterozygous for 2 frameshift mutations in NF1. HCT-116 and DLD-1 are heterozygous for the PIK3CA**H1047R**-activating mutation. All mutations were confirmed by DNA sequence analysis using published primers flanking each mutated exon. After treatment with GDC-0879 (a kind gift from Genentech) and/or NVP-BEZ235 (LC Labs) for 48 hours, cell viability was measured by MTS assay, as previously described (24). All experiments were carried out for a minimum of 3 times in quadruplicate.

**Immunoblotting**

Cells were lysed in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1 mmol/L EDTA, and protease and phosphatase inhibitors), incubated on ice for 10 minutes and centrifuged at 14,000 rpm for 10 minutes. Tumors were homogenized with a TH tissue homogenizer (Omni International) in lysis buffer, incubated for 10 minutes on ice, and vigorously shaken for 30 minutes at 4°C. Concentrations of whole cell or tumor lysates were determined by Bio-Rad Protein Assay (Bio-Rad). Ten and 25 μg protein lysate for whole cell and tumor, respectively, were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane, blocked in 1% bovine serum albumin for 1 hour, incubated at room temperature for 2 hours with primary antibody and 1 hour with secondary antibody. Detection was conducted using the Amersham ECL Western Blot Detection Reagents (GE Healthcare). p-MEK, total mitogen-activated protein/ extracellular signal–regulated kinase (MEK), p-ERK, total extracellular signal–regulated kinase (ERK), p-AKT Thr473, total AKT, p-S6 Ser240/244, and total S6 were obtained from Cell Signaling Technologies.

**Apoptosis assay**

A total of 10⁶ VACO432 and RKO cells were treated with 2 μmol/L GDC-0879, 100 mmol/L NVP-BEZ235, and...
combination in 0.1% FBS for 16 hours. Cells were washed, pelleted, and resuspended in Cell Lysis Buffer (Invitrogen). Caspase-3 activity was measured by the CaspACE Assay System, Colorimetric (Promega), as per manufacturer’s instructions. All experiments were carried out for a minimum of 3 times in duplicate.

**BrafV600E GEMM**

Mice with conditional ApC alleles (ApC CKO) were crossed to those with a latent BrafV600E allele (BrafCA; a kind gift from M. McMahon, UCSF Cancer Center, San Francisco, CA) to generate ApCCKO/CKO; BrafCA/+ mice (ApC-Braf; refs. 26, 27). Colonized tumors were induced using adenovirus-expressing Cre recombine (AdCre) and followed by optical colonoscopy, as previously described (23). As a tumor size metric, the Tumor Size Index (TSI) was calculated as (tumor area/colonic lumen area) × 100 (%), as we have validated previously (24). Ten tumors were genotyped for the recombinated BrafV600E allele using the following primers and cycling conditions: Braf:AD FwdA1, 5'-TGGATATTATTGTGCGAAGTCC-3', and Braf:AD RevB1, 5'-CTGCTGGAGGAACGCGG-3'; 94°C for 2 minutes, 94°C for 30 seconds, 60°C for 90 seconds, 72°C for 1 minute, 72°C for 10 minutes. The product sizes are as follows: wild-type Braf is 185 bp, conditionally targeted Braf is 308 bp, and BrafV600E is 335 bp (26). The 10 tumors were sequenced for mutations in exons 9 and 20 of the Braf gene using the following primers: 9F: 5'-GGCTCACAACCATCGTAACAAGA-3', reverse: 5'-TGGGCCAATCTTGTATTGTTG-3', 20F: 5'-ACTGCCGAAGCTTCCTATAC-3', and 5'-TGATGCTGCTGAAATGGATC-3'. Tumor-bearing mice were treated with 100 mg/kg GDC-0879 and/or 25 mg/kg NVP-BEZ235 in 0.5% methylcellulose/0.2% Tween 80 by daily gavage for 28 days. All protocols were approved by the Tufts Institutional Animal Care and Use Committee.

**Statistical analysis**

Data are expressed as mean ± SD. Comparisons of tumor size, proliferation, and apoptosis between control and treated cohorts were calculated using two-tailed independent-samples t tests. Percentage of adenoma versus carcinoma in ApC and ApC-Braf group was compared using Fisher exact test. P < 0.05 was considered significant for all analyses. All analyses were calculated using SPSS 18.0 for Windows (IBM, Inc.).

