Response to BRAF-targeted Therapy Is Enhanced by Cotargeting VEGFRs or WNT/β-Catenin Signaling in BRAF-mutant Colorectal Cancer Models

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ABSTRACT

The fact that 10% of colorectal cancer tumors harbor BRAF V600E mutations suggested targeting BRAF as a potential therapy. However, BRAF inhibitors have only limited single-agent efficacy in this context. The potential for combination therapy has been shown by the BEACON trial where targeting the EGF receptor with cetuximab greatly increased efficacy of BRAF inhibitors in BRAF-mutant colorectal cancer. Therefore, we explored whether efficacy of the mutant BRAF inhibitor vemurafenib could be enhanced by cotargeting of either oncogenic WNT/β-catenin signaling or VEGFR signaling. We find the WNT/β-catenin inhibitors pyrvinium, ICG-001 and PKF118-310 attenuate growth of colorectal cancer cell lines *in vitro* with BRAF-mutant lines being relatively more sensitive. Pyrvinium combined with vemurafenib additively or synergistically attenuated growth of colorectal cancer cell lines *in vitro*. The selective and potent VEGFR inhibitor axitinib was most effective against BRAF-mutant colorectal cancer cell lines *in vitro*, but the addition of vemurafenib did not significantly increase these effects. When tested *in vivo* in animal tumor models, both pyrvinium and axitinib were able to significantly increase the ability of vemurafenib to attenuate tumor growth in xenografts of BRAF-mutant colorectal cancer cells. The magnitude of these effects was comparable with that induced by a combination of vemurafenib and cetuximab. This was associated with additive effects on release from tumor cells and tumor microenvironment cell types of substances that would normally aid tumor progression. Taken together, these preclinical data indicate that the efficacy of BRAF inhibitor therapy in colorectal cancer could be increased by cotargeting either WNT/β-catenin or VEGFRs with small-molecule inhibitors.

Introduction

Colorectal cancer is the second leading cause of cancer mortality and morbidity worldwide. In 2017, colorectal cancer caused 896,000 deaths, 18 million years of life lost, and 877,000 years lived with disability (1). There were 1.8 million people diagnosed with colorectal cancer, of which approximately 10% had colorectal cancer tumors harboring BRAF mutations. BRAF-mutant colorectal cancer has unique molecular characteristics, which make this subtype more aggressive and with a poorer prognosis (2). Compared with other patients with colorectal cancer, patients with BRAF-mutant colorectal cancer are also less responsive to current treatment options (3–5). Previously, BRAF/MEK targeted drugs have been investigated for colorectal cancer treatment. However, it was shown that targeting only the BRAF/MEK signaling produced very limited effects against the growth of colorectal cancer cells and tumors (6, 7). Despite this lack of success, the BRAF/MEK signaling remains a promising target for colorectal cancer treatment, as BRAF/MEK signaling mediates essential survival activities of all cancer cells, especially BRAF-mutant colorectal cancer cells. The recent success of the BEACON trial has shown that the efficacy of a BRAF inhibitor (encorafenib) can be significantly increased by combination therapy with cetuximab, which targets the EGF receptor (8, 9). However, there remains significant room for improvement and in any case resistance to this combination will arise in some patients. This suggests the need to investigate how other drug combinations might increase efficacy of BRAF/MEK targeted therapies in BRAF-mutant colorectal cancer.

A wide range of signaling pathways are known to contribute to the development of colorectal cancer and to resistance of current drug therapies (10). This article investigates two such pathways as candidates for combination therapy with BRAF inhibitors in colorectal cancer. The first is WNT/β-catenin signaling as this is frequently upregulated in colorectal cancer tumors (11) and promotes resistance to BRAF inhibitors in colorectal cancer (12). Targeting VEGFR signaling is another possible candidate, as it is a key regulator in the colorectal cancer tumor microenvironment (13). Although the anti-VEGFA drug bevacizumab is used as part of a standard-of-care combination therapy in colorectal cancer (14, 15), targeting this pathway alone has not been clinically effective in treating colorectal cancer (16). Therefore, the main goal of this study is to understand whether targeting WNT/β-catenin or VEGFR signaling could enhance the efficacy of BRAF inhibitor treatment.

Materials and Methods

Compounds

Vemurafenib and axitinib were procured from LC Laboratories. PKF118-310 (17) and ICG-001 (18) were acquired from SelleckChem. Pyrvinium pamoate (19) was purchased from Sigma-Aldrich. CI-1040

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was synthesized at the Auckland Cancer Society Research Centre. Cetuximab was obtained and used as described previously (20).

Cell lines and culture

RAW264.7, 3T3L1, MRC5, and colorectal cancer cell lines were sourced from the ATCC. MRC5 and colorectal cancer cell lines were maintained in Eagle's Minimal Essential Medium (Life Technologies); and RAW264.7 and 3T3L1 were maintained in DMEM (Life Technologies). Culture media were supplemented with 5% FBS (Moregate Biotech), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Life Technologies). Cells were cultured at 37°C in humidified 5% CO_2 incubators. Cell lines were authenticated by short tandem repeat profiling (DNA Diagnostics) and confirmed to be *Mycoplasma* negative by PlasmoTest (InvivoGen).

