Assessment of the In Vivo Activity of PI3K and MEK Inhibitors in Genetically Defined Models of Colorectal Cancer

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Abstract

The objective of tailoring medicines for cancer patients according to the molecular profile of their disease holds great promise for the improvement of cancer therapy. Nevertheless, this approach has been limited, in part, due to the lack of predictive and informative preclinical studies. Herein, we describe an assessment of the therapeutic potential of targeting PI3K/mTOR and MAPK signaling in genetically defined mouse models of colorectal cancer mirroring disease subtypes targeted for novel therapy in the FOCLS4 trial. Our studies demonstrate that dual PI3K/mTOR inhibition is highly effective in invasive adenocarcinoma models characterized by combinatorial mutations in Apc and Pten; Apc and Kras; and Apc, Pten and Kras. MEK inhibition was effective in the combinatorial Apc and Kras setting, but had no impact in either Apc Pten mutants or in Apc Pten Kras triple mutants. Furthermore, we describe the importance of scheduling for combination studies and show that although no additional benefit is gained in Apc Pten mice, combination of PI3K/mTOR and MAPK inhibition leads to an additive benefit in survival in Apc Kras mice and a synergistic increase in survival in Apc Pten Kras mice. This is the first study using robust colorectal cancer genetically engineered mouse models to support the validity of PI3K/mTOR and MEK inhibitors as tailored therapies for colorectal cancer and highlight the potential importance of drug scheduling in the clinic. Mol Cancer Ther; 14(10); 2175–86. ©2015 AACR.

Introduction

The notion of stratifying therapy according to the molecular profile of a given tumor is considered to hold great promise for the improvement of therapeutic outcome. Despite this, failures of certain novel targeted therapeutic agents in clinical trials have limited the progress of this approach. This is largely attributed to a lack of accurate guidance from preclinical studies and it is thought that the increased use of reliable genetically engineered mouse models (GEMM) may lead to improvements in predicting therapeutic outcome. Nevertheless, this approach has been limited, in part, due to the lack of predictive and informative preclinical studies. Herein, we describe an assessment of the therapeutic potential of targeting PI3K/mTOR and MAPK signaling in genetically defined mouse models of colorectal cancer mirroring disease subtypes targeted for novel therapy in the FOCLS4 trial. Our studies demonstrate that dual PI3K/mTOR inhibition is highly effective in invasive adenocarcinoma models characterized by combinatorial mutations in Apc and Pten; Apc and Kras; and Apc, Pten and Kras. MEK inhibition was effective in the combinatorial Apc and Kras setting, but had no impact in either Apc Pten mutants or in Apc Pten Kras triple mutants. Furthermore, we describe the importance of scheduling for combination studies and show that although no additional benefit is gained in Apc Pten mice, combination of PI3K/mTOR and MAPK inhibition leads to an additive benefit in survival in Apc Kras mice and a synergistic increase in survival in Apc Pten Kras mice. This is the first study using robust colorectal cancer genetically engineered mouse models to support the validity of PI3K/mTOR and MEK inhibitors as tailored therapies for colorectal cancer and highlight the potential importance of drug scheduling in the clinic. Mol Cancer Ther; 14(10); 2175–86. ©2015 AACR.
will often remain in situ in the absence of attributable symptoms; an approach being examined in the SYNCHRONOUS trial (15). Given this, we would argue that the GEMMs used in this study directly serve to re-emulate patients with colorectal cancer.

Mutations in genes that encode components of PI3K/AKT and RAS/MEK/ERK signaling frequently occur in colorectal cancer and coexist in approximately a third of all cases (16). In addition, sequencing analysis reveals that mutations in adenomatous polyposis coli (APC), KRAS, and PI3K are the most frequently observed genetic events in human colorectal cancer (17). Loss of the tumor-suppressor protein PTEN has been shown to occur in 12% to 80% of human colorectal cancer cases (18–20). Moreover, PTEN loss is associated with poor prognosis and predicts nonresponse to anti-EGFR agents currently available for KRAS wild-type colorectal cancer patients (21, 22). In light of this, we used three previously established and described GEMMs with combinatorial mutations in APC and Pten; APC and Kras; and APC, Pten, and Kras (12–14). We have used these models of intestinal tumorigenesis, all of which lead to invasive intestinal adenocarcinoma, to investigate the effects of PI3K/mTOR inhibition using NVP-BEZ235; MEK1/2 inhibition using MEK162 (Novartis Pharma); and combinatorial therapy using both NVP-BEZ235 and MEK162, as stratified therapeutic strategies. Although it is widely known that TP53 mutations also contribute to the pathogenesis of human colorectal cancer, this study aimed to directly emulate current clinical trials evaluating PI3K and MAPK pathway inhibitors by using only the relevant pathway status as a stratified approach to targeted therapy.