**Microsatellite instability testing**

MSI was assessed using a fluorescently labeled primer mBat-59 (forward: 5’-GATACCTTTATTCATTTAGCA-3’, reverse: 5’-GGCTCACAACCATCGGTAACAGA-3’) and Platinum PCR SuperMix High Fidelity (Invitrogen), as previously described (28). Ten tumors were genotyped as MSI-positive if one or more novel alleles were present in tumor but not the matched tail.

**Histopathology and immunohistochemistry**

Please see Supplementary Data for details about histopathology and immunohistochemistry.

**Results**

**BRAF inhibition decreases cell viability in BrafV600E CRC**

The BRAF inhibitors PLX4032, PLX4720, and GDC-0879 have comparable mechanism and efficacy across different BRAFV600E cancer cell lines (29, 30). We used the tool compound GDC-0879 to examine BRAF inhibition in BRAF-mutant (VACO432 and RKO), BRAF/KRAS wild-type (VT1 and T29), and KRAS-mutant (DLD-1 and HCT-116) CRC cell lines. GDC-0879 is a potent BRAF inhibitor in the nanomolar range and has a high degree of selectivity, as shown by screening of a representative panel of 140 full-length protein kinases (31). These studies revealed that BRAF inhibition decreases viability in BrafV600E (IC50 < 1.5 μmol/L), whereas BRAF/KRAS wild-type and KRAS-mutant CRC are significantly less sensitive (IC50 > 20 μmol/L; Fig. 1A). Western blot analysis of VACO432 and RKO after 0, 2, and 10 μmol/L GDC-0879 treatment for 2 hours revealed decreased p-MEK and p-ERK (Fig. 1B), with a rebound in signal seen at 24 hours. GDC-0879 treatment in a BRAF/KRAS wild-type cell line showed an induction of p-MEK and p-ERK signaling likely through activation of CRAF as reported by others (19), whereas a KRAS-mutant cell line was not affected by BRAF inhibitor treatment. These results show that in vitro BRAF inhibition decreases MAPK signaling and cell viability in a BrafV600E-specific manner.

**Isolated BRAF inhibition does not induce apoptosis**

As previous studies have shown that BRAF inhibition induces apoptosis in BrafV600E melanoma cells, we examined caspase-3 activity after treatment of BRAF-mutant (VACO432 and RKO) and BRAF/KRAS WT (VT1 and T29) cells with 2 μmol/L GDC-0879 for 16 hours, which revealed no significant induction of caspase-3 activity in the BRAF mutants (Fig. 1C). Similarly, others have confirmed that BRAF inhibitor treatment in BRAF-mutant CRC cell lines is not sufficient to induce apoptosis, as these cells show minimal sensitivity to single-agent treatment (32).

**P38K/mTOR pathway sustains signaling after BRAF inhibition**

To identify signaling pathways that might promote resistance to BRAF inhibition, we examined the P38K/mTOR pathway. We conducted Western blot analyses to assess p-AKT Thr473 and p-S6. Although it was expected that RKO would show activation of p-AKT and p-S6 given the presence of an activating PI3KCA mutation, these studies revealed sustained p-AKT and p-S6 after BRAF inhibition in VACO432, as well, suggesting that continued P38K/mTOR may limit the effectiveness of BRAF inhibition (Fig. 2A). BRAF wild-type and KRAS-mutant cells, VT1 and DLD-1, respectively, also showed sustained p-AKT and p-S6. Recent publications have indicated that receptor tyrosine kinases (RTK), such as EGFR and IGFR, can become activated in the BRAFV600E cell lines following BRAF inhibitor treatment (14, 19, 20), and
our studies revealed when the GDC-0879 treatment for 48 hours (as KRAS-mutant cell lines were insensitive to BRAF inhibition, formal IC50 values could not be calculated and are presented simply as >10 μmol/L). B, p-MEK and p-ERK were examined by Western blot analysis in BRAF-mutant (VACO432 and RKO), BRAF/KRAS wild-type (VT1 and T29), and KRAS-mutant (DLD-1) cells after treatment with 0, 2, and 10 μmol/L GDC-0879 for 2 hours. C, caspase-3 activity was assessed after treatment of BRAF-mutant (VACO432 and RKO) and BRAF/KRAS wild-type (VT1 and T29) cells with 2 μmol/L GDC-0879 for 16 hours, P < 0.05.