Cell proliferation assay

Colorectal cancer cells were seeded into 96-well plates (5,000 cells per well in 100 μL of medium) and left to settle for 24 hours at 37°C with 5% CO_2. Cells were treated with compounds for 72 hours. Cell viability was determined using the sulforhodamine B (SRB) assay as described previously (21, 22). Briefly, cells were fixed in 10% trichloroacetic acid at 4°C for 1 hour and left to dry at room temperature. Cells were then stained with 0.4% SRB (Sigma-Aldrich) in 1% acetic acid for 30 minutes in the dark at room temperature. Excessive SRB was washed off with 1% (vol/vol) acetic acid and the cell plate left to dry at room temperature. Cells were incubated with 10 mmol/L of unbuffered Tris base (Sigma-Aldrich) for 1 hour on a plate shaker in the dark at room temperature. The plate was read on a BioTek Synergy 2 microplate reader using absorbance mode at 565 nm with a reference wavelength of 690 nm. Optical densities of cells when the treatment started (0 hour), were subtracted from optical densities of cells at 72 hours. Data were expressed as percent of control from at least two independent experiments conducted in duplicates. A value of 100% inhibition indicates a cytostatic effect and values greater than 100% indicate cell death induction by the treatment. EC_{50} values were calculated by a four-parameter logistic nonlinear regression using GraphPad Prism 8.0 (GraphPad Software).

Cytokine assays

HT-29, Colo-205, and HCT-116 cells were seeded into 24-well plates (2.5×10^5 cells per well in 500-μL serum-free medium). Cells were treated with a single compound (vemurafenib, axitinib, or pyrvinium), combination of vemurafenib and axitinib, or combination of vemurafenib and pyrvinium for 12 hours. Culture supernatants were collected and centrifuged at $1,000 \times g$ for 5 minutes to remove cell debris. Supernatants were assayed for VEGF-A using a VEGF-A Human ProcartaPlex Simplex Kit (Invitrogen) and for MIF, IL8, and TGFα using a Human Luminex Discovery Assay KIT (R&D Systems) following the manufacturers' instructions. Data were normalized by cell counts relative to the control wells.

Conditioned media

RAW264.7, 3T3L1, and MRC5 cells were cultured in complete media in T175 flasks. When cell densities in the flasks reached 75% confluence, cells were incubated with axitinib (0.1 μmol/L), pyrvinium (0.1 μmol/L), or vehicle control (0.1% DMSO) for 12 hours. The cells were then washed twice with PBS and were allowed to grow in serum-free media for 24 hours. Conditioned media were collected, centrifuged at $1,000 \times g$ for 5 minutes to remove cell debris, and stored at −20°C.

Western blotting

Untreated cells were lysed in lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich). Lysates were extracted from the samples by centrifugation at $14,000 \times g$ for 15 minutes at 4°C. Protein content in the lysates was measured using a bicinchoninic acid assay (Thermo Fisher Scientific). Thirty micrograms of each lysate was loaded onto 8% polyacrylamide gels and separated by SDS-PAGE at 125 V for 90 minutes. Gels were transferred onto nitrocellulose membranes (Bio-Rad) at 25 V for 15 minutes. The membranes were incubated with blocking buffer [3% BSA in TBS with 0.5% Tween-20 (Sigma-Aldrich)]) for 1 hour at room temperature. Membranes were incubated overnight at 4°C on a mini-rocker with primary antibodies against ERK, pERK, APC, β-catenin, GSK3-α, Wnt-3a, Axin1, LRP5 (all Cell Signaling Technology), and β-actin (Sigma-Aldrich). All antibodies were diluted at 1:1,000 in blocking buffer, except for β-actin with 1:20,000 dilution. Membranes were washed three times, 5 minutes each, in TBS with 0.5% Tween-20 (TBST) and incubated with anti-rabbit (1:7,500 dilution; Dako) or anti-mouse (1:25,000 dilution; Sigma-Aldrich) goat IgG horseradish peroxidase–conjugated secondary antibody in blocking buffer for 1 hour on a mini-rocker at room temperature. Secondary antibody was removed and membranes washed again three times, 5 minutes each, in TBST. The membranes were incubated with Bio-Rad Clarity ECL for 4 minutes before imaging on a ChemiDocMP Imaging System (Bio-Rad). Equal loading of lysates was controlled using β-actin.

