Assessment of the in vivo activity of PI3K and MEK inhibitors in genetically defined models of colorectal cancer

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Abbreviation List:
PI3K: Phosphoinositide 3-kinase
mTOR: mechanistic target of rapamycin
MAPK: Mitogen activated protein kinase
MEK: MAP kinase ERK kinase
APC: Adenomatous Polyposis Coli
KRAS: Kirsten rat sarcoma viral oncogene homolog
PTEN: Phosphatase and tensin homolog
CRC: Colorectal cancer
GEMM: Genetically engineered mouse model
EGFR: Epidermal Growth Factor Receptor
ERK: Extracellular regulated MAP kinase
BrdU: Bromodeoxyuridine
DPI: Days post induction

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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 pre-clinical studies. Herein, we describe an assessment of the therapeutic potential of targeting PI3K/mTOR and MAPK signalling in genetically defined mouse models of colorectal cancer (CRC) mirroring disease subtypes targeted for novel therapy in the FOCUS4 trial. Our studies demonstrate that dual PI3K/mTOR inhibition is highly effective in invasive adenocarcinoma models characterised 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 utilising robust CRC GEMMs to support the validity of PI3K/mTOR and MEK inhibitors as tailored therapies for CRC and highlight the potential importance of drug scheduling in the clinic.

INTRODUCTION

The notion of stratifying therapy according to the molecular profile of a given tumour 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 pre-clinical studies and it is thought that the increased use of reliable genetically engineered mouse models (GEMM) may lead to improvements in predicting therapeutic response (1). The FOCUS 4 clinical trial is currently the largest molecularly stratified multi-site randomised trial to include targeted anti-cancer strategies against PIK3CA mutant or PTEN deficient and KRAS or BRAF mutant metastatic colorectal cancer (CRC) (For More Information: http://www.focus4trial.org/). Our pre-clinical study has therefore mirrored some of the major tumour genetic backgrounds of the FOCUS 4 trial using GEMMs and matched novel targeted therapeutic agents to help determine the rationale for treatment combinations likely to ultimately improve patient outcome. The genetically engineered mouse models used in this study faithfully recapitulate molecular pathways involved in the initiation and progression of tumourigenesis and are therefore powerful tools for the assessment of novel agents and proof-of-principle studies for molecularly targeted therapies.

Mutational activation of the KRAS oncogene is found in 40-50% of patients with CRC and, similarly, mutations activating the closely associated PI3K pathway occur in 40% of CRC cases (2). Both are known to be associated with poor prognosis and also predict poor outcome to conventional chemotherapy (3, 4). Moreover, KRAS and PI3K pathway mutations are established predictors of non-response to anti-EGFR targeted therapies in CRC (5, 6). This has led to a surge in the development of targeted agents against PI3K and MAPK pathways and their assessment in pre-clinical studies. A number of recent studies have evidenced the benefits of targeting these pathways utilising potent agents such as dual PI3K/mTOR and MEK1/2 inhibitors as single-agents and in combination therapy utilising robust in vivo models of human cancer (7-10). For CRC however, these evaluations have been restricted to cell based assays and xenotransplantation studies which carry certain limitations, such as the lack of an intact immune system or tumour microenvironments (11). To augment these
approaches, we have used three GEMMs of CRC, each bearing combinatorial mutations in Apc, Pten and Kras (12-14), to evaluate the benefits of targeted agents against the relevant pathways activated in these tumours. Despite many advantages, one argument against the use of GEMMS as pre-clinical models is the impracticability of primary bowel resection prior to palliative drug therapy. However, for many patients with metastatic CRC, the primary site of the disease 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 reflect patients with CRC.

Mutations in genes which encode components of PI3K/AKT and RAS/MEK/ERK signalling frequently occur in colorectal cancer (CRC) and co-exist in approximately a third of all cases (16). Additionally, sequencing analysis reveals that mutations in APC, KRAS and PI3K are the most frequently observed genetic events in human CRC (17). Loss of the tumour suppressor protein PTEN (phosphatase and tensin homologue) has been shown to occur in 12-80% of human CRC cases (18-20). Moreover, PTEN loss is associated with poor prognosis and predicts non-response to anti-EGFR agents currently available for KRAS wild-type CRC patients (21, 22). In light of this, we employed 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 tumourigenesis, 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 CRC, 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 harbouring conditional deletion of Apc and Pten and oncogenic activation of Kras utilising the AhCreER<sup>T</sup> and VillinCreER<sup>T</sup> 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 randomised to receive either 0.5% Methyl Cel lulose (Vehicle), 35mg/kg NVP-BEZ235 twice-daily (Novartis Pharmaceuticals), 30mg/kg MEK162 twice-daily (Novartis Pharmaceuticals) or combination at doses described in the text. Mice were treated daily until symptomatic of disease or and end point of 500 days post induction 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), 35mg/kg NVP-BEZ235, 30mg/kg 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 BrdU 2hrs 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 tumours and western blot analysis was carried out as previously described (12). Equivalent protein (30µg) from at least 3-6 tumours from n ≥ 3 mice was pooled per cohort and run in triplicate. Antibodies against pAKT Ser473 (4060),
pAKT Thr308 (4056), pS6RP (4858), p4EBP1 (2855), pERK (4370), Total ERK (4377) from Cell Signaling Technology and β-actin (A5216) from Sigma were used. Appropriate 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 (5µm), stained with haematoxylin and eosin (H&E) for tumour number analysis or, used for immunohistochemistry (IHC). Total tumour number was scored blind from 3 H&E stained slides for each sample. IHC against cleaved caspase 3 (1:200, 9664, Cell Signaling Technology) and BrdU (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 all tumours 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 CRC models**