Therefore, the residual activity of p-AKT and p-S6 could be a result of RTK activation. To address the possibility that continued PI3K/mTOR may limit the effectiveness of GDC-0879 treatment, we examined the effects of concomitant PI3K/mTOR and BRAF inhibition by treating VACO432 and RKO with GDC-0879 and the dual PI3K/mTOR inhibitor NVP-BEZ235. NVP-BEZ235 is a potent PI3K/mTOR inhibitor in the low nanomolar range that is highly specific, as evidenced by its poor inhibition (IC50 > 5 μmol/L) of a representative panel of protein kinases (33). Our studies revealed when the BRAFV600E cell lines VACO432 and RKO were treated with GDC-0879 at their relative IC50 (0.5 and 1 μmol/L, respectively), cell viability was reduced by 50%. When 1 nmol/L of NVP-BEZ235 was added in combination with GDC-0879, the overall cell viability was further reduced to 29% and 43%, respectively, as compared with untreated cells (Fig. 2B). Comparison of combination drug therapy to single-agent treatment revealed a significant difference (P < 0.05) in both cell lines. Western blot analyses indicated that combination treatment is associated with decreased MAPK and PI3K/mTOR signaling in the BRAFV600E cell lines, whereas combination treatment has no effect on MAPK signaling in BRAF wild-type or KRAS-mutant cells (Fig. 2C). These findings suggest that PI3K/mTOR signaling provides resistance to BRAF inhibition, which can be abrogated by concurrent BRAF and PI3K/mTOR blockade.

Concurrent BRAF and PI3K/mTOR blockade results in induction of apoptosis

To assess the effects of combined PI3K/mTOR and BRAF inhibition on apoptosis, we examined caspase-3 activity after treatment of BRAFV600E (VACO432 and RKO) and BRAF/KRAS wild-type (VT1 and T29) cells with 2 μmol/L GDC-0879 and 100 nmol/L NVP-BEZ235 for 16 hours. This revealed a significant 1.8- and 3.0-fold induction of caspase-3 activity as compared with untreated cells after combination treatment in the BRAF mutants VACO432 and RKO, respectively (P < 0.05; Fig. 2D). Comparison of each single-agent treatment to the combination drug treatment revealed a significant difference in the RKO cell line (GDC-0879 vs. combination, P < 0.001 and NVP-BEZ235 vs. combination, P < 0.001). However, similar analysis of the VACO432 line revealed a trend toward increased apoptosis that was not statistically significant (GDC-0879 vs. combination, P = 0.09 and NVP-BEZ235 vs. combination, P = 0.1). These findings suggest that concurrent BRAF and PI3K/mTOR inhibition decreases viability through apoptotic induction.
Development of a GEMM for MSS BRAF<sup>V600E</sup> CRC

Preclinical drug studies are problematic in most CRC GEMMs, as they present with mainly small intestinal tumors, thereby precluding the use of optical colonoscopy to follow dynamic treatment responses. Because of this, we have described the development of multiple GEMMs for sporadic CRC based on surgical administration of AdCre to the distal colon of floxed mice (23). To develop a GEMM for MSS BRAF<sup>V600E</sup> CRC, we administered AdCre to mice bearing floxed Apc and latent <i>Braf</i> alleles (Apc-Braf; Supplementary Fig. S1; refs. 26, 27). We conducted this procedure in 192 mice, which resulted in distal colonic tumor formation in 178 animals (93%). Following AdCre injection, we used colonoscopy to monitor development of individual tumors, which revealed significantly faster growth in Apc-Braf than in mice bearing floxed Apc alleles alone (Apc; <i>P</i> < 0.0001; Fig. 3A). Furthermore, we observed that the mean tumor multiplicities in Apc-Braf and Apc mice were 2.25 and 1.45, respectively (<i>P</i> < 0.0001; Fig. 3B). MSI testing in 10 individual colonic tumors using pyrosequencing of a microsatellite locus after PCR amplification for a specific mononucleotide repeat revealed no allelic size variations, showing that these tumors are MSS (data not shown). Western blot analysis and immunohistochemistry confirmed increased MAPK signaling in Apc-Braf tumors (Fig. 3C). These results suggest that our strategy results in a reproducible, robust GEMM for MSS BRAF<sup>V600E</sup> CRC.