Materials and Methods

Experimental animals and tissue harvesting

Procedures were carried out in accordance with UK Home Office regulations. All mice were maintained on an outbred background and genotyped by PCR for appropriate alleles. Mouse strains harboring conditional deletion of APC and PTEN and villinCreERT transgenes were induced as described previously (12, 13). For long-term survival studies, mice were aged until a defined treatment start point, at which they were randomized to receive either 0.5% methyl cellulose (vehicle). 35 mg/kg NVP-BEZ235 twice daily (T;D, Novartis Pharmaceuticals), 30 mg/kg of MEK162 twice daily (Novartis Pharmaceuticals), or combination at doses described in the text. Mice were treated daily until symptomatic of disease or to an endpoint of 500 days after induction (DPI) whereupon they were harvested 4 hours after final dose. Kaplan–Meier survival analysis was performed using SPSS Statistics 18.0. For short-term pharmacodynamic experiments, induced mice were aged until symptomatic of disease. Mice were then administered a single dose of vehicle (equivalent volume per weight). 35 mg/kg of NVP-BEZ235, 30 mg/kg of MEK162, or combinations (as outlined in the text) and harvested 4 hours following the final dose. Mice used for short-term experiments were also administered a dose of bromodeoxyuridine (BrdUrd) 2 hours prior to killing. Intestinal tissues were flushed with water, opened longitudinally, rolled into swiss-roll structures, and fixed in 10% neutral buffered formalin (Sigma) overnight before processing and embedding in paraffin wax.

Western blot analysis

Proteins were extracted from snap-frozen tumors and Western blot analysis was carried out as previously described (12). Equivalent protein [30 μg] from at least 3 to 6 tumors from n ≥ 3 mice were pooled per cohort and run in triplicate. Antibodies against pAKT Ser473 (4060), pAKT Thr308 (4056), pS6RP (4858), p4EBP1 (2855), pERK (4370), total extracellular signal–regulated kinase (ERK; 4377) from Cell Signaling Technology and β-actin (A5216) from Sigma were used. Appropriate horseradish peroxidase (HRP)–linked secondary antibodies (GE Healthcare) and ECL reagents (GE Healthcare) were used to develop blots.

Histology, immunohistochemistry, and scoring

Formalin-fixed paraffin-embedded tissues were sectioned (5 μm), stained with hematoxylin and eosin (H&E) for tumor number analysis or, used for immunohistochemistry (IHC). Total tumor number was scored blind from three H&E-stained slides for each sample. IHC against cleaved caspase-3 (1:200; 9664; Cell Signaling Technology) and BrdUrd (1:150; C47580; BD Biosciences) was performed using standard methodologies. Positive staining was scored as a percentage of total epithelial cells per field of view. Typically, five fields were assessed in each tumor in a minimum of 3 mice per cohort. Error bars represent standard deviation of the mean. Statistical analysis was performed using the Mann–Whitney U test. All analysis on scoring were two-tailed tests where n equals the number of lesions scored.

Results

Description and validation of colorectal cancer models

Inactivation of APC and Pten was used to mimic activation of Wnt signaling and PI3K signaling, respectively. Activation of oncogenic Kras was used to mimic activation of MAPK signaling. Conditional mutation of these genes within the intestinal epithelium was achieved using tamoxifen and β-naphthoflavone (BNF) for APCfl/fl Ptenfl/fl mice bearing the AhCreERT transgene; or tamoxifen dissolved in corn oil for APCfl/fl KrasLSL+/− and APCfl/fl Ptenfl/fl KrasLSL+/− mice bearing the VillinCreERT transgene. These mice are referred hereon as, APCfl/fl Ptenfl/fl, APCfl/fl KrasLSL+/−, and APCfl/fl Ptenfl/fl KrasLSL+/−, respectively. Left untreated, APCfl/fl Ptenfl/fl mice develop invasive intestinal malignancies with median survival of 100, 160, and 40 DPI, respectively. Although APCfl/fl Ptenfl/fl and APCfl/fl Ptenfl/fl KrasLSL+/− mice develop lesions within the small intestine only, APCfl/fl KrasLSL+/− mice present with colon lesions additionally (12–14). Analysis of the molecular pathways in tumors harvested from these mice confirms activation of relevant pathways compared with APCfl/fl controls (Supplementary Fig. S1), reinforcing credence of these models for therapeutic intervention. A cohort of mice for each tumor subgroup was also euthanized at the relevant treatment start points to confirm the presence of tumors with varying degrees of severity. As such, APCfl/fl Ptenfl/fl mice presented with adenomas and early invasive adenocarcinomas characterized by sub-mucosal invasion, whereas APCfl/fl KrasLSL+/− and APCfl/fl Ptenfl/fl KrasLSL+/− mice primarily presented with adenomas at the start of treatment (Supplementary Figs. S2–S5).