Inactivation of Apc and Pten was used to mimic activation of Wnt signalling and PI3K signalling respectively. Activation of oncogenic Kras was used to mimic activation of MAPK signalling. Conditional mutation of these genes within the intestinal epithelium was achieved using Tamoxifen and beta-napthoflavone (BNF) for Apc<sup>−/+</sup> Pten<sup>−/−</sup> mice bearing the AhCreER<sup>T</sup> transgene; or Tamoxifen dissolved in corn oil for Apc<sup>−/+</sup> Kras<sup>V12DLSL/+</sup> and Apc<sup>−/+</sup> Pten<sup>−/−</sup> Kras<sup>V12DLSL/+</sup> mice bearing the VillinCrER<sup>T</sup> transgene. These mice are referred hereon
as, Apc$^{v+}$ Pten$^{ff}$, Apc$^{v+}$ Kras$^{LSL/+}$ and Apc$^{v+}$ Pten$^{ff}$ Kras$^{LSL/+}$, respectively. Left untreated, mice develop invasive intestinal malignancies with median survivals of 100, 160 and 40 days post induction, respectively. Whilst Apc$^{v+}$ Pten$^{ff}$ and Apc$^{v+}$ Pten$^{ff}$ Kras$^{LSL/+}$ mice develop lesions within the small intestine only, Apc$^{v+}$ Kras$^{LSL/+}$ mice present with colon lesions additionally (12-14). Analysis of the molecular pathways in tumours harvested from these mice confirms activation of relevant pathways compared to Apc$^{v+}$ controls (Supplementary figure 1), reinforcing credence of these models for therapeutic intervention. A cohort of mice for each tumour sub-group was also euthanized at the relevant treatment start points to start time to confirm presence of tumours with varying degrees of severity. As such, Apc$^{v+}$ Pten$^{ff}$ mice presented with adenomas and early invasive adenocarcinomas characterised by sub-mucosal invasion, whereas Apc$^{v+}$ Kras$^{LSL/+}$ and Apc$^{v+}$ Pten$^{ff}$ Kras$^{LSL/+}$ mice primarily presented with adenomas at the start of treatment (Supplementary figures 2-5).

Antagonism of PI3K/mTOR signalling in CRC models

Given that PI3K signalling is increased in all three tumour models (Supplementary figure 1), we first antagonised the pathway using the dual PI3K/mTOR inhibitor NVP-BEZ235. At 4 hours following a single dose of NVP-BEZ235, PI3K/mTOR signalling was reduced in Apc$^{v+}$ Pten$^{ff}$ tumours (Figure 1a). At this time point we observed increased apoptosis (Figure 1d, $p\leq0.05$, Mann Whitney U test) and reduced proliferation (Figure 1e, $p\leq0.05$, Mann Whitney U test) as scoring by cleaved caspase 3 and BrdU positivity respectively (Supplementary figure 2, 3). Continuous daily treatment from 77 days post induction was found to significantly increase survival of Apc$^{v+}$ Pten$^{ff}$ mice from a median of 99 days to 266 days post induction (Figure 2a, $n\geq15$ mice per cohort, $p\leq0.0001$, Log-Rank test). Taken together, these data illustrate favourable anti-tumour activity of NVP-BEZ235 in Pten deficient tumours.
We next evaluated the effect of NVP-BEZ235 exposure in the Kras mutant setting, given that Kras is known to activate PI3K signalling through direct interaction with p110α (23), and our confirmatory observation of this activation (Supplementary figure 1) in Apc^{+/+} Kras^{LSL/+} colon tumours. Western analysis of short term treatment of NVP-BEZ235 revealed modest inhibition of AKT signalling at Ser473 and Thr308, but substantial inhibition of signalling downstream of mTOR complex 1 as assessed by levels of pS6RP (Figure 1b). These molecular changes were accompanied by increased apoptotic signalling through cleaved caspase 3 (Figure 1d, p≤0.05, Mann Whitney U test, Supplementary figure 2), suggesting a significant anti-tumour effect despite variable inhibition of PI3K signalling. In the long term setting, NVP-BEZ235 treatment from 100 days post induction significantly increased survival of Apc^{+/+} Kras^{LSL/+} mice from a median of 153 days to 343 days post induction (Figure 2b, n≥12 per cohort, p≤0.0001, Log-Rank test), indicating considerable dependence of Kras mutant tumours on PI3K/mTOR signalling. These data highlight the potential benefit of targeting PI3K signalling in Kras mutant CRCs.