Apc-Braf tumors recapitulate the BRAF<sup>V600E</sup> sessile serrated CRC pathway

Although it has been shown that intestinal expression of mutant BRAF<sup>V600E</sup> is sufficient to induce serrated epithelium and crypt hyperplasia, it does not reliably result in adenoma or carcinoma formation (34). Because human BRAF<sup>V600E</sup>-mutant adenomas display evidence of Wnt activation, we sought to determine if tumors from our Apc-Braf GEMM would recapitulate the sessile serrated CRC pathway in humans. We examined 36 tumors from Apc-Braf mice at 8 to 24 weeks after AdCre infection and observed (i) discrete foci with architectural abnormalities of gland serration, gland branching, basal dilatation, lateral gland extension, and dystrophic goblet cells, which are consistent with human SSA/P, and (ii) SSA/P with cytologic dysplasia, which is characteristic of SSA progression in humans (35, 

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Figure 2. Concomitant PI3K/mTOR blockade increases the effectiveness of in vitro BRAF inhibition. A, PI3K/mTOR signaling in BRAF-mutant (VACO432 and RKO), BRAF/KRAS wild-type (VT1), and KRAS-mutant (DLD-1) cells was examined by Western blot analysis for p-AKT Thr473 and p-S6 Ser240/244 after treatment with 0, 2, and 10 μmol/L GDC-0879 for 2 hours. B, cell viability in BRAF-mutant (VACO432 and RKO) and BRAF/KRAS wild-type (VT1 and T29) cells was examined after treatment with GDC-0879 (0.5 and 1 μmol/L for VACO432/VT1 and RKO/T29, respectively), NVP-BEZ235 (1 and 10 nmol/L for VACO432/VT1 and RKO/T29, respectively), and GDC-0879 + NVP-BEZ235 for 48 hours. C, MAPK, PI3K, and mTOR signaling in BRAF-mutant (VACO432 and RKO), BRAF/KRAS wild-type (VT1), and KRAS-mutant (DLD-1) cells was examined by Western blot analysis for p-MEK, p-ERK, p-AKT, and p-S6 after treatment with 2 μmol/L GDC-0879 and 100 nmol/L NVP-BEZ235 for 2 hours. D, caspase-3 activity was assessed after treatment of VACO432 and RKO cells with 2 μmol/L GDC-0879 and 100 nmol/L NVP-BEZ235 for 16 hours. ** <i>P</i> < 0.01, * <i>P</i> < 0.05.

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**Clin Cancer Res; 19(10) May 15, 2013**

**Clinical Cancer Research**

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Published OnlineFirst April 2, 2013; DOI: 10.1158/1078-0432.CCR-12-2556

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We examined 20 tumors from mice at 25 or more weeks after AdCre infection and in 10 (50%) of these mice observed SSA/P with adenocarcinoma (Fig. 4A). Of these, 16 tumors were flat or laterally spreading with central depression on endoscopic and gross examination (Fig. 4B). Four of these tumors exhibited evidence of invasive adenocarcinoma (Fig. 4C). Finally, we observed lesions with epithelial serration, mucin depletion in the upper polyp, and straight, mucin-depleted bases, which are consistent with hyperplastic polyps (Fig. 4D). No serration was seen in 40 tumors from mice bearing only the modified Apc allele (Supplementary Fig. S2). Taken together, these results suggest that our GEMM model is a robust surrogate for the human sessile serrated adenoma-carcinoma pathway.

**In vivo BRAF blockade inhibits tumor growth in Apc-Braf mice**

We next ascertained the effect of *in vivo* BRAF inhibition. After AdCre infection, we used colonoscopy to monitor tumor development and to randomize comparably sized tumors for treatment with either control or 100 mg/kg of GDC-0879 for 28 days. We monitored growth and/or regression of individual tumors during treatment (Fig. 5A). Although the mean TSI in controls significantly increased during treatment (32.6% vs. 51.4%; 

*P* = 0.017), the mean TSI in the GDC-0879–treated cohort did not (27.5% vs. 27.7%; 

*P* = 0.95; Fig. 5B). These results show that *in vivo* BRAF blockade results in stasis of *BrafV600E* tumors.

**In vivo BRAF blockade results in inhibition of MAPK and sustained PI3K/mTOR signaling**

We next ascertained if the resistance mechanisms we had identified *in vitro* would have *in vivo* significance in our GEMM for *BRAFV600E* CRC. To examine the effects of *in vivo* BRAF inhibition on MAPK and PI3K/mTOR signaling, we conducted Western blot analysis and immunohistochemistry for p-ERK, p-AKT, and p-S6 in Apc-Braf tumors. These studies revealed that a decrease in p-ERK levels is seen in tumor lysates following treatment with GDC-0879 but sustained p-AKT and p-S6 levels (Fig. 5C). These results show that *in vivo* BRAF inhibition results in MAPK blockade and sustained PI3K/mTOR signaling.