Antagonism of PI3K/mTOR signaling in colorectal cancer models

Given that PI3K signaling is increased in all three tumor models (Supplementary Fig. S1), we first antagonized the pathway using the dual PI3K/mTOR inhibitor NVP-BEZ235. At 4 hours, following a single dose of NVP-BEZ235, PI3K/mTOR signaling was reduced in APCfl/fl Ptenfl/fl tumors (Fig 1A). At this time point, we
also observed increased apoptosis (Fig. 1D; \( P \leq 0.05 \); Mann–Whitney U test) and reduced proliferation (Fig. 1E; \( P \leq 0.05 \); Mann–Whitney U test) as scoring by cleaved caspase-3 and BrdUrd positivity, respectively (Supplementary Figs. S2 and S3). Continuous daily treatment from 77 DPI was found to significantly increase survival of Apcf/+ Ptenf/f mice from a median of 99 to 266 DPI (Fig. 2A; \( n \geq 15 \) mice per cohort; \( P \leq 0.0001 \); log-rank test). Taken together, these data illustrate favorable antitumor activity of NVP-BEZ235 in Pten-deficient tumors.

We next evaluated the effect of NVP-BEZ235 exposure in the Kras-mutant setting, given that Kras is known to activate PI3K signaling through direct interaction with p110\( \alpha \) (23), and our confirmatory observation of this activation (Supplementary Fig. S1) in Apcf/+ KrasLSL/+ colon tumors. Western blot analysis of short-term treatment of NVP-BEZ235 revealed modest inhibition of AKT signaling at Ser473 and Thr308, but substantial inhibition of signaling downstream of mechanistic target of rapamycin (mTOR) complex 1 as assessed by levels of pS6RP (Fig. 1B). These molecular changes were accompanied by increased apoptotic signaling through cleaved caspase-3 (Fig. 1D; \( P \leq 0.05 \); Mann–Whitney U test; Supplementary Fig. S2), suggesting a significant antitumor effect despite variable inhibition of PI3K signaling. In the long-term setting, NVP-BEZ235 treatment from 100 DPI significantly increased survival of Apcf/+ KrasLSL/+ mice from a median of 153 to 343 DPI (Fig. 2B; \( n \geq 12 \) per cohort; \( P \leq 0.0001 \); log-rank test), indicating considerable dependence of Kras-mutant tumors on PI3K/mTOR signaling. These data highlight the potential benefit of targeting PI3K signaling in Kras-mutant colorectal cancers.

Mutations in KRAS and those activating PI3K signaling coexist in a third of all colorectal cancers. In our Apcf/+ Ptenf/f KrasLSL/+ mouse model, although neither activation of MAPK signaling nor...
hyper-activation of PI3K signaling was observed in intestinal tumors (Supplementary Fig. S1), mice have a significantly reduced lifespan and present with more tumors at death (13). Despite the increased number, Apcf/+/Ptenf/f KrasLSL/+/ mice predominantly present with noninvasive adenomas similar to Apcf/+/KrasLSL/+/ mice (Supplementary Figs. S5A, S6A, and S7A), and as reflected in BrdUrd scoring of vehicle-treated tumors (Fig. 1e). Short-term exposure to NVP-BEZ235 resulted in minimal perturbation of pAKT473 and pAKT308 levels, but pS6RP and p4EBP1 were suppressed as similarly observed in the Apc Pten and Apc Kras models. Our data therefore show that concurrent activation of both PI3K and Kras diminishes the ability of NVP-BEZ235 to reduce signaling immediately downstream of PI3K (Fig. 1C). Nevertheless, levels of apoptosis as scored through cleaved caspase-3 were found to be increased (Fig. 1D; \( P \leq 0.05 \); Mann–Whitney U test; Supplementary Fig. S2). Long-term intervention using NVP-BEZ235 from 22 DPI in this tumor model was found to significantly increase survival of mice from a median of 40 to 104 DPI (Fig. 2C; \( n \geq 12 \) per cohort; \( P \leq 0.0001 \); log-rank test).

Despite the favorable effects on survival in all three tumor models, NVP-BEZ235 only reduced the total number of lesions in Apcf/+/Ptenf/f mice as assessed at the time of death (Fig. 2D). However, such tumors were of increased size as assessed by total area (Supplementary Fig. S4A) and an increased proportion were "advanced invasive adenocarcinomas", defined as showing invasion through the muscle wall (Supplementary Fig. S4B). Although these observations may indicate a phenotype of resistant tumor

