Sensitivity of KRAS-Mutant Colorectal Cancers to Combination Therapy That Cotargets MEK and CDK4/6

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Abstract

Purpose: The emerging need for rational combination treatment approaches led us to test the concept that targeting MEK and CDK4/6 would prove efficacious in KRAS-mutant (KRASmt) colorectal cancers, where upregulated CDK4 and hyperphosphorylated retinoblastoma (RB) typify the vast majority of tumors.

Experimental Design: Initial testing was carried out in the HCT-116 tumor model, which is known to harbor a KRAS mutation. Efficacy studies were then performed with five RB+ patient-derived colorectal xenograft models, genomically diverse with respect to KRAS, BRAF, and PIK3CA mutational status. Tolerance, efficacy, and pharmacodynamic evaluation of target modulation were evaluated in response to daily dosing with either agent alone or concurrent coadministration.

Results: Synergy was observed in vitro when HCT-116 cells were treated over a broad range of doses of trametinib and palbociclib. Subsequent in vivo evaluation of this model showed a higher degree of antitumor activity resulting from the combination compared to that achievable with single-agent treatment. Testing of colorectal patient-derived xenograft (PDX) models further showed that combination of trametinib and palbociclib was well tolerated and resulted in objective responses in all KRASmt models tested. Stasis was observed in a KRAS/BRAF wild-type and a BRAFmt model.

Conclusions: Combination of trametinib and palbociclib was well tolerated and highly efficacious in all three KRAS-mutant colorectal cancer PDX models tested. Promising preclinical activity seen here supports clinical evaluation of this treatment approach to improve therapeutic outcome for patients with metastatic colorectal cancer. Clin Cancer Res; 22(2); 405–14. ©2015 AACR.

Introduction

Aberrant hyperactivation of KRAS plays a prominent role in tumor initiation and progression in a broad spectrum of human cancers. KRAS mutations comprise >80% of all RAS mutations and are associated with the highest frequency, roughly 20%, of all human malignancies (1). The incidence of KRAS mutations is especially high in colorectal cancer, where the frequency is >40%. Treatment options for patients with metastatic colorectal cancer (mCRC) harboring a KRAS mutation who have failed first-line chemotherapy are limited, reflected by this disease being the third leading cause of cancer deaths in the United States (2). The monoclonal antibody cetuximab was the first EGFR inhibitor approved for clinical use against mCRC but provides no benefit to patients whose cancers harbor a KRAS mutation (3, 4). Efforts to develop drugs directly targeting mutant KRAS protein remain challenging due to issues with target specificity. Consequently, efforts aimed at pharmacologic intervention of KRAS signaling have focused intensively in recent years on downstream targets in the RAS–MAPK cascade, including BRAF and MEK. Inhibitors of MEK were shown to exert in vitro antiproliferative effects in roughly half of the KRASmt tumors tested (5). However, MEK inhibitors have not shown significant clinical activity in patients with colorectal cancer when used as single agents. Adaptive signaling mechanisms contribute to the failure of MEK inhibitors in a monotherapy setting, dictating the need for rational combination treatment approaches.

The present study was undertaken to explore the therapeutic merits of impairing cell cycle progression by combining agents that target MEK and cyclin-dependent kinase 4 (CDK4). Progression through the G1–S phase is dependent upon phosphorylation of the retinoblastoma (RB) protein by CDK4 (6) or the highly homologous CDK6 (7). In support of this approach, a synthetic lethal interaction between KRAS and CDK4 has been reported in a mouse model of non–small cell lung carcinoma (8). Furthermore, combined inhibition of MEK and CDK4 was found to elicit significant synergy in NRAS-mutant melanoma, where a gradient model of oncogenic RAS signaling was proposed to explain the observed decoupling of proliferation and survival (9). We believe that there exists a strong scientific rationale for exploration of this combination strategy in colorectal cancer, where RB, the main
**Translational Relevance**