**In vivo BRAF inhibition blocks proliferation but does not induce apoptosis**

We next sought to determine whether the observed tumor stasis after *in vivo* BRAF inhibition derived from effects on tumor proliferation and/or induction of apoptosis. To assess the effects of *in vivo* BRAF inhibition on cellular proliferation, we conducted immunohistochemistry for the proliferation marker Ki-67, which revealed a 71% decrease ( 

*P* < 0.05). To assess the effects of *in vivo* BRAF inhibition on cellular apoptosis in colonic tumors, we used an *in situ* terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay that revealed a nonsignificant 1.9-fold increase ( 

*P* = 0.08; Fig. 5D). These results suggest that *in vivo* BRAF blockade results in tumor stasis primarily through a decrease in tumor proliferation.

**Concomitant in vivo BRAF and PI3K/mTOR blockade results in tumor regression**

We next sought to examine if concomitant PI3K/mTOR blockade enhances the effects of *in vivo* BRAF inhibition. Following AdCre induction, we used optical colonoscopy to randomize comparably sized tumors for treatment with either control, 100 mg/kg of GDC-879, 25 mg/kg of NVP-BEZ235, or both GDC-0879 and NVP-BEZ235 for...
28 days (Fig. 5A). We did not observe any in vivo effects on the histopathology of the normal mucosa or the overall health of the mice following either single or combination drug treatment. GDC-0879 (27.5% in pretreatment vs. 27.7% in posttreatment; \( P = 0.95 \)) and NVP-BEZ235 (33.7% in pretreatment vs. 36.5% in posttreatment; \( P = 0.68 \)) treated cohorts showed tumor stasis (Fig. 5B). However, the combination GDC-0879- and NVP-BEZ235–treated cohort showed significant tumor regression (35.4% in pretreatment vs. 19.8% in posttreatment; \( P = 0.003 \); Fig. 5B). These results show that concomitant PI3K/mTOR blockade enhances the effects of in vivo BRAF inhibition.

Concomitant in vivo BRAF and PI3K/mTOR blockade results in inhibition of MAPK and PI3K/mTOR signaling

To examine the effects of in vivo BRAF and PI3K/mTOR inhibition on MAPK and PI3K/mTOR signaling, we conducted Western blot analyses that revealed decreased p-ERK, p-AKT, and p-S6 levels in tumor lysates following treatment. The continuous downregulation of p-S6 likely plays an important role in the impressive therapeutic responses seen in combination treated tumors. These findings were confirmed by subsequent immunohistochemistry analysis (Fig. 5C). These results suggest that concomitant BRAF and PI3K/mTOR blockade induces in vivo tumor regression.

Concomitant in vivo BRAF and PI3K/mTOR inhibition blocks proliferation and induces apoptosis

We next sought to determine whether the observed colonic tumor regression after in vivo BRAF inhibition and PI3K/mTOR inhibition derives from changes in proliferation and/or apoptosis. To assess the effects of in vivo BRAF and PI3K/mTOR inhibition on proliferation, we used immunohistochemistry analysis for the proliferation marker Ki-67, which revealed a 87% decrease (\( P < 0.01 \)). To assess the effects of in vivo BRAF inhibition on apoptosis, we used an in situ TUNEL assay that revealed a statistically significant 5-fold induction with concomitant BRAF and PI3K/mTOR blockade (\( P < 0.05 \); Fig. 5D). These results suggest that the observed tumor regression after concomitant BRAF and PI3K/mTOR inhibition derives from a significant decrease in proliferation and induction of apoptosis.

Discussion

Because of the extremely poor prognosis for patients with BRAF\(^{V600E}\) CRC, the development of novel therapies for this clinical segment is urgently needed (3, 4). The limited...
The response of BRAFV600E CRC to vemurafenib suggests the presence of de novo or rapidly acquired resistance mechanisms (17). Besides the de novo resistance seen in BRAFV600E CRC toward single-agent BRAF-inhibition, the MAPK pathway can be reactivated by additional mechanisms. For example, 2 publications have reported it is the increased activity of phospho-EGFR that follows BRAF-inhibition in CRC that leads to reactivation of p-ERK (19, 20). Similar findings have been observed in in vivo melanoma xenograft models of acquired resistance and in in vitro CRC systems (14, 16). In addition, elevated levels of RAS-GTP, CRAF, and p-AKT have been found in melanoma that is resistant to BRAF-inhibition (37). Here, we show the requirement for concomitant targeting of BRAF and PI3K/mTOR for effective treatment of BRAFV600E CRC.