Animal tumor models

All experiments were in compliance with the New Zealand Welfare Act 1999 and the Animal Ethics Committee of the University of Auckland (Auckland, New Zealand; ethical approval R1781). Age-matched, pathogen-free female NIH-III mice (NIH-Lyst$^{bg\text{-}J}$Foxn^{nu}Btk^{xid}; Charles River) and C57/BL6 mice were purchased from the Vernon Jansen Unit at the University of Auckland (Auckland, New Zealand). Mice were kept in a pathogen-free facility with controlled temperature (22°C) and a 12-hour light/dark cycle. Colorectal cancer cells growing in culture flasks were trypsinized and washed in PBS. Trypsin was removed by centrifugation at $800 \times g$ at room temperature. Cells were washed twice with PBS and then resuspended at 50×10^6 cells/mL in PBS (HT-29, Colo-205, and HCT-116) or 5×10^6 cells/mL (CT-26). Cell suspension was subcutaneously injected into the right flank of mice. The number of cells inoculated into each mouse was 5×10^6 cells in 100 μL of PBS (HT-29, Colo-205, and HCT-116) or 5×10^5 cells in 100 μL of PBS (CT-26). Mouse body weight was monitored daily by an electronic scale. Tumor volumes were assessed from two orthogonal dimensions (length and width) using the formula: volume $= 0.5 \times \text{length} \times \text{width}^2$. Drug treatment started when tumors reached an average volume of 100 mm^3. Vemurafenib and axitinib were formulated in 0.4% methylcellulose and administered via oral (p.o.) gavage. Pyrvinium was administered intraperitoneally. To determine whether drugs were acting synergistically, the Bliss index was calculated (23).

Data availability

The data generated in this study are available within the article and its Supplementary Figures and Tables.

Results

Role of BRAF mutations in the proliferation of colorectal cancer cells

We studied a range of colorectal cancer cell lines with and without BRAF mutations (Supplementary Table S1). First, we examined the

expression in colorectal cancer cell lines of proteins important to BRAF/MEK signaling, WNT/β-catenin signaling, and VEGFR signaling (Supplementary Fig. S1). APC was absent in all three BRAF-mutant cell lines and four of nine BRAF WT lines tested. β-catenin was strongly expressed in all the human colorectal cancer lines. Furthermore, higher expression levels of LRP5, a coreceptor for the transduction of the WNT/β-catenin signals, were also observed in the three BRAF-mutant cell lines and the BRAF WT SW620. These results indicate a highly active level of WNT/β-catenin pathway in colorectal cancer cells.

Growth of BRAF-mutant cell lines was more sensitive to both the BRAF-mutant selective inhibitors vemurafenib (24) and dabrafenib (25) compared with BRAF WT cell lines (**Fig. 1A** and **B**). Of the WNT/β-catenin pathway inhibitors PKF118-310, ICG-001, and pyrvinium, the pyrvinium had the greatest and most consistent effect on cell growth (**Fig. 1C–E**). Axitinib was chosen as an example of a VEGFR inhibitor because it is a highly potent and selective inhibitor of VEGFR1, 2, and 3 (26) and is used in the clinic, mainly to treat renal cancer (27). Axitinib had some effect in attenuating growth of colorectal cancer cell lines, but notably this effect was greatest in the BRAF-mutant cell lines (**Fig. 1F**).

These findings lead us to investigate whether adding either pyrvinium or axitinib could improve the efficacy of a BRAF inhibitor. The studies above showed that the EC_{50} for effects of pyrvinium on cell growth was in the region of 1 μmol/L. Therefore, concentrations below this (300 nmol/L) and above this (3 μmol/L) were chosen for combination studies. These were used in combination with a concentration of vemurafenib of 1 μmol/L as this was above the concentration where efficacy was seen in the BRAF-mutant colorectal cancer cell lines but at which effects were still not seen in most BRAF WT cell lines. The combination of pyrvinium and vemurafenib inhibited the growth of all colorectal cancer cell lines tested (**Fig. 2**). The most notable finding was a clear combinatorial effect which was at least as great as the combined effect of each drug alone but in most lines was greater than the additive effect of each drug alone. This was even observed in BRAF WT lines. Together, these data indicated that pyrvinium enhanced the inhibitory effects of the BRAF inhibitor vemurafenib on the growth of colorectal cancer cells.

Both vemurafenib and axitinib were effective at attenuating growth of the three BRAF-mutant colorectal cancer cell lines tested, but in most of the BRAF WT cells, each drug alone was relatively ineffective (**Fig. 3**). The growth of BRAF-mutant colorectal cancer cell lines was effectively suppressed by the drug combination in these cells (**Fig. 3**). In contrast with the pyrvinium/vemurafenib combination, the axitinib/vemurafenib combination did not induce additive effects in most of the BRAF WT colorectal cancer cell lines.

We went on to explore the effects of these drug combinations in animal models as in addition to direct effects of the drugs on the tumor cells there are also possible additional effects that could be generated by effects of drugs on the tumor microenvironment. We first investigated the combination of vemurafenib and pyrvinium. The effects observed *in vitro* could be reproduced *in vivo* using BRAF-mutant HT-29 and Colo-205 xenograft models, and there was evidence that these were acting synergistically, particularly in the Colo-205 model (**Fig. 4A** and **B**). Notably, the reduced growth of the vemurafenib/pyrvinium combination was very similar to reductions caused by the combination of vemurafenib and cetuximab, the latter now being a standard of care for BRAF-mutant colorectal cancer (ref. 8; **Fig. 4A** and **B**).