Activating mutations in the RAS/RAF/MEK pathway present a therapeutic challenge to clinical management of a broad spectrum of solid tumors, including colorectal cancers. Despite advancement of a multitude of MEK inhibitors into clinical trials, they have failed to elicit sufficient activity to significantly impact outcome in patients with metastatic colorectal cancer, thus dictating the need for rational combination treatment approaches. We demonstrate that the CDK4/6 inhibitor palbociclib may have great promise beyond breast cancer as it leads to improved therapeutic outcome when combined with the MEK inhibitor trametinib to treat patient-derived colorectal xenografts. We describe efficacy testing of five heterogeneous colorectal cancers and report that the KRASmut subset was most responsive to this combination strategy. Promising preclinical activity seen here suggests that dual inhibition of MEK and CDK4/6 is a viable treatment strategy to target the subpopulation of colorectal cancers exhibiting hyperactivated KRAS signaling.

Target of CDK4, is rarely mutated. Inactivation of the Apc gene, which is a key early event in colorectal tumorigenesis, is accompanied by upregulation of cyclin D2 along with increased expression of CDK4/6 and hyperphosphorylated Rb (10). A query of cancer microarray databases in Oncomine (http://www.oncomine.org) provides evidence of CDK4 upregulation in colorectal tumors compared with corresponding normal mucosa in 13 of 37 analyses (P < 0.0001). Inactivation of p16 via promoter methylation also occurs at a significant frequency in colorectal tumors (20%–50% of cases), resulting in loss of the negative regulatory role of this protein that normally serves as a tumor suppressor to inhibit the assembly of cyclin D–CDK4/6 complexes (11, 12). On the basis of the collective supporting rationale, we hypothesized that dual inhibition of CDK4/6 and MEK is a viable treatment strategy to target the subpopulation of colorectal cancers exhibiting hyperactivated KRAS signaling. Here we report that combined therapy with palbociclib and trametinib did indeed result in tumor regressions in all three KRASmut patient-derived xenograft models tested. This was achieved with concurrent dosing of both agents at their single-agent maximum tolerated doses (MTD).

**Materials and Methods**

**Cells**

HCT-116 cells were purchased from ATCC and were maintained in McCoy 5A medium with 10% FBS and 1% penicillin/streptomycin/glutamine (PSG) at 37°C in 5% CO2.

**Drugs**

Trametinib was purchased from LC Laboratories. Palbociclib was obtained under a Material Transfer Agreement from Pfizer Global R & D. For cellular studies, drugs were dissolved in DMSO at a concentration of 10 mmol/L and stock solutions stored at –20°C.

**Cell proliferation assays**

For growth inhibition analysis, cells were seeded in white-walled/clear-bottom tissue culture treated 96-well plates at 2,000 cells per well and allowed to adhere for 24 hours followed by addition of growth media containing serial dilutions of trametinib, palbociclib, or both drugs in combination. Control wells received DMSO at a final concentration of 0.2%. Cells were incubated for 3 days in the continuous presence of drug or DMSO and viability was measured using CellTiter-Glo (Promega). Viability was calculated as a percentage of the DMSO-treated cells. Three replicates were performed for each of the different drug treatment conditions. Data were modeled using a nonlinear regression curve fit with a sigmoidal dose–response using GraphPad Prism 5 (GraphPad Software). Synergy calculations were performed using Combenfit software (Cancer Research UK Cambridge Institute).