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**Figure 2.** Continuous NVP-BEZ235 treatment extends survival of all tumor models and reduces tumor number in Apcf/+/Ptenf/f mice. The Kaplan–Meier survival analysis of Apcf/+/Ptenf/f (A), Apcf/+/KrasLSL/+/ (B), and Apcf/+/Ptenf/f KrasLSL/+/ (C) mice on vehicle (blue line) or 35-mg/kg NVP-BEZ235 (green line) twice daily by oral gavage revealed significantly increased survival across all genotypes (median survivals: Apcf/+/Ptenf/f vehicle = 99 days vs. NVP-BEZ235 = 266 DPI, Apcf/+/KrasLSL/+/ vehicle = 153 vs. NVP-BEZ235 = 343 DPI, Apcf/+/Ptenf/f KrasLSL/+/ vehicle = 40 vs. NVP-BEZ235 = 104 DPI; \( P \leq 0.0001 \); log-rank test) Treatment start points: Apcf/+/Ptenf/f = 77DPI, Apcf/+/KrasLSL/+/ = 100DPI, Apcf/+/Ptenf/f KrasLSL/+/ = 22DPI. D, tumors at death were scored from H&E stained intestinal sections for each cohort. A significant reduction in tumor number was observed for Apcf/+/Ptenf/f mice on NVP-BEZ235 (N-B) treatment compared with vehicle (V). * \( P \leq 0.05 \); \( n \geq 12 \) per cohort, Mann–Whitney U test.

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growth in response to chronic NVP-BEZ235 dosing, this interpretation is confounded by the animals’ longevity—treated mice survived much longer than controls (median, 266 vs. 100 days). In contrast, analysis of tumors arising in Apcf/ Ptenf/ KrasLSL/ mice revealed that while NVP-BEZ235 treatment had no effect on the total burden of colon tumors, it was associated with a significant reduction in the total tumor area of small intestinal tumors, albeit without any change in the total number of lesions present or in their invasive characteristics (Supplementary Figs. S5A, S5B, S6A, and S6B). The later was also the case for Apcf/ Ptenf/ KrasLSL/ lesions following long-term NVP-BEZ235 exposure (Supplementary Fig. S7A). Interestingly, histologic examination showed that many of the intestinal tumors in the treated Apcf/ Ptenf/ KrasLSL/ cohort contained areas of incipient or frank ulceration (Supplementary Fig. S7B and S7C). A small minority of these were ulcerating adenocarcinomas, but most were noninvasive adenomas showing a spectrum of degenerative changes that were interpreted to represent progressive tumor destruction/regression leading to loss of mucosal barrier integrity and ulceration.

**MEK inhibition through MEK162 in models of colorectal cancer**

Given that activation of oncogenic Kras reduced the ability of NVP-BEZ235 to completely inhibit PI3K signaling, we next investigated the consequences of inhibiting MAPK signaling though the MEK1/2 inhibitor MEK162 in all three tumor models. In Apcf/ Ptenf/ mice, short-term exposure to MEK162 reduced levels of pERK and surprisingly also pAKT308 (Fig. 3A), highlighting the complexity of targeting closely associated signaling

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**Figure 3.** MEK inhibition reduces MAPK signaling but results in differential modulation of PI3K signaling across the tumor models. Western blot analysis of tumor lysates from Apcf/ Ptenf/ small intestine tumors (SITs; A), Apcf/ KrasLSL/ colon polyps and SITs (B), and Apcf/ Ptenf/ KrasLSL/ SITs (C) exposed to vehicle or 30-mg/kg MEK162 for 4 hours. Immunoblotting with antibodies against the MAPK effector pERK revealed decreased signaling across the genotypes. An increase in PI3K/mTOR signaling was observed in some Apcf/ KrasLSL/ tumors; however, a reduction in signaling was observed in Apcf/ Ptenf/ KrasLSL/ tumors. Cleaved caspase-3 (D) and BrdUrd-positive-cells (E) per microscopic field were scored from short-term (4 hours) vehicle (blue bar) and MEK162 (red bar)-treated tumors. A significant increase in cleaved caspase-3 staining was observed in Apcf/ KrasLSL/ colon polyps and Apcf/ Ptenf/ KrasLSL/ SITs. A reduction in BrdUrd-positive-cells was observed in Apcf/ Ptenf/ tumors, and a significant increase in BrdUrd-positive-cells in Apcf/ Ptenf/ KrasLSL/ tumors. Data represent average of five different fields per tumor; n ≥ 3 mice per cohort; error bars, standard deviation. *P ≤ 0.05, Mann-Whitney U test.
cascades. Although acute MEK inhibition significantly reduced proliferation, (Fig. 3E; P ≤ 0.05; Mann–Whitney U test; Supplementary Fig. S3), there was no effect on survival of Apc<sup>+/+</sup> Pten<sup>-/-</sup> mice when administered MEK162 continuously from 77 DPI (Fig. 4A, median survival: vehicle = 99 days vs. MEK162 = 101 DPI; n ≥ 15; P > 0.05, log-rank method)

In Apc<sup>+/+</sup> Kras<sup>LSL/</sup> mice, short-term MEK162 exposure increased apoptosis through cleaved caspase-3 in Apc<sup>+/+</sup> Kras<sup>LSL/</sup> colon tumors (Fig. 3D; P ≤ 0.05; Mann–Whitney U test) and abolished detectable ERK signaling (Fig. 3B). Interestingly, Apc<sup>+/+</sup> Kras<sup>LSL/</sup> colon tumors displayed variable alterations in PI3K/mTOR signaling following MEK inhibitor treatment as assessed by Western blot analysis of pAKT<sub>308</sub> and pS6RP, suggesting heterogeneous compensatory activation of the closely associated PI3K signaling cascade (Fig. 3B) and may reflect inter-tumor heterogeneity. Long-term MEK162 administration from 100 DPI significantly increased survival of mice from a median of 153 to 287 days (Fig. 4B; P = 0.0001; n ≥ 12; log-rank test).