We next tested the combination of vemurafenib and axitinib. In all the colorectal cancer tumor models tested, axitinib alone displayed a significant effect in delaying the tumor growth (**Fig. 4C–F**). In general, there was more effect of axitinib as a single agent in the animal models compared with the *in vitro* assays, which probably reflects effects on blood vessels or other cells in the microenvironment of the tumors. Response to vemurafenib of each tumor model was very similar to its effect *in vitro*. BRAF WT tumors of HCT-116 and CT-26 did not respond to vemurafenib at all (**Fig. 4C** and **D**), while BRAF-mutant HT-29 and Colo-205 tumors responded well to vemurafenib (**Fig. 4E** and **F**). Notably, in the BRAF-mutant tumor models, there was evidence of a synergistic effect of the two inhibitors on tumor growth (**Fig. 4C** and **D**).

We next investigated the effects of the components of the drug combination on interactions between the cancer cells and the cell types that make up the tumor microenvironment. In these experiments, we investigated whether molecules secreted from these cells would have an impact on the ability of the BRAF-mutant colorectal cancer cells to respond to vemurafenib. These studies show that conditioned media from either mouse fibroblasts (3T3L1), human fibroblasts (MRC5), or mouse macrophage/monocytes (RAW264) could reduce the ability vemurafenib to attenuate growth of either HT-29 cells (**Fig. 5A–C**) or Colo-205 cells (**Fig. 5D–F**). This effect was not observed in BRAF WT colorectal cancer cell lines (Supplementary Fig. S2). Importantly, if these fibroblasts had been pretreated with axitinib or pyrvinium, these effects were lost. This suggested that these cell types found in the microenvironment were secreting substances that induce vemurafenib resistance and that part of the mechanism of action of axitinib or pyrvinium was to regulate the secretion of such factors.

We also investigated the impact of the drug combinations on cytokine production in the BRAF-mutant lines (HT-29 and Colo-205) and found additive reductions in the secretion of MIF, IL8, TGFα, and VEGFA (**Fig. 6A**, **B**, **D**, **E**, **G**, and **H**), while this was not seen in the BRAF WT cell line (**Fig. 6C**, **F**, and **I**). To assess the impact of the combination on cell signaling pathways, we used Western blotting for phosphor-ERK in BRAF-mutant HT-29 and BRAF WT HCT-116 cells. As expected, vemurafenib increased ERK phosphorylation in BRAF WT cells and the drug combinations were unable to attenuate this increase (Supplementary Fig. S3). In contrast, the drug combinations were able to attenuate insulin-induced increases in ERK phosphorylation in BRAF-mutant HT-29 cells.

Discussion

The Ras/Raf/MAPK pathway is long known as a major driver of colorectal cancer biology, and approximately 10% of patients with colorectal cancer have BRAF-mutant tumors (28, 29). Targeting the BRAF pathway in melanomas driven by BRAF mutations has been shown to achieve significant clinical benefit although resistance develops over time (30, 31). Preclinical studies show that pharmacologic inhibition of this pathway can significantly attenuate growth of colorectal cancer cell lines *in vitro* (12, 32–37) and as single agents in animal models of colorectal cancer (12, 33, 36). It was therefore somewhat surprising that targeting this pathway with single-agent BRAF inhibitors or with a combination of BRAF/MEK inhibitors has achieved limited success for colorectal cancer (38, 39). This suggested that resistance pathways already existed in colorectal cancer cells that could override inhibition of the RAF/MEK/ERK pathway. This in turn suggested that targeting these resistance pathways along with the BRAF pathway could improve outcomes. This has been shown in the BEACON trial, which showed that efficacy of BRAF inhibitors can be significantly improved when used in combination with inhibitors targeting the EGF receptor (8, 9).