**Patient samples and establishment of patient-derived colorectal xenograft models**

Tumor and matched normal specimens were obtained from patients undergoing liver metastasectomies or colon resections of primary disease at the University of Michigan University Hospital (Ann Arbor, MI). All patients provided informed written consent and samples were procured with approval of the University of Michigan Institutional Review Board. Specimens were obtained within four hours of surgery and immediately transferred to DMEM/F12 media supplemented with 10% FBS and 1% PSG at 4°C. A portion of normal colon specimens was fixed in 10% neutral-buffered formalin (NBF) and the remainder snap-frozen in liquid nitrogen. Portions of tumor specimens were either fixed in 10% NBF; snap-frozen in liquid nitrogen, or divided into fragments approximately 3 × 3 mm2 for subcutaneous implantation into female 6- to 7-week-old CIEA NOG mice (NOD.Cg-Prkdcscid Ii2rgtm1Sug/JicTac from Taconic) using an 11G Trocar needle. Tumor-implanted mice were monitored for tumor growth for up to 4 months following implantation. Xenografted tumors from the NOG mice were passaged into female 6- to 7-week-old NCR nude mice (CrTac:NG-Foxn1nu from Taconic) for model expansion. Patient-derived xenograft (PDX) models were maintained in nude mice for no more than four passages before fresh material from the freezer was used to regenerate the line.

**Xenograft studies**

Female 6- to 7-week-old NCR nude mice (CrTac:NG-Foxn1tm1 from Taconic) were implanted subcutaneously with low-passage PDX tumor fragments (~30 mg) into the region of the right axilla. Mice were randomized into treatment groups and treatments initiated when tumors reached 100 to 200 mg. Trametinib and palbociclib were administered daily for 10 to 14 days by oral gavage as a fine suspension in 0.5% HPMC with 0.2% Tween-80 or saline, respectively, based upon individual animal body weight (0.2 mL/20 g). Subcutaneous tumor volume and body weights were measured two to three times a week. Tumor volumes were calculated by measuring two perpendicular diameters with calipers and using the formula: tumor volume = (length × width2)/2. Mice were held following cessation of treatment until tumor burdens reached about 1,000 mg to allow for calculation of tumor growth delay. Percent treated/control (%T/C) was calculated by dividing the median treated tumor weight by the median control tumor weight and multiplying by 100 on the last day of treatment. Tumor growth delay (T–C) was calculated by subtracting the median time to reach evaluation size (750 mg) of the treated group by the median time to evaluation size of the control.
group. A partial regression (PR) is defined as a tumor that regressed to ≤50% of the baseline tumor volume. A complete response (CR) is defined as a tumor below the limits of palpation (<40 mm). All procedures related to the handling, care, and treatment of animals were conducted in accordance with University of Michigan’s Committee on the Use and Care of Animals guidelines.

Mutation detection

Initial mutational screening was performed using the qBiomarker Somatic Mutation PCR Array for Human Colon Cancer (Qiagen). Mutational status was confirmed by Sanger sequencing.

Flow cytometry

For cell-cycle experiments, HCT-116 cells were seeded into 6-well plates at 300,000 cells per well and allowed to adhere overnight. Cells were cultured with either DMSO (0.1%), trametinib (10 nmol/L), palbociclib (1 μmol/L), or combination of the two agents at these concentrations for 24 hours. Cells were harvested with 0.05% Trypsin, spun down, washed with PBS, fixed with 70% ethanol, and stored at 4°C for at least 24 hours. Once ready for analysis, the fixed cells were spun down, washed with PBS, and allowed to incubate for 30 minutes prior to analysis with a propidium iodide solution: 50 μg/mL propidium iodide (Life Technologies), 0.1% Triton X-100 (Sigma-Aldrich), 50 μg/mL RNase A (Qiagen), and PBS. Data were collected on a Cyan ADP Analyzer (Beckman Coulter), with collection of 10,000 events. Histograms were generated and cell-cycle analyses were performed using flow cytometry analysis software ModFit LT V4.0.5 (Verity Software House).

p16 promoter methylation

Following bisulfite conversion of gDNA, a methylation-specific PCR (MSP) assay was carried out as described by Herman and colleagues (13). MSP using primers selectively recognize a fully methylated and unmethylated sequence in the promoter region of p16 that contains numerous CpG islands. A fully methylated control (enzymatically methylated human MLH1 mismatch repair gene) was probed in every assay to ensure bisulfite conversion fidelity.