Figure 4.

Long-term MEK inhibition only is beneficial for Apc Kras mice. The Kaplan–Meier survival analysis of Apc<sup>+/+</sup> Pten<sup>-/-</sup> (A), Apc<sup>+/+</sup> Kras<sup>LSL/</sup> (B), and Apc<sup>+/+</sup> Pten<sup>-/-</sup> Kras<sup>LSL/</sup> (C) mice on vehicle (blue line) or 30-mg/kg MEK162 (red line) twice daily by oral gavage revealed significantly increased survival of Apc<sup>+/+</sup> Kras<sup>LSL/</sup> mice only (median survivals: Apc<sup>+/+</sup> Pten<sup>-/-</sup> vehicle = 99 days vs. MEK162 = 101 DPI, Apc<sup>+/+</sup> Kras<sup>LSL/</sup> vehicle = 153 vs. MEK162 = 287 DPI; P ≤ 0.0001, log-rank test, Apc<sup>+/+</sup> Pten<sup>-/-</sup> vehicle = 40 vs. MEK162 = 36 DPI). Treatment start points: Apc<sup>+/+</sup> Pten<sup>-/-</sup> = 77DPI, Apc<sup>+/+</sup> Kras<sup>LSL/</sup> = 100DPI, Apc<sup>+/+</sup> Pten<sup>-/-</sup> Kras<sup>LSL/</sup> = 22DPI. Tumors at death were scored from H&E-stained intestinal sections for each cohort (D). A significant reduction in colon and small intestinal tumor number was observed for Apc<sup>+/+</sup> Kras<sup>LSL/</sup> mice on MEK162 treatment (M) compared with vehicle (V). * P ≤ 0.05; n ≥ 12 per cohort, Mann–Whitney U test.
p4EBP1 levels (Fig. 3C). However, this was also accompanied by increased cellular proliferation at this time point (Fig. 3e; \( P \leq 0.05 \); Mann–Whitney U test, Supplementary Fig. S3). Long-term MEK162 exposure from 22 DPI did not deliver a significant survival benefit (Fig. 4c, median survival = 40 days vs. MEK162 = 36 days; \( P > 0.05 \); \( n > 13 \); Mann–Whitney U test), indicating that additional Pten loss negated the beneficial effect of MEK162 observed in Apc\(^{f/+}\) Kras\(^{LSL/+}\) tumors.

Analysis of tumor burden at death following MEK162 treatment in all three tumor models echoed the observations from survival analysis, in that a significant reduction in the total number of lesions was only observed in the responding Apc\(^{f/+}\) Kras\(^{LSL/+}\) mice (Fig. 4D). Furthermore, significant alterations in tumor area were only detected in the small intestine despite the increase in invasive lesions (Supplementary Figs. S5A, S5B, S6A, and S6B). For Apc\(^{f/+}\) Pten\(^{f/+}\) mice, MEK162 had no significant effect on tumor number, tumor area, or invasive characteristics (Fig. 4D; Supplementary Fig. S4A and S4B), whereas for Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice, although total tumor number appears to be reduced, the proportion of invasive lesions following MEK162 treatment is slightly increased (Fig. 4D and Supplementary Fig. S7A), potentially attributable to the increased proliferation in tumors observed from acute MEK inhibition.

**Combinatorial therapy in models of colorectal cancer**

The observations from long-term therapeutic and short-term antitumor experiments of NVP-BEZ235 and MEK162 as single agents, suggest combinatorial inhibition could provide additional therapeutic benefits in all tumor models, in particular for Apc\(^{f/+}\) Kras\(^{LSL/+}\) and Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice, as elements of crosstalk were more apparent in these settings. First, however, it was crucial to establish an effective method of administering the two compounds to achieve the most favorable antitumor effects. Three combination strategies were chosen which involved administration of 30 mg/kg MEK162 1 hour prior to (combo 1), after (combo 2), or at the same time (combo 3) as 35 mg/kg NVP-BEZ235. For all cohorts, mice were euthanized 4 hours following the final dose and samples were collected for evaluation of short term pharmacodynamics and antitumor activity. Probing of tumor lysates for effectors downstream of PI3K/mTOR and MAPK signaling revealed that Apc\(^{f/+}\) Pten\(^{f/+}\) tumors were particularly sensitive to the order of drug sequencing. In this tumor setting, combination strategy 2 (combo 2) resulted in maximal inhibition of pERK and PI3K/mTOR effectors (Fig. 5A), in addition, NVP-BEZ235 elicited effects in a dose-dependent manner in Apcf\(^{f/+}\) and Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice, as elements of cross-talk were more apparent in this setting. In any case, the pharmacodynamic effects observed in Apc\(^{f/+}\) Kras\(^{LSL/+}\) colon polyps may be due to an additive effect of combining inhibitors, given that MEK inhibition results in increased levels of pAKT (Fig. 5b) and PI3K/mTOR inhibition in this setting predominantly alters signaling downstream of mTOR (Fig. 1b), further providing rationale for long-term combination therapy in this tumor setting.