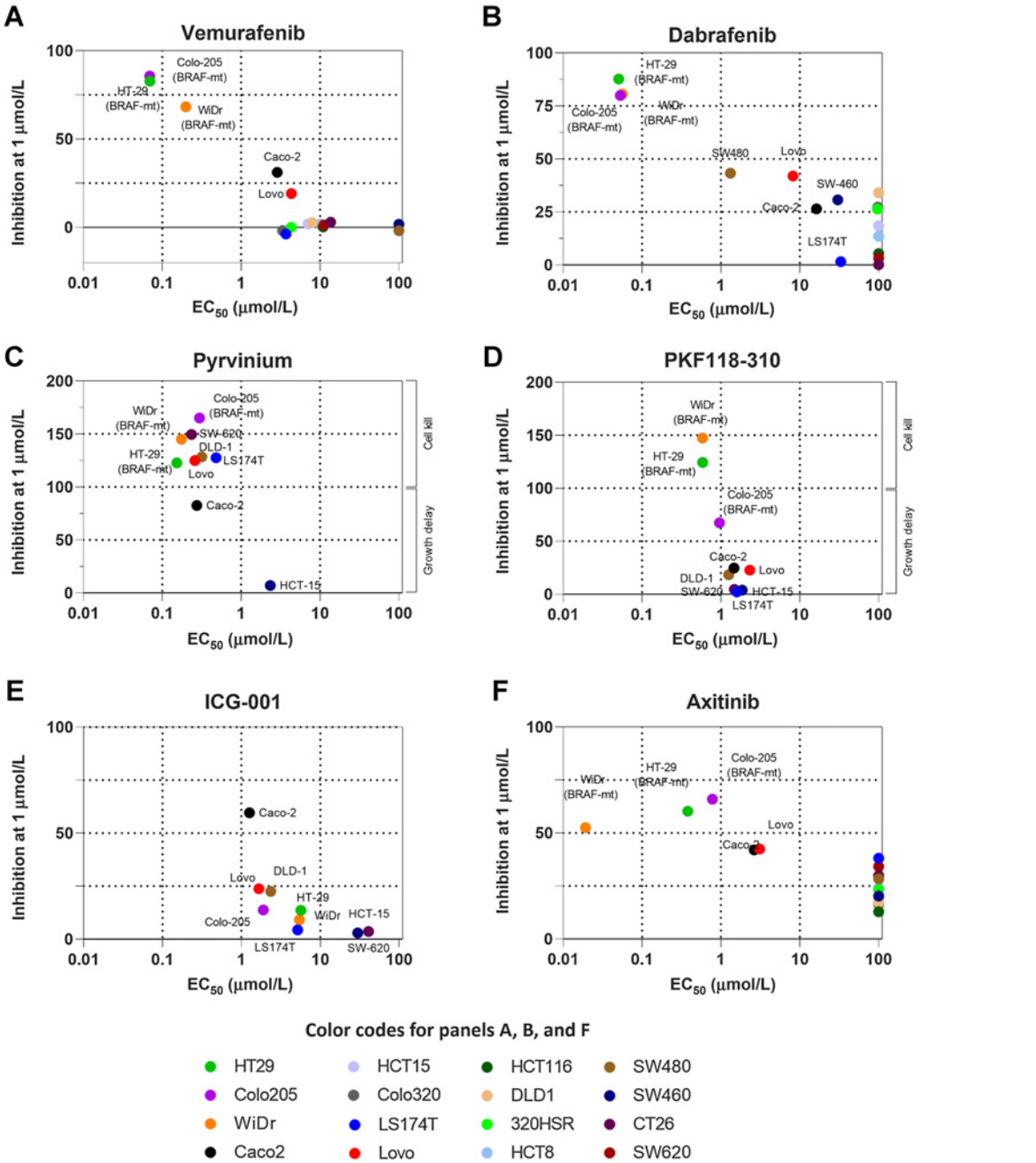

Figure 1.

Role of the BRAF/MEK, WNT/β-catenin, and VEGFRs signaling pathways in colorectal cancer cell growth. Colorectal cancer cells were seeded in 96-well plates (5,000 cells/well) and treated 24 hours later with vemurafenib (**A**), dabrafenib (**B**), pyrvinium pamoate (**C**), PKF118-310 (**D**), ICG-001 (**E**), and axitinib (**F**). Concentrations ranged from 0.1 to 100 µmol/L. After 72 hours of treatment, cell viability was determined by the SRB assay as described in the Materials and Methods. The *x*-axis represents cell-based EC_{50} of each inhibitor for each cell line, which was analyzed from the cell viability data. The *y*-axis represents the magnitude of inhibition at 1 µmol/L for each inhibitor in each cell line. Each color-coded dot in the graphs represents a cell line. Magnitudes between 0% and 100% indicate cell growth was slowed down, and magnitudes greater than 100% indicate a cell kill effect.

The APC/β-catenin pathway is upregulated in many colorectal cancers and is an obvious pathway that could contribute to resistance to BRAF inhibitors (12). This pathway has been suggested as a target in colorectal cancer but it has proven difficult due to the lack of extracellular targets accessible to mAbs and the lack of targets in the pathway with structures amenable to classical small drug development approaches (40, 41). However, a limited range of proven compounds are available that target this pathway; of those tested here, the most effective was pyrvinium. This has been used as an anthelmintic for over 60 years (42) but more recently was identified as an activator of casein kinase-1 and thus an agent that drives rapid turnover of β-catenin (19). Our study builds on previous studies that indicated that targeting