Western blotting

Tumors were manually homogenized with a Teflon pestle (Bel-Art) in lysis buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% Nonidet P-40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and protease and phosphatase inhibitors], rocked for 30 minutes at 4°C, and centrifuged at 14,000 rpm for 20 minutes at 4°C. Protein concentration was determined by BioRad Protein Assays and lysates were subsequently subjected to SDS gel electrophoresis. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and probed with primary antibodies recognizing p-ERK1/2 (thr202/tyr204), ERK1/2, p-RB (ser780), and RB (all from Cell Signaling Technology) and GAPDH (Abcam). After incubation with anti-rabbit HRP-linked secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), proteins were detected using chemiluminescence (GE Healthcare).

Immunohistochemistry

Tissues were fixed in 10% NBF, embedded in paraffin, and sectioned in accordance with standard procedures. Embedding, sectioning, hematoxylin and eosin (H&E), and all other staining were performed by the University of Michigan Cancer Center Histopathology Core. The Ki67, pRB, and cleaved caspase-3 antibodies for immunohistochemistry (IHC) were obtained from Cell Signaling Technology and the total RB antibody for IHC from Abcam. Images were taken with a Nikon E-800 microscope, Olympus DP71 digital camera, and DP Controller software. For quantification of staining, representative images were obtained from the stained slides at ×40 objective magnification for ImmunoRatio analysis. For each treatment condition (vehicle, trametinib, palbociclib, and combination), five representative fields of view from four individual tumors were analyzed. The images were analyzed using the basic mode in the ImmunoRatio software. Quantification is presented as mean ± SEM. In assessing two different groups, 2-tailed Student t test (unpaired) was used for statistical analysis.

Statistical analysis of PDX efficacy studies

ANOVA was applied on the outcomes of tumor volume and the time to reach a tumor burden of 750 mg under the log normal assumption. This was followed by post hoc pairwise comparison with Bonferroni adjusted significance threshold set at P = 0.05/6 = 0.008, accounting for the six possible pairwise differences. Tumor growth delay data (time for tumors to reach 750 mg) for the CRM 13–180 study was analyzed using censored log normal data, as roughly 15% of the values were right censored in the sense that tumor volume did not reach 750 mg at the time the animals were sacrificed. P values indicating statistical significance are provided in the figure legends.

Results

Evaluation of toxicity of trametinib and palbociclib in combination

Informed selection of agents is crucial for exploring the central hypothesis that dual targeting of MEK and CDK4/6 holds therapeutic promise for the treatment of KRASmut colorectal cancer. We selected palbociclib to inhibit CDK4/6 activity on the basis of its known ability to selectively target these kinases (14) and its promising clinical activity in patients with breast cancer that has led to its regulatory approval. A multitude of MEK inhibitors is available with proven ability to effectively impair ERK activation in vivo (15). The potential for overlapping toxicities when combining palbociclib and the MEK inhibitor PD0325901 proved problematic in early work carried out to evaluate MEK inhibitor–based combinations (Sebolt-Leopold, unpublished data). However, we found that trametinib at its reported single-agent MTD of 3 mg/kg (16) can be administered for a full course of 10 daily treatments in combination with palbociclib administered daily at its single-agent MTD (150 mg/kg) with weight loss in the combination group never surpassing 10% (Supplementary Fig S1). The absence of overlapping toxicities upon combination of these two agents at their reported single-agent MTD provided the basis for subsequent pharmacologic evaluation of this approach.