Mutations in KRAS and those activating PI3K signalling co-exist in a third of all CRCs. In our Apc^{+/+} Pten^{+/+} Kras^{LSL/+} mouse model, although neither activation of MAPK signalling nor hyper-activation of PI3K signalling was observed in intestinal tumours (Supplementary figure 1), mice have a significantly reduced lifespan and present with more tumours at death (13). Despite the increased number, Apc^{+/+} Pten^{+/+} Kras^{LSL/+} mice predominantly present with non-invasive adenomas similar to Apc^{+/+} Kras^{LSL/+} mice (Supplementary figure 5a,6a,7a), and as reflected in BrdU scoring of vehicle treated tumours (Figure 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 signalling immediately downstream of PI3K (Figure
Nevertheless, levels of apoptosis as scored through cleaved caspase 3 were found to be increased (Figure 1d, p≤0.05, Mann Whitney U test, Supplementary figure 2). Long term intervention using NVP-BEZ235 from 22 days post induction in this tumour model was found to significantly increase survival of mice from a median of 40 days to 104 days post induction (Figure 2c, n≥13 per cohort, p≤0.0001, Log-Rank test).

Despite the favourable effects on survival in all three tumour models, NVP-BEZ235 only reduced the total number of lesions in Apc^{f/+} Pten^{f/f} mice as assessed at the time of death (Figure 2d). However, such tumours were of increased size as assessed by total area (Supplementary figure 4a) and an increased proportion were ‘advanced’ carcinomas, defined as showing invasion through the muscle wall (Supplementary figure 4b). While these observations may indicate a phenotype of resistant tumour 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 days vs 100 days). By contrast, analysis of tumours arising in Apc^{f/+} Kras^{LSL/+} mice revealed that while NVP-BEZ235 treatment had no effect on the total burden of colon tumours, it was associated with a significant reduction in the total burden of small intestinal tumours, albeit without any change in the total number of lesions present or in their invasive characteristics (Supplementary figure 5a, 5b and 6a, 6b). The later was also the case for Apc^{f/+} Pten^{f/f} Kras^{LSL/+} lesions following long term NVP-BEZ235 exposure (Supplementary figure 7a). Interestingly, histological examination showed that many of the intestinal tumours in the treated Apc^{f/+} Pten^{f/f} Kras^{LSL/+} cohort contained areas of incipient or frank ulceration (Supplementary figure 7b, 7c). A small minority of these were ulcerating adenocarcinomas, but most were non-invasive adenomas showing a spectrum of degenerative changes that were interpreted to represent progressive tumour destruction/regression leading to loss of mucosal barrier integrity and ulceration.
MEK inhibition through MEK162 in models of CRC

Given that activation of oncogenic Kras reduced the ability of NVP-BEZ235 to completely inhibit PI3K signalling, we next investigated the consequences of inhibiting MAPK signalling though the MEK1/2 inhibitor MEK162 in all three tumour models.

In Apc\(^{\text{f/+}}\) Pten\(^{\text{f/f}}\) mice, short term exposure to MEK162 reduced levels of pERK and surprisingly also pAKT308 (Figure 3a) highlighting the complexity of targeting closely associated signalling cascades. Although acute MEK inhibition significantly reduced proliferation, (Figure 3e, \(p \leq 0.05\), Mann Whitney U test, Supplementary figure 3) there was no effect on survival of Apc\(^{\text{f/+}}\) Pten\(^{\text{f/f}}\) mice when administered MEK162 chronically from 77 days post induction, (Figure 4a, median survival: vehicle = 99 days vs MEK162 = 101 days post induction, \(n \geq 15\), \(p \geq 0.05\), Log-Rank method)

In Apc\(^{\text{f/+}}\) Kras\(^{\text{LSL/+}}\) mice, short term MEK162 exposure increased apoptosis through cleaved caspase 3 in Apc\(^{\text{f/+}}\) Kras\(^{\text{LSL/+}}\) colon tumours (Figure 3d, \(p \leq 0.05\), Mann Whitney U test) and abolished detectable ERK signalling (Figure 3b). Interestingly, Apc\(^{\text{f/+}}\) Kras\(^{\text{LSL/+}}\) colon tumours displayed variable alterations in PI3K/mTOR signalling following MEK inhibitor treatment as assessed by western analysis of pAKT308 and pS6RP, suggesting heterogeneous compensatory activation of the closely associated PI3K signalling cascade (Figure 3b) and may reflect inter-tumour heterogeneity. Long term MEK162 administration from 100 days post induction significantly increased survival of mice from a median of 153 days to 287 days (Figure 4b, \(p \leq 0.0001\), \(n \geq 12\), Log-Rank test). Although median survival of Apc\(^{\text{f/+}}\) Kras\(^{\text{LSL/+}}\) mice exposed to NVP-BEZ235 is approximately 60 days (343 days post induction; Figure 2b) longer, the difference is not statistically significant (\(p \geq 0.05\), Log-Rank test), indicating equipotent effects of NVP-BEZ235 and MEK162 with regard to long term survival in this genetic setting.
Short term exposure to MEK162 in Apc\textsuperscript{f/+} Pten\textsuperscript{f/f} Kras\textsuperscript{LSL/+} tumour-bearing mice led to a reduction in ERK signalling (Figure 3c), an induction of apoptosis (Figure 3d, p≤0.05, Mann Whitney U test, Supplementary figure 2) and also a reduction in PI3K/mTOR signalling indicated by reduced pAKT308, pS6RP and p4EBP1 levels (Figure 3c). However, this was also accompanied by increased cellular proliferation at this time point (Figure 3e, P≤0.05, Mann Whitney U test, Supplementary figure 3). Long term MEK162 exposure from 22 days post induction did not deliver a significant survival benefit (Figure 4c, median survival vehicle = 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\textsuperscript{f/+} Kras\textsuperscript{LSL/+} tumours.