Similarly, Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) tumors displayed parallels in response to the varied combination strategies. Although levels of pERK and pS6RP were found to be reduced with all three strategies, pAKT473 was only reduced following combo 3 (Fig. 5D). A marked increase in apoptosis through cleaved caspase-3 was observed for all three strategies (Fig. 5E), indicating proapoptotic signaling. Proliferation was increased with both combo 1 and 2, similar to our observations with single-agent MEK162, possibly signifying an adaptive response (Fig. 5F). Nevertheless, given that Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice responded dramatically to single-agent NVP-BEZ235 in the long term but acutely showed only modest pathway inhibition, the lack of complete pathway inhibition from the short-term combination studies was not regarded as unfavorable. Therefore, despite some differences in response to the combination strategies between the tumor models, combination strategy 2, which appeared to be beneficial for all cohorts in terms of favorable pharmacodynamics and antitumor activity, was chosen as the strategy for long-term combination treatment.

In the Apc\(^{f/+}\) Pten\(^{f/+}\) setting, mice were exposed using a daily regimen of combo 2 (NVP-BEZ235 1 hour prior to MEK162), followed 8 hours later by an additional daily administration of NVP-BEZ235. This regimen was termed combo 3 (Fig. 2) and was used to ensure tolerability, as twice-daily combo 2 exposure was found to cause weight loss. Although combo R1 was found to significantly increase survival of mice compared with vehicle controls, (Figure 6a, median survival: vehicle = 99 days vs. combo = 270 DPI; \( n \geq 15 \); \( P \leq 0.0001 \); log-rank test) this did not provide any additional benefit to single agent NVP-BEZ235 (Fig. 6A, median survival: NVP-BEZ235 = 266 days vs. combo = 270 DPI; \( n \geq 15 \); \( P > 0.05 \); log-rank test). Assessment of tumors at death revealed further similarities to single-agent NVP-BEZ235: total tumor numbers (Fig. 6D, \( P \leq 0.05 \); Mann–Whitney U test), tumor area (Supplementary Fig. S4A), and the proportion of invasive lesions (Supplementary Fig. S4B) were found to be similar to NVP-BEZ235. In this tumor setting, combo 3 provided no additional benefits for survival or tumor burden. These data strongly suggest that PI3K and mTOR inhibition is effective and sufficient for a therapeutic response in PTEN-deleted intestinal tumors.

For combination treatment experiments in Apc\(^{f/+}\) Kras\(^{LSL/+}\) and Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice, the dosing regimen was further reduced to only a single daily combo 2 regimen (NVP-BEZ235 1 hour prior to MEK162) to ensure tolerability. This is referred to as combo R2. Subsequently, additional control arms of single agents were undertaken to establish the full effects of combination treatment. In Apc\(^{f/+}\) Kras\(^{LSL/+}\) mice, the reduced once-daily (O-D) NVP-BEZ235 and MEK162 remained equipotent (Fig. 6b). In addition, NVP-BEZ235 elicited effects in a dose-dependent manner, but MEK162 reached maximal effects with the once-daily dosing regimen, in terms of survival benefit (Figs. 2B, 4B, and 6B). Although the reduced single-agent doses resulted in similar colon tumor severity profiles, the total tumor area appears increased compared with higher dose regimens, suggesting that single treatments are more permissive for colon tumor growth.
In contrast, small intestinal lesions from once daily–treated mice appeared similar with regard to tumor area and severity in comparison with tumors from mice on twice-daily treatments, suggesting comparable sensitivities of small intestinal tumors to the long-term treatments (Supplementary Fig. S6A and A6B).