Synergy of trametinib and palbociclib combination therapy in HCT-116 model

To explore the therapeutic potential of combining trametinib and palbociclib to treat KRASmut colorectal cancer, we first carried out studies with the HCT-116 colorectal cancer cell line, which...
carries an activating KRAS mutation (G13D), and is conducive to evaluating the correlation of in vitro to in vivo data for predicting sensitivity. A 5 × 5 matrix combination dose–response screen of trametinib and palbociclib was performed in HCT-116 cells to assess both single-agent activity and evaluate additive, synergistic, or antagonistic interactions across a range of doses. As single agents, both trametinib and palbociclib exhibited dose-dependent antiproliferative activity with IC50 values of 5.7 nmol/L and 5.9 μmol/L, respectively (data not shown). A heat map of the excess over highest single agent (EOHSA) is shown depicting the doses at which the drug combinations achieve better than predicted inhibition of cell viability (Fig. 1A, top). The combination of trametinib and palbociclib was synergistic over the majority of dose combinations tested. Additional synergy evaluation was performed by calculating the combination index (CI) values on the basis of the median effect principle (17). Consistent with the EOHSA method, we observed synergy at the majority of doses tested and importantly at doses that are thought to be clinically relevant (Fig. 1A, bottom).

Subsequent in vivo evaluation of HCT-116 tumor–bearing mice provided additional evidence that this drug combination produced a higher degree of antitumor activity than that achievable from single-agent treatment. Combination treatment with trametinib and palbociclib resulted in a 14-day tumor growth delay (Fig. 1B), which was significantly longer than the 7-day tumor growth delay elicited from either single agent alone. Treatment with the combination also resulted in partial tumor regressions in 2 of 5 mice, whereas single-agent treatment resulted in no objective responses.

Trametinib and palbociclib combination therapy potentiates G1 arrest

To characterize the mechanism of growth inhibition incurred in response to trametinib and palbociclib combination therapy,
cell-cycle distribution studies were carried out. After 24 hours of treatment, a significant increase in the percentage of HCT-116 cells arrested in G0–G1 was found to occur in response to inhibition of both MEK and CDK4/6 (Fig. 1C). As expected, treatment with trametinib or palbociclib alone led to a G1 block (61% to 66% compared with 35% of DMSO-treated cells). Combination of the two agents led to 84% of cells being arrested in G1 and was accompanied by decreases in the percentages of cells in S and G2–M.

Colorectal PDX commonly are positive for phosphorylated RB expression

A heterogeneous panel of colorectal cancer PDX models was established from fresh biospecimens procured from patient surgeries (Supplementary Table S1). Of the 18 PDX models established, 11 originated from colon resections, 6 resulted from liver metastasectomies, and 1 was established from an ovarian metastasis. Both the original patient tissue and the xenograft tissue were analyzed by H&E staining, whereupon colorectal cancer PDX tissues were found to exhibit a similar histologic phenotype to that of patient tissue from which they were derived (Fig. 2A). Genomic profiling revealed that the most prevalent oncogenic mutations occur in KRAS, PIK3CA, and BRAF as reflected by incidence rates of 44%, 22%, and 11%, respectively. Overall, the frequency of these mutations in our panel is consistent to that reported in the clinical colorectal cancer population. Targeted exome sequencing was also carried out comparing the genomic profile of the xenograft tumors with the original patient samples. In each case, no difference in the major oncogenic drivers was found between these two sets of samples (data not shown). This suggests that our panel is indeed an accurate representation of clinically relevant disease.

When analyzed for pRB expression, all 18 models were found to be RB-positive (Supplementary Fig. S2). Five of these models (Table 1) were selected on the basis of diverse genotypes to explore the in vivo consequences of combined MEK and CDK4/6 inhibition: three models that are KRASmt, one model that is BRAFmt and a fifth model that is BRAFWTKRASWT. All five models were characterized for expression levels of CDK4, pERK, and pRB compared with matching normal tissue from the same patient from whom the tumor specimen originated. Normal tissue was not available for the CRM 12-1159 model, as it originated from a patient undergoing hepatic metastasectomy. As shown in Fig. 2B, there were no discernable differences in CDK4 and pERK expression among this subset of PDX models when comparing tumor models to each other or to normal tissue. This result is not
The combination of trametinib and palbociclib is efficacious in KRAS\textsuperscript{mt} patient-derived colorectal cancer xenografts