Robust GEMMs should recapitulate the appropriate histopathologic spectrum observed in humans. BRAFV600E CRC derives from the recently described serrated pathway associated with characteristic histologic features and Wnt activation (34). A recent BratF600E GEM model based on the AcreER system has been reported to have similar serrated features and subsequent activation of the Wnt pathway (38). However, as BratF600E is activated along the entire gastrointestinal tract, these animals die prematurely from overwhelming tumor burden before the individual lesions evolve to advanced carcinoma. To develop a tractable model that is amendable to assessment of experimental therapeutics, we focally activated BratF600E and inactivated Apc in the distal colon of floxed mice using AdCre, as we have previously described (23, 24). In these mice, we observed early SSA/P, SSA/P with cytologic dysplasia, SSA/P with adenocarcinoma, and invasive carcinoma. These findings show that we are able to recapitulate the histopathologic spectrum of the sessile serrated adenoma–carcinoma pathway seen in humans, making this a robust surrogate for BRAFV600E CRC.

Cancer GEMMs that are robust preclinical drug testing platforms should present with the following features: (i)
tumors develop rapidly along a reproducible time line, and (ii) tumors can be continuously monitored throughout drug treatment. Because of the association between BRAF mutation and activation of the Wnt pathway in human CRC, we began with conditional Apc-Braf mice to accelerate tumorigenesis (36, 38, 39). To enable the use of optical colonoscopy for longitudinal tumor evaluation, we focally modify floxed genes in the distal colon using AdCre, as we have previously described (23, 24). As such, these tumors reproducibly progress in the distal colon, permitting monitoring by optical colonoscopy throughout experimental drug treatments. Our GEMM fulfills the above requirements, making it a robust platform for evaluation of experimental therapeutics.

In both our in vitro and our in vivo data using our novel BRAFV600E GEMM, we corroborate the findings seen in human patients: BRAF-mutant tumors are not sensitive to single-agent BRAF inhibition. As tumor growth is modulated by complex, interdigitating signaling networks, blockade at a single node often results in compensation through a parallel pathway. Recently, publications have indicated that in the presence of a BRAF inhibitor, the MAPK pathway can be re-activated by the expression of RTKs (14, 19). In contrast, the PI3K pathway shows sustained activity in 60% of treatment-naïve BRAFV600E CRC, which may explain the ineffectiveness of BRAF inhibitors (22). Consequently, concurrent inhibition of both pathways may be required. For example, chronic BRAF inhibition has been suggested to confer drug resistance in BRAFV600E melanoma through recruitment of PI3K signaling, whereas simultaneous blockade of both signaling pathways results in effective treatment (27, 29).

Furthermore, the requirement for concomitant inhibition of the MAPK and PI3K/mTOR pathways has been shown in other cancer models (40–42).

To examine if this feature were present in our model for BRAFV600E CRC, we interrogated PI3K/mTOR pathway signaling both in vitro and in vivo after BRAF inhibition, revealing the presence of p-AKT Thr473 and p-S6 Ser240/244 indicating activity through the PI3K/mTOR pathway (Figs. 2A and 5C). Although one BRAFV600E CRC cell line carries the activating PIK3CA mutation H1047R (RKO), the other (VACO432) does not. Although VACO432 is heterozygous for a P124T mutation, this has to our knowledge not been characterized as an activating mutation. Taken together with the significant proportion of patients with BRAFV600E CRC with sustained PI3K/mTOR activity (22), we believe that the need for combination therapy is independent of PIK3CA mutational status.