Figure 5. Sequencing of combination is crucial for effective inhibition of MEK–ERK and PI3K–AKT signaling in Apc Pten tumors. Western blot analysis of tumor lysates from Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} small intestinal tumors (SITs; A), Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} colon polyps and SITs (B and C), and Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} Kras\textsuperscript{LSL/+} SITs (D) mice exposed to the different combination strategies. Immunoblotting with antibodies against downstream effectors of MAPK and PI3K/mTOR pathways revealed complete inhibition of signaling only with combo 2 in Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} tumors. Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} and Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} Kras\textsuperscript{LSL/+} tumors were less sensitive to the order of sequencing and all combinations reduced signaling MAPK and PI3K/mTOR signaling through pERK and pS6RP, respectively. Scoring of cleaved caspase-3 (E) and BrdUrd-positive cells (F) in tumors from mice exposed to vehicle (blue bar), combo 1 (red bar), 2 (green bar), and 3 (purple bar). A significant increase in cleaved caspase-3 scoring was observed with combo 2 and 3 in Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} tumors, with combo 2 and 3 in Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} colon polyps and with all three combination strategies in Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} and Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} Kras\textsuperscript{LSL/+} SITs. A significant reduction in BrdUrd-positive cells was observed with combo 1 and 2 in Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} tumors and a significant increase in staining with combo 2 and 3 in Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} and combo 1 and 2 in Apc\textsuperscript{f/+} Pten\textsuperscript{-/-} Kras\textsuperscript{LSL/+} SITs. Data represent average of five different fields per tumor, n ≥ 3 mice per cohort, error bars represent standard deviation. *, P ≤ 0.05, Mann–Whitney U test.
Combination treatment in Apcf/+ Ptenf/f KrasLSL/+ mice was found to be well tolerated and resulted in an additive increase in median survival compared with single agents (Fig. 6b, median survivals: vehicle = 153 days vs. NVP-BEZ235 O-D = 238 days vs. MEK162 O-D = 286 days vs. combo R2 = 389 DPI, all comparisons are significant at P ≤ 0.0001 except for MEK162 O-D vs. NVP-BEZ235 O-D P ≥ 0.05; log-rank method). In the colon, combination treatment was found to result in reduced tumor number, but no significant change in tumor area or severity, despite the increase in elapsed time from treatment start point, potentially indicating that treatment results in restriction of both tumor growth and progression (Fig. 6D and Supplementary Fig. S5A and A5B).

Contrastingly, in the small intestine, combined NVP-BEZ235 and MEK162 administration resulted in reduced tumor number as well as reduced tumor area, indicating potent effects on tumor growth (Fig. 6D and Supplementary Fig. S6A and S6B).

Finally, combination treatment (combo R2) administered to Apcf/+ Ptenf/f KrasLSL/+ mice also resulted in an increase in survival when compared with vehicle cohorts (Fig. 6C, median survivals: vehicle = 40 days vs. combo R2 = 125 DPI; P ≤ 0.0001; log-rank test). Analysis of tumors at death indicated no difference in the number of lesions (Fig. 6D), but did also uncover the presence of tumor

Figure 6.
Long-term evaluation of combination treatment in Apc Pten, Apc Kras, and Apc Pten Kras mice. The Kaplan–Meier survival analysis of Apcf/+ Ptenf/f (A), Apcf/+ KrasLSL/+ (B), and Apcf/+ Ptenf/f KrasLSL/+ (C) mice on vehicle (blue line) or combination (purple line) with control NVP-BEZ235 (green line) and MEK162 (red line) treatments. Administration of combination treatment significantly increased survival of Apcf/+ Ptenf/f mice compared with vehicle, however not compared with single-agent NVP-BEZ235. For Apcf/+ KrasLSL/+ mice, combination treatment prolonged longevity and resulted in an additive increase in survival when compared with single-agent MEK162 and NVP-BEZ235. In Apcf/+ Ptenf/f KrasLSL/+ mice, combined treatment synergistically increased survival of mice compared with NVP-BEZ235 and MEK162 alone. (Median survivals: Apcf/+ Ptenf/f vehicle = 153 vs. combo R1 = 270 DPI; P ≤ 0.0001, log-rank test; Apcf/+ KrasLSL/+ vehicle = 99 days vs. combo R1 = 270 DPI; P ≤ 0.0001, log-rank test; Apcf/+ Ptenf/f KrasLSL/+ vehicle = 40 vs. combo R2 = 125 DPI; P ≤ 0.0001, log-rank test.)

Treatment start points: Apcf/+ Ptenf/f = 77DPI, Apcf/+ KrasLSL/+ = 100DPI, Apcf/+ Ptenf/f KrasLSL/+ = 22DPI. Scoring of tumors from H&E-stained sections of intestines (D) revealed a significant reduction in tumor number in those receiving combination therapy (C) compared with vehicle (V) in the Apcf/+ Ptenf/f and Apcf/+ KrasLSL/+ cohorts (*, P ≤ 0.05; n ≥ 12 per cohort, Mann–Whitney U test).
pathway inhibition was coupled with favorable increases in apoptotic signaling and reduced proliferation (Fig. 1A, D, and E). Interestingly, Kras-mutant tumors exhibited greater sensitivity for TORC1 inhibition than PI3K inhibition (Fig. 1B), which may be a result of promiscuous Kras activation of PI3K signaling through interactions with PI10 (23). Furthermore, this effect was exacerbated in the presence of concurrent Pten deletion and suggests that oncogenic Kras prevents the ability of NVP-BEZ235 to completely reduce AKT signaling (Fig. 1C). If indeed this effect is attributable to the presence of oncogenic Kras signaling, this highlights the potential advantages of MEK inhibition in these tumor settings.