The three KRAS\textsuperscript{mt} colorectal cancer PDX models advanced for pharmacological evaluation were selected on the basis of diverse genotypes (KRAS\textsuperscript{G12V} PIK3CA\textsuperscript{WT}, KRAS\textsuperscript{G12D} PIK3CA\textsuperscript{Q546E}, and KRAS\textsuperscript{Q61H} PIK3CA\textsuperscript{E542K}). Subcutaneous tumors of these three models (CRM 13-180, CRC 13-1333, and CRC 14-136) were implanted into NCR nude mice. Treatment was initiated when the mean tumor volume reached about 150 mm\textsuperscript{3}. For each model, administration of the single agents was modestly inhibitory as reflected by tumor growth inhibition (% T/C) values that ranged from 29% to 72% for trametinib and 41% to 72% for palbociclib (Fig. 3A and Table 2). Single-agent treatment resulted in tumor growth delay values that were also not significant when compared with the vehicle control group. As anticipated, the two PIK3CA-mutated models were more refractory to trametinib treatment alone than the PIK3CA\textsuperscript{WT} model. Combination treatment resulted in enhanced tumor growth inhibition (% T/C, 17%–29%) in all three models. Very importantly, objective responses were seen in all three KRAS\textsuperscript{mt} models treated with the combination (Table 2 and Fig. 3B). Whereas stable disease or progression was observed for the single-agent cohorts, partial regressions were observed in the combination groups in all three models. Intragroup variability in the combination groups was generally quite low as reflected by SEM values that ranged from 6% to 21%. Statistically significant tumor growth delay was observed in two of the models (20 and >90 days for CRC 13-1333 and CRM 13-180 models, respectively). However, the response of CRC 14-136 tumors to combination treatment was clearly less durable than the other two KRAS\textsuperscript{mt} models as reflected by resumption of tumor growth as soon as treatment was terminated. Importantly, efficacy derived from the combination of trametinib and palbociclib at their respective single-agent MTD doses was not accompanied by any apparent adverse clinical signs of toxicity. The maximum amount of treatment-related weight loss in the combination group did not exceed 10%, consistent with earlier toxicity testing carried out in non–tumor-bearing animals (Supplementary Fig. S5).

The combination of trametinib and palbociclib potentiates suppression of RB phosphorylation

Pharmacodynamic evaluation of pERK and pRb expression levels revealed significant target modulation by each agent. Representative data are shown in Fig. 4 for the CRM 13-180 tumor model as measured by immunoblotting (Fig. 4A) and IHC (Fig. 4B). Total Rb and ERK expression levels are shown in
Supplementary Fig. S6. Interestingly, tumors from the animals in the combination arm exhibited a significant reduction in pRB levels compared with palbociclib treatment alone, consistent with enhanced antitumor activity as well as reduced Ki67 staining in this group (Fig. 4C). IHC staining of cleaved caspase-3 revealed no induction of apoptosis in any of the four experimental groups (Supplementary Fig. S7).

Trametinib and palbociclib combination treatment of KRAS wild-type and BRAF-mutant PDX models is not superior to palbociclib monotherapy

The effectiveness of this combination treatment strategy was additionally tested in two colorectal cancer PDX models that are wild-type with respect to RAS. Chosen for study were the CRM 12-1159 model, which harbors a PIK3CA mutation, and CRC 13-893, which is a BRAF-mutant model. As summarized in Table 2, CRM 12-1159 tumors proved to be refractory to MEK inhibitor single-agent treatment as reflected by both T/C and T/C0 parameters (72% and 3 days, respectively). Growth of this KRAS/BRAF wild-type model was modestly impaired by CDK4/6 inhibitor single-agent treatment, with T/C and T/C0 values of 45% and 16 days, respectively. Furthermore, the combination of trametinib and palbociclib did not elicit a meaningful increase in therapeutic activity compared with palbociclib alone against CRM 12-1159 tumors, as reflected by comparable or equivalent activity parameters between the two groups.
The combination of these two agents was also not superior to palbociclib alone when treating BRAF-mutant CRC 13-983 tumors (23- vs. 25-day growth delay for the combination and palbociclib single-agent groups, respectively). However, CRC 13-893 tumors were notably more responsive to both trametinib and palbociclib as single agents than all other models tested, including the KRAS-mutant models. Whereas no objective responses were observed with CRM 12-1159 and CRC 13-983 tumors in any of the treatment arms, combination treatment with trametinib and palbociclib did appear to lead to stasis (Table 2 and Fig. 3A).