Analysis of tumour burden at death following MEK162 treatment in all three tumour models echo the observations from survival analysis in that a significant reduction in the total number of lesions was only observed in the responding Apc\textsuperscript{f/+} Kras\textsuperscript{LSL/+} mice (Figure 4d). Furthermore, significant alterations in tumour area were only detected in the small intestine despite the increase in invasive lesions (Supplementary figure 5a, 5b, 6a and 6b). For Apc\textsuperscript{f/+} Pten\textsuperscript{f/f} mice, MEK162 had no significant effect on tumour number, tumour area or invasive characteristics (Figure 4d, Supplementary figure 4a, 4b) whereas for Apc\textsuperscript{f/+} Pten\textsuperscript{f/f} Kras\textsuperscript{LSL/+} mice, although total tumour number appears to be reduced, the proportion of invasive lesions following MEK162 treatment is slightly increased (Figure 4d, Supplementary figure 7a), potentially attributable to the increased proliferation in tumours observed from acute MEK inhibition.

**Combinatorial therapy in models of CRC**

The observations from long term therapeutic and short term anti-tumour experiments of NVP-BEZ235 and MEK162 as single-agents, suggest combinatorial inhibition could provide
additional therapeutic benefits in all tumour models, in particular for Apc\textsuperscript{E+} Kras\textsuperscript{LSL+/} and Apc\textsuperscript{E+} Pten\textsuperscript{fl} Kras\textsuperscript{LSL+/} mice as elements of cross-talk were more apparent in these settings. Firstly however, it was crucial to establish an effective method of administering the two compounds to achieve the most favourable anti-tumour effects. Three combination strategies were chosen which involved administration of 30mg/kg MEK162 1hr prior to (combo 1), post (combo 2) or at the same time (combo 3) as 35mg/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 anti-tumour activity. Probing of tumour lysates for effectors downstream of PI3K/mTOR and MAPK signalling revealed that Apc\textsuperscript{E+} Pten\textsuperscript{fl} tumours were particularly sensitive to the order of drug sequencing. In this tumour setting, combination strategy 2 (combo 2) resulted in maximal inhibition of pERK and PI3K/mTOR effectors (Figure 5a), as well as increased apoptosis and reduced proliferation (Figure 5e, 5f). Interestingly, MEK inhibition prior to or concurrently with NVP-BEZ235, reduced its ability to inhibit PI3K and mTOR signalling in PTEN deficient tumours (Figure 5a). Additionally, these findings are unlikely due to feedback activation of PI3K signalling as single agent MEK inhibition in Apc\textsuperscript{E+} Pten\textsuperscript{fl} mice did not lead to compensatory activation of PI3K signalling in tumours (Figure 3a).

Apc\textsuperscript{E+} Kras\textsuperscript{LSL+/} tumours responded to the different combination strategies in similar ways through comparably reduced levels of pERK and pS6RP (Figure 5b, 5c), and increased levels of cleaved caspase 3 (Figure 5e). No discernible alterations in the levels of pAKT at either Ser473 or Thr308, or in the levels of p4EBP1 were observed (Figure 5b, 5c). One potential explanation for this apparent contradiction is that the inhibition of pS6RP may be through interactions between terminal ERK signalling and mTOR rather than through AKT, as the level of p4EBP1 (which is also downstream of AKT/mTOR signalling) was unaltered. Nevertheless, the pharmacodynamic effects observed in Apc\textsuperscript{E+} Kras\textsuperscript{LSL+/} colon polyps may be
due to an additive effect of combining inhibitors, given that MEK inhibition results in increased levels of pAKT (Figure 3b) and PI3K/mTOR inhibition in this setting predominantly alters signalling downstream of mTOR (Figure 1b), further providing rationale for long term combination therapy in this tumour setting.