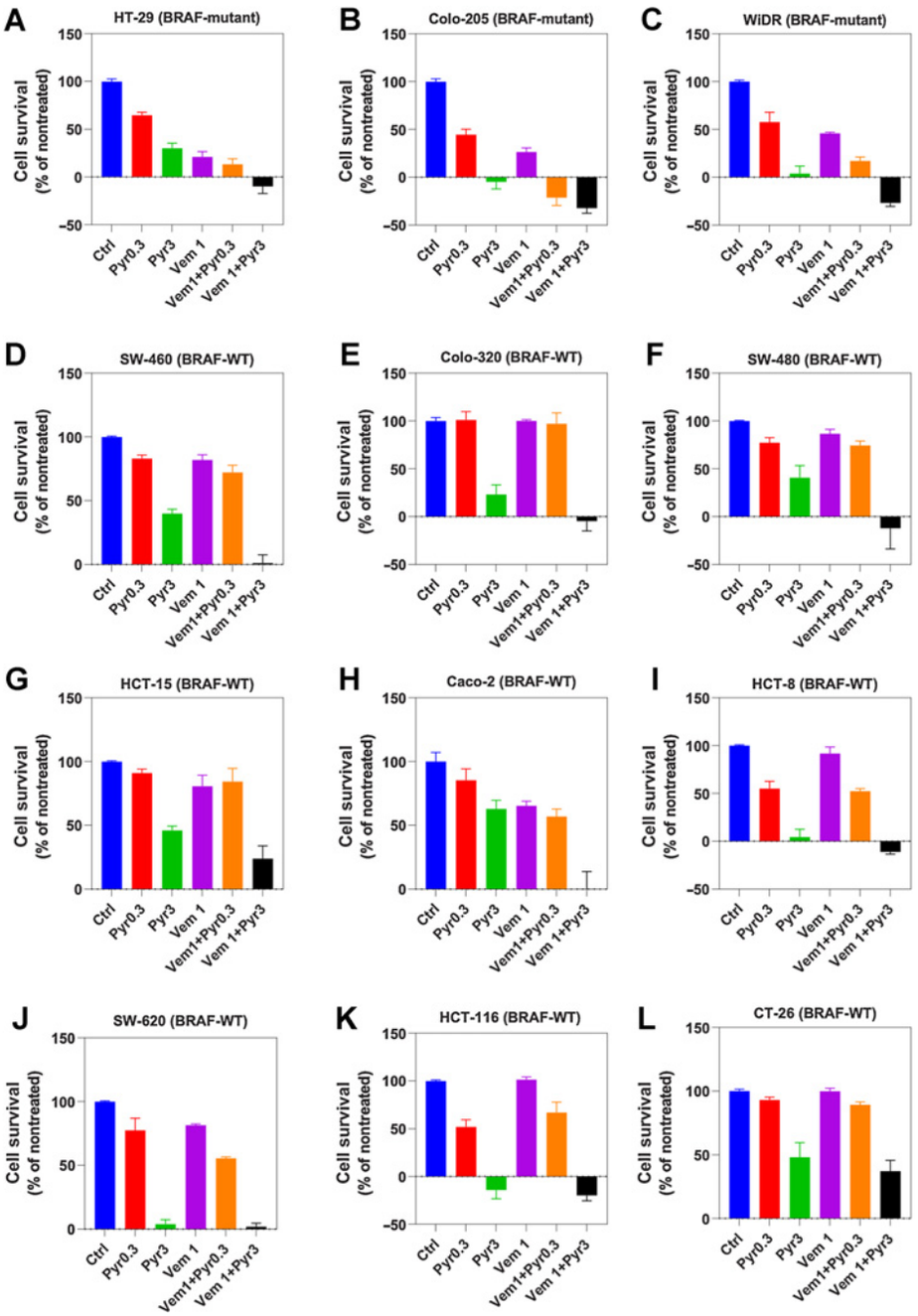

Figure 2.

Effect of cotargeting the BRAF/MEK and WNT/β-catenin signaling pathways in colorectal cancer cell growth. Colorectal cancer cells were seeded in 96-well plates (5,000 cells/well) and treated 24 hours later with vemurafenib (1 μmol/L), pyrvinium (0.3 or 3 μmol/L), and both vemurafenib (1 μmol/L) and pyrvinium (0.3 or 3 μmol/L). After 72 hours of treatment, cell viability was determined by the SRB assay as described in the Materials and Methods. HT-29 cell (**A**), Colo-205 (**B**), WiDR (**C**), SW-460 (**D**), Colo-320 (**E**), SW-480 (**F**), HCT-15 (**G**), Caco-2 (**H**), HCT-8 (**I**), SW-620 (**J**), HCT-116 (**K**), CT-26 (**L**).

pyrvinium can attenuate growth of colorectal cancer cell lines (19, 36, 43). Our finding extends those by showing pyrvinium was more effective than the other two WNT/β-catenin inhibitors tested and was more efficacious in BRAF-mutant colorectal cancer cell lines. However, we find that in all colorectal cancer lines tested, there was an additive or synergistic effect of vemurafenib and pyrvinium when used in combination. Simultaneously targeting WNT/β-catenin pathway and vemurafenib has additive effects that are similar to the combination of cetuximab and vemurafenib in two BRAF-mutant colorectal cancer animal xenograft models. Our experiments show that the effects of the combination *in vivo* are not only directly on proliferation of the colorectal cancer cells but most likely due to a combination of effects on secretion of factors required for tumor viability as a whole from cell types typically found in the tumor microenvironment and the colorectal cancer cell themselves. It is possible that adding pyrvinium may overcome some of the paradoxical tumor-promoting effects of BRAF inhibitors because these inhibitors are known to activate β-catenin (12). It is of note that pyrvinium also potentiates effects of doxorubicin in colorectal cancer animal models (43) further highlighting the benefits of simultaneously targeting two different pathways in colorectal cancer. Pyrvinium is an FDA-approved drug (42), paving an easier pathway for further studies to