Our subsequent in vitro and in vivo studies showed that combined BRAF and PI3K/mTOR inhibition is required for effective treatment of BRAFV600E CRC. Although it has been suggested that NVP-BEZ235 is a more potent mTOR inhibitor than a PI3K inhibitor (33), inhibition of this particular signaling node seems to be critical to the successful treatment of BRAFV600E CRC. As we did not test this directly, we cannot say whether pure PI3K inhibitors or pure mTOR inhibitors will be as or more effective in combination with BRAF inhibition. However, the more potent mTOR inhibition seen by the down-regulation of p-S6 with NVP-BEZ235 may suggest this to be a more important component. To our knowledge, we are the first to show a BRAF inhibitor–based combination therapy that effectively downregulates TORC1, as evidenced by downregulation of p-S6. In addition, others have also reported the need for a combinational approach. It has been reported that combination treatment with a RAF inhibitor (RAF265) and a dual PI3K/mTOR inhibitor (BEZ) is effective in thyroid cancer (43). Future efforts are underway to further refine which targets along the PI3K/mTOR axis provide the strongest results. Collectively, these results suggest that patients with BRAFV600E CRC may benefit from such combination therapy.

To maximize tumor regression, robust strategies for induction of apoptosis are critical. Although BRAF inhibition alone is sufficient to induce apoptosis in melanoma (44), such treatment results in only growth arrest of BRAFV600E thyroid and colon cancers (43, 45–48) with resistance to cell death (41). This suggests that differential apoptotic responses are one underlying mechanism of the disparate clinical outcomes seen in patients with melanoma and CRC treated with BRAF inhibitors. In our experiments, the effects on apoptosis may have appeared more pronounced in RKO because it harbors a prototypic PIK3CA activating mutation (H1047R), whereas VACO432 contains a rare and uncharacterized PIK3CA mutation (P124T) that might possess lower intrinsic activity. Furthermore, we examined apoptosis after 16 hours, as this has been reported to be the time of maximal apoptotic induction by agents such as staurosporine. It is possible that examination at a different time point might further augment the differences in VACO432 cells to achieve statistical significance. Nonetheless, our data suggest that concomitant treatment of BRAFV600E CRC cell lines with NVP-BEZ235 may be important to tip the scales from inhibition of proliferation toward induction of apoptosis. Furthermore, concomitant BRAF and PI3K inhibition has been observed to increase the sensitivity of CRC to TRAIL treatment (34). This indicates the need for both growth inhibition along with apoptosis. Our studies show that concomitant BRAF and PI3K/mTOR inhibition results in both growth inhibition and an induction of apoptosis leading to in vivo tumor regression.

The approach to targeted therapy in BRAFV600E cancers has been shifting and is seen in the design of recent clinical trials. The identification of the need for BRAF inhibitor–based treatments to include additional inhibitors has led to trials that include a BRAF inhibitor given in combination with additional drugs such as MEK-inhibitors, PI3K-specific inhibitors, and monoclonal antibodies against the protein death receptor ligand-1 (PD-L1). In addi...
we have shown that combination treatment of BRAF\(^{V600E}\) CRC with a BRAF inhibitor and PI3K/mTOR inhibitor downregulates both p-ERK and p-S6, reduces cellular viability, and induces tumor regression in BRAF\(^{V600E}\) GEMMs. It remains unknown whether EGFR inhibition in combination with BRAF inhibition is capable of downregulating TORC1 and achieving the same in vivo tumor regression.

In summary, the establishment of robust therapeutic strategies for BRAF\(^{V600E}\) CRC is an unmet clinical need. Collectively, our findings provide the preclinical rationale for further examination of BRAF and PI3K/mTOR inhibitors in clinical trials for BRAF\(^{V600E}\) CRC. Furthermore, our novel GEMM recapitulates the sessile serrated adenoma–carcinoma pathway in humans, making it a robust platform to interrogate BRAF\(^{V600E}\) CRC biology and refine further combinatorial therapeutic regimens.

**Disclosure of Potential Conflicts of Interest**

J.A. Engelman has commercial research grants from Novartis and Sanofi-Aventis and is a consultant/advisory board member of Novartis, Genentech, Sanofi-Aventis, and GSK. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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References


Correction: Concomitant BRAF and PI3K/mTOR Blockade Is Required for Effective Treatment of BRAF<sup>V600E</sup> Colorectal Cancer

In this article (Clin Cancer Res 2013;19:2688–98), which was published in the May 15, 2013, issue of Clinical Cancer Research (1), in Fig. 1B, the phospho-MEK and total-MEK for the RKO cell line are incorrect and are a copy of the phospho-MEK and total-MEK from the Vaco 432 blot above it. The corrected figure appears below.

The authors regret this error.

Reference


Published OnlineFirst June 18, 2013.
doi: 10.1158/1078-0432.CCR-13-1433
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