Similarly, Apc<sup>+</sup> Pten<sup>+</sup> Kras<sup>LSL+</sup> tumours 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 (Figure 5d). A marked increase in apoptosis through cleaved caspase 3 was observed for all three strategies (Figure 5e) indicating pro-apoptotic signalling. Proliferation was increased with both combo 1 and 2, similar to our observations with single agent MEK162, possibly signifying an adaptive response (Figure 5f). Nevertheless, given that Apc<sup>+</sup> Pten<sup>+</sup> Kras<sup>LSL+</sup> mice responded dramatically to single-agent NVP-BEZ235 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 unfavourable. Therefore, despite some differences in response to the combination strategies between the tumour models, combination strategy 2, which appeared to be beneficial for all cohorts in terms of favourable pharmacodynamics and anti-tumour activity, was chosen as the strategy for long term combination treatment.

In the Apc<sup>+</sup> Pten<sup>+</sup> setting, mice were exposed using a daily regimen of combo 2 (NVP-BEZ235 1hr prior to MEK162), followed 8 hours later by an additional daily administration of NVP-BEZ235. This regimen is termed Combo R1 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 to vehicle controls, (Figure 6a, median survival: vehicle = 99 days vs combo = 270 days post induction, n≥15, p≤0.0001, Log-Rank method) this did not provide any additional benefit to single agent NVP-BEZ235 (Figure 6a, median survival: NVP-BEZ235 = 266 days vs combo = 270 days post induction,
Assessment of tumours at death revealed further similarities to single agent NVP-BEZ235: total tumour numbers (Figure 6d, p≤0.05, Mann Whitney U test), tumour area (Supplementary figure 4a), and the proportion of invasive lesions (Supplementary figure 4b) were found to be similar to NVP-BEZ235 indicating that the addition of MEK162 in this setting provided no additional benefits for survival or tumour burden. These data strongly suggest that PI3K and mTOR inhibition is effective and sufficient for a therapeutic response in PTEN deleted intestinal tumours.

For combination treatment experiments in Apc\(^{f/+}\) Kras\(^{LSL+/c}\) and Apc\(^{f/+}\) Pten\(^{f/+}\) Kras\(^{LSL/+}\) mice, the dosing regimen was further reduced to only a single daily combo 2 regimen (NVP-BEZ235 1hr 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 NVP-BEZ235 and MEK162 remained equipotent (Figure 6b). Additionally, 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 (Figure 2b, 4b, 6b). Although the reduced single agent doses resulted in similar colon tumour severity profiles, the total tumour area appears increased compared to higher dose regimens suggesting that single treatments are more permissive for colon tumour growth (Supplementary figure 5a, 5b). In contrast, small intestinal lesions from once daily treated mice appeared similar with regard to tumour area and severity in comparison to tumours from mice on twice daily treatments, suggesting comparable sensitivities of small intestinal tumours to the long term treatments (Supplementary figure 6a, 6b).

Combination treatment in Apc\(^{f/+}\) Kras\(^{LSL/+}\) mice was found to be well tolerated and resulted in an additive increase in median survival compared to single agents (Figure 6b, median survivals: vehicle = 153 days vs NVP-BEZ235 O-D = 238 days vs MEK162 O-D = 286 days
vs combo R2 = 389 days post induction, all comparisons are significant at p≤0.0001 except for MEK162 O-D vs NVP-BEZ235 O-D p value≥0.05, Log-Rank method). In the colon, combination treatment was found to result in reduced tumour number, but no significant change in tumour area or severity, despite the increase in elapsed time from treatment start point, potentially indicating restriction of both tumour growth and progression (Figure 6d, Supplementary figure 5a and 5b). Contrastingly, in the small intestine, combined NVP-BEZ235 and MEK162 administration resulted in reduced tumour number as well as reduced tumour area indicating potent effects on tumour growth (Figure 6d, Supplementary figure 6a, 6b).

Finally, combination treatment (combo R2) administered to Apc^{E+} Pten^{ff} Kras^{LSL/+} mice also resulted in an increase in survival when compared to vehicle cohorts (Figure 6c, median survivals: vehicle = 40 days vs combo = 125 days post induction, p≤0.0001, Log-Rank test). Analysis of tumours at death indicated no difference in the number of lesions (Figure 6d), but did also uncover the presence of tumour ulceration suggestive of signs of tumour destruction/regression, as described above (Supplementary fig 7b, 7c). Despite this, proportionally more invasive lesions characterised by sub-mucosal invasion were observed in the combination treated cohort, which may be due to either resistant tumour growth or simply the increase in time elapsed (Supplementary figure 7a). Nevertheless, combination therapy resulted in a synergistic benefit in survival when compared to single-agents NVP-BEZ235 and MEK162, as when reduced to once-daily (O-D) administration NVP-BEZ235 no longer elicited any survival benefit in Apc^{E+} Pten^{ff} Kras^{LSL/+} mice (Figure 6c, median survival: vehicle = 40 days vs NVP-BEZ235 O-D = 36 days post induction, p≥0.05, Log-Rank test). These data further suggest dose-dependent effects of reduced NVP-BEZ235 similar to those seen in the Apc^{E+} Kras^{LSL/+} mice. Furthermore, analysis of tumours at the end of treatment revealed that proportionally more superficially invasive carcinomas were present in NVP-
BEZ235 O-D treated mice in comparison with vehicle and NVP-BEZ235 T-D mice. Together, these data suggest the dose of NVP-BEZ235 used for therapeutic antagonism is critical.