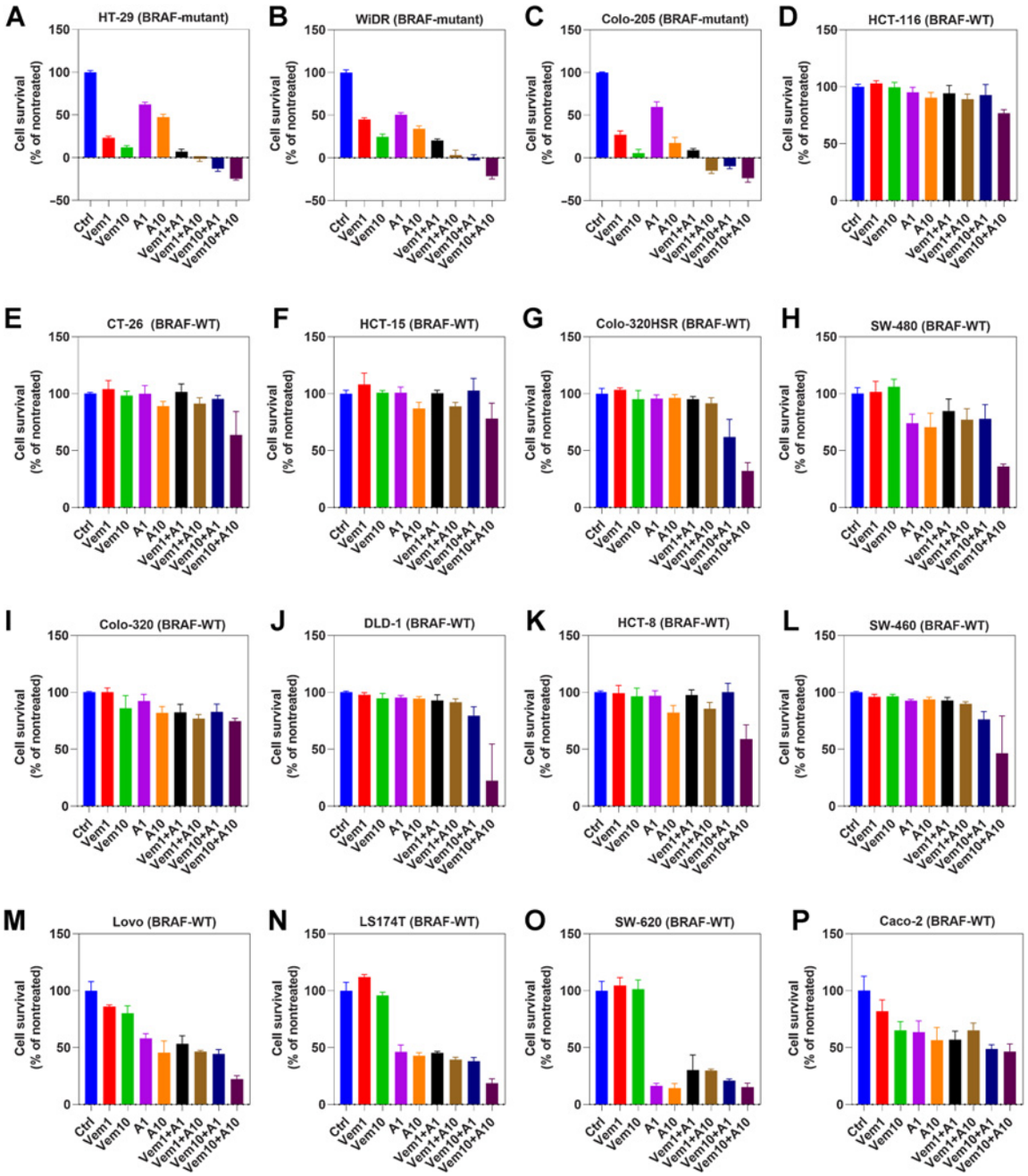

Figure 3.