**Discussion**

Following introduction of the first orally active MEK inhibitor CI-1040 into clinical trials, the anticancer drug potential of this target class has been intensely investigated (15, 18, 19). A number of research programs were subsequently launched around the CI-1040 template in efforts to optimize the pharmacologic properties of this mechanistic class of molecular targeted agents. Trametinib emerged as the first MEK inhibitor to win regulatory approval for the treatment of metastatic BRAFmut melanoma (20). However, MEK inhibitors as a target class have not shown demonstrable clinical activity in patients with colorectal cancer when used as single agents. This result is not unexpected on the basis of preclinical studies showing tumor stasis at best against KRASmt tumors, likely reflecting the limitations of single-agent targeted approaches for colorectal cancer.

Reactivation of CRAF has been reported to limit the ability of MEK inhibitors to inhibit ERK signaling in KRAS-mutant tumors that arises from the induction of RAF–MEK complexes (21). Activated ERK feedback serves to impair signaling through the RAF/MEK/ERK pathway in KRASmt cells by directly phosphorylating and inhibiting CRAF kinase activity (22). Consequently, release of feedback inhibition of CRAF kinase activity results in induction of MEK phosphorylation. However, Lito and colleagues showed that not all allosteric MEK inhibitors behave similarly; newer agents like trametinib act to target catalytic activity of MEK and further impair its reactivation by CRAF by disrupting RAF–MEK complexes (21). They further showed that trametinib was less affected than PD0325901 to reactivated CRAF signaling.

**Table 2.** Comparison of the efficacy of monotherapy and combination therapy in five colorectal cancer PDX models

<table>
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<th>Model</th>
<th>Mutational status</th>
<th>Trametinib</th>
<th>Palbociclib</th>
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<tr>
<td>CRM 13-983</td>
<td>BRAF (V600E)</td>
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<td>5</td>
<td>0</td>
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</tbody>
</table>

NOTE: T/C represents the relative size of the median tumor burden of the treated versus vehicle control groups on the last day of treatment. Tumor growth delay (T – C) represents the difference in days for the treated (T) and vehicle control (C) tumor burden to reach a median size of 750 mg.
suggested that it results in more durable inhibition of ERK signaling by increasing the dissociation rate of MEK from RAF, an important feature when targeting KRASmut tumors. On the basis of these considerations, trametinib emerged from the multitude of MEK inhibitors currently available as a strong candidate for inclusion in combination treatment studies.

Exome sequencing conducted on a large population of patients diagnosed with colorectal cancer showed that the G1–S checkpoint is genetically altered in approximately half of these cases (23). Consequently, this subgroup of patients with KRASmut colorectal cancer emerges as potential candidates for dual targeting of CDK4/6 and MEK. Along these lines, data reported here provide further support for this combination approach, as cotargeting these kinases led to a significant increase in KRASmut colorectal cancer cells arrested in G1–G0, compared with either single targeted approach.