**DISCUSSION**

Although the promise of stratified medicine is currently far from fulfilled, support for the notion of tailoring medicines for a specific patient population remains strong, for example as evidenced by the development of Imatinib to specifically treat chronic myeloid leukaemia and gastrointestinal stromal tumours. Here, we use robust and reliable GEMMs to assess the effect of genetic variation on drug response, in the context of murine intestinal neoplasia, with likely relevance to human CRC to inform clinical trials such as the FOCUS 4 trial. Given that mutations activating PI3K/AKT and RAS/MEK/ERK signalling co-exist in a third of all CRCs, the efficacy of the dual PI3K/mTOR inhibitor NVP-BEZ235 and the MEK1/2 inhibitor MEK162 as single agents and in combination was addressed in three autochthonous tumour models with differing combinatorial mutations in Apc, Pten and Kras.

We have shown that dual PI3K and mTOR inhibition using NVP-BEZ235 is beneficial therapeutically in the Apc\(^{E+}\) Pten\(^{ff}\) setting in particular, but also for Apc\(^{E+}\)Kras\(^{LSL/+}\) and Apc\(^{E+}\) Pten\(^{ff}\) Kras\(^{LSL/+}\) mice, evidenced by increased median survivals (Figure 2a-c). This may be attributable to the role of PI3K/mTOR signalling in a number of crucial cellular processes including cell survival and proliferation through activation of Akt. Phosphorylation and activation of Akt through PDK1 at Thr308 and at Ser473 by mTOR complex 2 (TORC2) leads to subsequent activation of TORC1 signalling which regulates protein synthesis through activation of ribosomal protein S6 kinases and the eukaryotic initiation factor 4E binding protein (4E-BP1) (24, 25). Additionally, Akt has inhibitory effects on negative regulators of the cell cycle including p27 (kip1) (26) and p21 (cip1) (27), but also elicits inhibitory effects
on GSK-3, thus activating c-Myc and cyclin D1 which promote progression of the cell cycle (28). PI3K signalling is also critical for regulating cell death through inhibition of the pro-apoptotic factors Fas, Bim and Bad (29-31) and regulating autophagy and cellular metabolism, processes implicated in mediating increased longevity. A number of these properties of PI3K signalling were evaluated immediately after exposure to NVP-BEZ235 in our three models to determine the pharmacodynamic and anti-tumour effects of NVP-BEZ235. Here, Apc^f/+ Pten^ff mice displayed substantial sensitivity to NVP-BEZ235 as complete pathway inhibition was coupled with favourable increases in apoptotic signalling and reduced proliferation (Figure 1a, 1d, 1e). Interestingly, Kras mutant tumours exhibited greater sensitivity for TORC1 inhibition than PI3K inhibition (Figure 1b), which may be a result of promiscuous Kras activation of PI3K signalling through interactions with P110 (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 signalling (Figure 1c). If indeed this effect is attributable to the presence of oncogenic Kras signalling, this highlights the potential advantages of MEK inhibition in these tumour settings.

Given the above observations, MEK inhibition was next investigated utilising MEK162 as single agent therapy in our three models of intestinal tumourigenesis. MEK inhibition in Apc^f/+Kras^LSL/+ mice was found to be equipotent with PI3K/mTOR inhibition in terms of survival (Figure 2b, 4b and 6b), suggesting equivalent dependence of KRAS mutant tumours on PI3K/AKT and RAS/ERK signalling. This property of KRAS mutant tumours 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 tumours 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 tumours non-responsive to MEK inhibition (Figure 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 MEK inhibition (32-34). Moreover, given that Apc<sup>+/−</sup> Pten<sup>+/−</sup> mice show no response to long term MEK inhibition (Figure 4a), mutations activating the PI3K pathway such as PTEN deletion, can also be used to predict non-response to MEK inhibitors in the KRAS wild-type tumour setting.

Whilst 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 whilst 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 which differed in the order of compound administration. Here, MEK162 was administered 1 hour prior to (combo 1), post (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<sup>+/−</sup> Pten<sup>+/−</sup> tumours whereby MEK162 administered prior to or at the same time as NVP-BEZ235 diminished sensitivity to complete PI3K and mTOR inhibition (Figure 5a). Given that the most favourable anti-tumour 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 (Figure 5e, 5f, 6a). It is possible that whilst MEK inhibition may lead to
favourable pharmacodynamic effects in combination acutely, MAPK signalling is not required for tumour maintenance in Apc$^{f+}$ Pten$^{ff}$ mice and therefore pathway inhibition does not lead to a synergistic effect in the long term. In direct contrast to the Apc$^{f+}$ Pten$^{ff}$ setting, Apc$^{f+}$Kras$^{LSL/+}$ and Apc$^{f+}$ Pten$^{ff}$ Kras$^{LSL/+}$ tumours in response to the combination strategies displayed less effective and less variation in inhibition of both pathways (Figure 5b, 5c, 5d). Nevertheless, the observation that long term combo 2 administration increased median survival additively in Apc$^{f+}$Kras$^{LSL/+}$ mice and synergistically in Apc$^{f+}$ Pten$^{ff}$ Kras$^{LSL/+}$ mice indicates that whilst some antagonism may be prevalent as suggested by the short term combination studies, the two pathways are essential in tumour maintenance (Figure 6b, 6c). These data suggest that combination therapy in KRAS mutant settings could provide substantial benefits.