Given the above observations, MEK inhibition was next investigated using MEK162 as single-agent therapy in our three models of intestinal tumorigenesis. MEK inhibition in Apc(Efi)/Pten(f/f) KrasLSL/+;Ptenf/f mice was found to be equipotent with PI3K/mTOR inhibition in terms of survival (Figs. 2B, 4B, and 6B), suggesting equivalent dependence of KRAS-mutant tumors on PI3K/AKT and RAS/ERK signaling. This property of KRAS-mutant tumors appears to be context dependent. Similarly to the observations we report here, independent MEK and PI3K inhibition have been shown to be effective in KRAS melanoma models (9); however, KRAS-mutant pancreatic and lung tumors were found to be more responsive to MEK rather than PI3K inhibition (8, 10). Our data also show that additional Pten deletion renders otherwise sensitive KRAS-mutant tumors nonresponsive to MEK inhibition (Fig. 4C). This finding is in accordance with previous studies and provides further evidence for the notion that KRAS mutational status alone is not sufficient as a prognostic marker for response to PI3K inhibition (32–34). Moreover, given that Apc(Efi)/Pten(f/f) mice show no response to long-term MEK inhibition (Fig. 4A), mutations activating the PI3K pathway, such as PTEN deletion, can also be used to predict nonresponsiveness to MEK inhibitors in the KRAS wild-type tumor setting.

Although single-agents NVP-BEZ235 and MEK162 substantially improved survival of mice as described previously, it was anticipated that combination therapy may further increase this benefit. Despite the attractiveness of combinatorial therapy, there is currently little data in the literature to direct the most appropriate dosing schedule for combination of PI3K and MEK inhibitors. This is crucial, as while agents may be effective as single agents, antagonism between two agents when combined, especially given cross-talk between the two pathways may be evident. Furthermore, due to overlapping sensitivities, the combination may result in no net clinical gain when administered jointly (35). To address some of these issues, we chose to investigate three varied combination strategies that differed in the order of compound administration. Here, MEK162 was administered 1 hour prior to (combo 1), after (combo 2), or at the same time (combo 3) as NVP-BEZ235 to determine whether scheduling is key to achieve concomitant pathway inhibition. Interestingly, sensitivity to the scheduling was primarily detected in Apc(Efi)/Pten(f/f) tumors, whereby MEK162 administered prior to or at the same time as NVP-BEZ235 diminished sensitivity to complete PI3K and mTOR inhibition (Fig. 5a). Given that the most favorable antitumor effects, in terms of increased apoptosis and reduced proliferation, were also observed by this strategy (combo 2), it was surprising that long-term administration provided no additional benefits to single-agent NVP-BEZ235 (Figs. 5E and F and 6A). It is possible that while MEK inhibition may lead to favorable pharmacodynamic effects in combination acutely, MAPK signaling is not
required for tumor maintenance in Apc\(^{-/-}\) Pten\(^{-/-}\) mice and therefore pathway inhibition does not lead to a synergistic effect in the long term. In direct contrast to the Apc\(^{-/-}\) Pten\(^{-/-}\) setting, Apc\(^{-/-}\) Kras\(^{LSL^{-/}}\) and Apc\(^{-/-}\) Pten\(^{-/-}\) Kras\(^{LSL^{-/}}\) tumors in response to the short-term combination strategies displayed less effective and less variation in inhibition of both pathways (Fig. 5B–D). Nevertheless, the observation that long-term combo 2 administration increased median survival additively in Apc\(^{-/-}\) Kras\(^{LSL^{-/}}\) mice and synergistically in Apc\(^{-/-}\) Pten\(^{-/-}\) Kras\(^{LSL^{-/}}\) mice indicates that while some antagonism may be prevalent as suggested by the short-term combination studies, the two pathways are essential in tumor maintenance (Fig. 6B and C). These data suggest that combination therapy in KRAS-mutant settings could provide substantial benefits.

In summary, we have performed a systematic preclinical study that effectively evaluates rational therapeutic strategies for Apc, Pten, and Kras mutant colorectal cancer with the aim of identifying optimal clinical strategies. Our data show that PI3K/mTOR inhibition enhances survival in both Kras-mutant and Pten-mutant settings. In contrast, MEK inhibition is only effective in a Kras-mutant background and this is overridden by additional Pten mutation. Critically, we also demonstrate true synergy between the two therapies, but only in the presence of all three (Apc, Kras, and Pten) mutations. Taken together, our data confirm the notion that specific pathway targeting is effective, at least with the GEMMs used here, and so support the general concept of stratified approaches to therapy. Our data also highlight both synergies and limitations in the use of therapeutic combinations, which occur in a genotype-specific manner. Such studies to identify stratified approaches have been previously conducted for lung and ovarian cancers (7, 33); however, this is the first such study conducted using GEMMs for colorectal cancer. As such, our studies should inform human clinical trials such as the FOCUS 4 trial (http://www.focus4trial.org/).

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