First, we felt it important to address the potential for overlapping toxicities when combining trametinib with a CDK4/6 inhibitor. We have found that other MEK inhibitors, namely PD0325901 and binimetinib, required dose lowering of their respective MTDs when they were coadministered with palbociclib (data not shown). Here, we report that trametinib at its single-agent MTD could be administered for a full course of 10 to 14 daily treatments in combination with palbociclib, also administered daily at its single-agent MTD. We are not suggesting that other MEK inhibitors do not possess therapeutic potential in combination with palbociclib. Rather, the present study provides support for broader testing of the MEK/CDK4/6 combination concept in colorectal cancer with the portfolio of agents available against each of these molecular targets. In this regard, it is encouraging that binimetinib in combination with the CDK4/6 inhibitor LEE011 has shown hints of early clinical activity in patients with NRAS-mutant melanoma (24).

Clinical application of this combination approach in the colorectal cancer population needs to address the multi-RAS signatures of colorectal cancer. While the majority of KRAS mutations occur within exon 2 at codons 12 or 13, activating mutations can also occur in exons 3 or 4 at codons 61 and 146. With an incidence rate that is considerably lower (1%–4%) than those in exon 2, these mutations as well as those of NRAS are also negative predictive biomarkers for anti-EGFR therapy (4, 25). It is encouraging that the combination of trametinib and palbociclib proved efficacious against a KRASmut PDX model mutated in codon 61 (UM CRC 14-136), as reflected by a T/C value of 29% on the last day of treatment. However, these tumors quickly regrew after treatment was terminated and did not result in a statistically significant tumor growth delay. Additional testing of codon 61 or 146 KRASmut models is warranted to further explore the therapeutic potential of this combination strategy against this rare subgroup of colorectal cancer tumors.

One of the major obstacles to successful deployment of MEK inhibitors to treat KRASmut colorectal cancer is the high frequency of PIK3CA mutations that occur in these tumors. These mutations serve to restore cyclin D1 expression and cell-cycle progression causing them to no longer be dependent on KRAS/MEK/ERK signaling (26). Major efforts have been expended in recent years to combine MEK and PI3K pathway inhibitors in the clinic only to be met with significant toxicity issues. Combination of MEK and PI3K inhibitors was further shown to result in disappointing activity in RASmut colorectal cancer PDX models as evidenced by disease stabilization but absence of overt tumor regression (27). Because virtually all colorectal cancer tumors are RAS+, an alternative strategy for blocking cell-cycle progression in PIK3CAmut tumors is to incorporate a CDK4/6 inhibitor in the treatment regimen. We report here that the CDK4/6 inhibitor palbociclib does in fact render MEK inhibitor–insensitive colorectal cancer PDX models sensitive to this combination treatment strategy in all three KRASmut models evaluated, two of which are also PIK3CAmut. It is noteworthy that the HCT-116 model, which we earlier showed to be highly sensitive to the trametinib/palbociclib combination, also harbors a PIK3CA mutation (H1047R). These results have important implications for future clinical testing of this combination treatment strategy in patients diagnosed with KRASmut colorectal cancer, for which there exist marginally effective therapies. It is especially encouraging that the combination of trametinib and palbociclib elicited tumor regressions in all of these models at doses that did not produce any weight loss for the duration of treatment.

Two PDX models were included in our analyses that are KRAS wild-type, one of which was BRAF-mutated (V600E). While the trametinib/palbociclib combination proved less effective in these models compared with its activity against KRASmut tumors, we were nonetheless surprised to observe stasis. Further investigation of additional KRAS wild-type and BRAFmut models is warranted to understand the significance of this early finding.

In conclusion, we have identified a promising combination strategy, namely dual targeting of MEK and CDK4/6, that merits consideration for clinical testing. Our data with PDX models additionally suggest that patients with coexistent PIK3CA mutations are candidates for this combination treatment strategy directed against KRASmut cancer. Further mouse trials are warranted with an expanded colorectal cancer PDX panel to capture the heterogeneity encountered in the clinic to optimize clinical trial design and further define a patient enrichment strategy.
References

Sensitivity of KRAS-Mutant Colorectal Cancers to Combination Therapy That Cotargets MEK and CDK4/6

Elizabeth K. Ziemke, Joseph S. Dosch, Joel D. Maust, et al.