In summary, we have performed a systematic pre-clinical study which 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 mutations. 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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Figure legends

**Figure 1. NVP-BEZ235 leads to differential inhibition of PI3K/mTOR signalling across tumour models and activation of apoptosis**

Western blot analysis of tumour lysates from Apc\(^{+/+}\) Pten\(^{+/+}\) small intestinal tumours (SITs) (a), Apc\(^{+/+}\) Kras\(^{LSL/+}\) colon polyps and SITs (b) and Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) SITs (c) exposed to vehicle or 35mg/kg NVP-BEZ235 (N-B) for 4 hours. Immunoblotting with antibodies against effectors of the PI3K/mTOR pathway revealed a marked reduction in signalling across the genotypes. Cleaved caspase 3 (d) and BrdU (e) positive cells per microscopic field were scored from short term (4 hours) vehicle and NVP-BEZ235 treated tumours. A significant increase in cleaved caspase 3 staining was observed for all genotypes and a significant reduction in BrdU positive cells was observed in Apc\(^{+/+}\) Pten\(^{+/+}\) tumours. Data represent average of 5 different fields per tumour, n≥3 mice per cohort, error bars represent standard deviation. (*p value ≤ 0.05, Mann Whitney U test)

**Figure 2. Continuous NVP-BEZ235 treatment extends survival of all tumour models and reduces tumour number in Apc\(^{+/+}\) Pten\(^{+/+}\) mice**
Kaplan-Meier survival analysis of Apc$^{+/-}$ Pten$^{+/−}$ (a), Apc$^{+/-}$ Kras$^{LSL/+}$ (b) and Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ (c) mice on Vehicle (blue line) or 35mg/kg NVP-BEZ235 (green line) twice-daily (T-D) by oral gavage revealed significantly increased survival across all genotypes (Median survivals: Apc$^{+/-}$ Pten$^{+/−}$ vehicle = 99 days vs NVP-BEZ235 = 266 days post induction (DPI), Apc$^{+/-}$ Kras$^{LSL/+}$ vehicle = 153 vs NVP-BEZ235 = 343 DPI, Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ vehicle = 40 vs NVP-BEZ235 = 104 DPI, p≤0.0001 Log-Rank test) Treatment start points: Apc$^{+/-}$ Pten$^{+/−}$ = 77DPI, Apc$^{+/-}$ Kras$^{LSL/+}$ = 100DPI, Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ = 22DPI. (d) Tumours at death were scored from H&E stained intestinal sections for each cohort. A significant reduction in tumour number was observed for Apc$^{+/-}$ Pten$^{+/−}$ mice on NVP-BEZ235 (N-B) treatment compared to vehicle (V). (*p value ≤ 0.05, n≥ 12 per cohort, Mann Whitney U test)

Figure 3. MEK inhibition reduces MAPK signalling but results in differential modulation of PI3K signalling across the tumour models

Western blot analysis of tumour lysates from Apc$^{+/-}$ Pten$^{+/−}$ small intestine tumours (SITs) (a), Apc$^{+/-}$ Kras$^{LSL/+}$ colon polyps and SITs (b) and Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ SITs (e) exposed to vehicle or 30mg/kg NVP-BEZ235 for 4 hours. Immunoblotting with antibodies against the MAPK effector pERK revealed decreased signalling across the genotypes. An increase in PI3K/mTOR signalling was observed in some Apc$^{+/-}$ Kras$^{LSL/+}$ tumours however, a reduction in signalling was observed in Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ tumours. Cleaved caspase 3 (d) and BrdU (e) positive cells per microscopic field were scored from short term (4 hours) vehicle and MEK162 treated tumours. A significant increase in cleaved caspase 3 staining was observed in Apc$^{+/-}$ Kras$^{LSL/+}$ colon polyps and Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ SITs. A reduction in BrdU positive cells was observed in Apc$^{+/-}$ Pten$^{+/−}$ tumours, and a significant increase in BrdU positive cells in Apc$^{+/-}$ Pten$^{+/−}$ Kras$^{LSL/+}$ tumours. Data represent average of 5 different fields per tumour, n≥3 mice per cohort, error bars represent standard deviation. (*p value ≤ 0.05, Mann Whitney U test)
Figure 4. Long term MEK inhibition only is beneficial for Apc Kras mice

Kaplan-Meier survival analysis of Apc^{fl/+} Pten^{fl/+} (a), Apc^{fl/+} Kras^{LSL/+} (b) and Apc^{fl/+} Pten^{fl/+} Kras^{LSL/+} (c) mice on Vehicle (blue line) or 30mg/kg MEK162 (red line) twice-daily (T-D) by oral gavage revealed significantly increased survival of Apc^{fl/+} Kras^{LSL/+} mice only (Median survivals: Apc^{fl/+} Pten^{fl/+} vehicle = 99 days vs MEK162 = 101 days post induction (DPI), Apc^{fl/+} Kras^{LSL/+} vehicle = 153 vs MEK162 = 287 DPI p≤0.0001 Log-Rank test, Apc^{fl/+} Pten^{fl/+} Kras^{LSL/+} vehicle = 40 vs MEK162 = 36 DPI) Treatment start points: Apc^{fl/+} Pten^{fl/+} = 77DPI, Apc^{fl/+} Kras^{LSL/+} = 100DPI, Apc^{fl/+} Pten^{fl/+} Kras^{LSL/+} = 22DPI. Tumours at death were scored from H&E stained intestinal sections for each cohort (d). A significant reduction in colon and small intestinal tumour number was observed for Apc^{fl/+} Kras^{LSL/+} mice on MEK162 treatment (M) compared to vehicle (V). (*p value ≤ 0.05, n ≥ 12 per cohort, Mann Whitney U test)

Figure 5. Sequencing of combination is crucial for effective inhibition of MEK-ERK and PI3K-AKT signalling in Apc Pten tumours

Western blot analysis of tumour lysates from Apc^{fl/+} Pten^{fl/+} small intestinal tumours (SITs) (a), Apc^{fl/+} Kras^{LSL/+} colon polyps and SITs (b, c) and Apc^{fl/+} Pten^{fl/+} Kras^{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 signalling only with combo 2 in Apc^{fl/+} Pten^{fl/+} tumours. Apc^{fl/+} Kras^{LSL/+} and Apc^{fl/+} Pten^{fl/+} Kras^{LSL/+} tumours were less sensitive to the order of sequencing and all combinations reduced signalling MAPK and PI3K/mTOR signalling through pERK and pS6RP respectively. Scoring of cleaved caspase 3 (e) and BrdU (f) positive cells in tumours from mice exposed to combo 1, 2 and 3. A significant increase in cleaved caspase 3 scoring was observed with combo 2 in Apc^{fl/+} Pten^{fl/+} tumours, with combo 2 and 3 in Apc^{fl/+} Kras^{LSL/+} colon polyps and with all three combination strategies in Apc^{fl/+} Kras^{LSL/+} and Apc^{fl/+} Pten^{fl/+} Kras^{LSL/+} SITs. A
significant reduction in BrdU positive cells was observed with combo 1 and 2 in Apc\(^{+/+}\) Pten\(^{+/+}\) tumours and a significant increase in staining with combo 1 and 2 in Apc\(^{+/+}\) Kras\(^{LSL/+}\) and Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) SITs. Data represent average of 5 different fields per tumour, \(n \geq 3\) mice per cohort, error bars represent standard deviation. (*p value \(\leq 0.05\), Mann Whitney U test).

Figure 6. Long term evaluation of combination treatment in Apc Pten, Apc Kras and Apc Pten Kras mice

Kaplan-Meier survival analysis of Apc\(^{+/+}\) Pten\(^{+/+}\) (a), Apc\(^{+/+}\) Kras\(^{LSL/+}\) (b) and Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) (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 Apc\(^{+/+}\) Pten\(^{+/+}\) mice compared to vehicle however, not compared to single agent NVP-BEZ235. For Apc\(^{+/+}\) Kras\(^{LSL/+}\) mice, combination treatment prolonged longevity and resulted in an additive increase in survival when compared to single agent MEK162 and NVP-BEZ235. In Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) mice, combined treatment synergistically increased survival of mice compared to NVP-BEZ235 and MEK162 alone. (Median survivals: Apc\(^{+/+}\) Pten\(^{+/+}\) vehicle = 99 days vs combo R1 = 270 days post induction (DPI) p value \(\leq 0.0001\) Log-Rank test, Apc\(^{+/+}\) Kras\(^{LSL/+}\) vehicle = 153 vs combo R2 = 389 DPI p value \(\leq 0.0001\) Log-Rank test, Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) vehicle = 40 vs combo = 125 DPI p value \(\leq 0.001\) Log-Rank test) Treatment start points: Apc\(^{+/+}\) Pten\(^{+/+}\) = 77DPI, Apc\(^{+/+}\) Kras\(^{LSL/+}\) = 100DPI, Apc\(^{+/+}\) Pten\(^{+/+}\) Kras\(^{LSL/+}\) = 22DPI. Scoring of tumours from H&E stained sections of intestines (d) revealed a significant reduction in tumour number in those receiving combination therapy (C) compared with vehicle (V) in the Apc\(^{+/+}\) Pten\(^{+/+}\) and Apc\(^{+/+}\) Kras\(^{LSL/+}\) cohorts (* p value \(\leq 0.05\), n \(\geq 12\) per cohort, Mann Whitney U test).
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Figure 6. Long term evaluation of combination treatment in Apc Pten, Apc Kras and Apc Pten Kras mice.
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Assessment of the in vivo activity of PI3K and MEK inhibitors in genetically defined models of colorectal cancer

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