Effect of cotargeting the BRAF/MEK and VEGFR signaling pathways in colorectal cancer cell growth. Colorectal cancer cells were seeded in 96-well plates (5,000 cells/well) and treated 24 hours later with vemurafenib (1 or 10 μmol/L), axitinib (1 or 10 μmol/L), and both vemurafenib (1 or 10 μmol/L) and axitinib (1 or 10 μmol/L). After 72 hours of treatment, cell viability was determined by the SRB assay as described in the Materials and Methods. HT-29 (**A**), WiDR (**B**), Colo-205 (**C**), HCT-116 (**D**), CT-26 (**E**), HCT-15 (**F**), Colo-320HSR (**G**), SW-480 (**H**), Colo-320 (**I**), DLD-1 (**J**), HCT-8 (**K**), SW-460 (**L**), Lovo (**M**), LS174T (**N**), SW-620 (**O**), Caco-2 (**P**).

examine the effects of pyrvinium in combination therapies for BRAF-mutant colorectal cancer. However, a limitation of using pyrvinium is that it has very poor oral bioavailability (44), which is why intraperitoneal (i.p.) administration was used in our study and in most other studies in animal cancer models (43, 45). However, oral administration of high doses of pyrvinium in mice can achieve measurable amounts to pyrvinium in circulation and achieve efficacy in animal tumor models without significant body weight loss (43, 46). To our knowledge, there have been no clinical trials using WNT/β-catenin pathway together with BRAF inhibitors to treat colorectal cancer, which raises the possibility that a therapeutic window could be identified for use of such treatment in colorectal cancer.

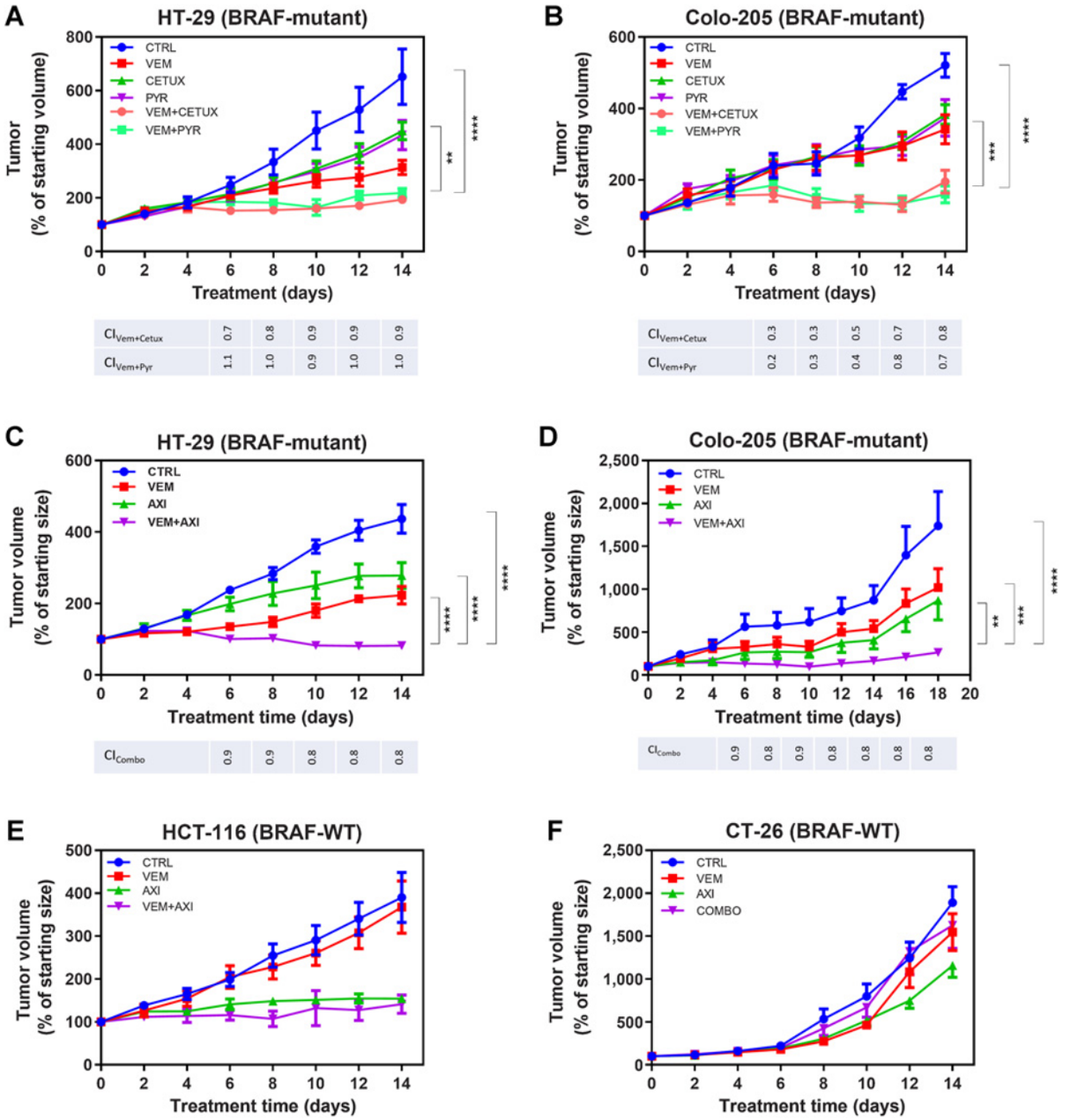

Figure 4.

Effect of the vemurafenib in combination with pyrvinium, cetuximab, or axitinib on BRAF-mutant colorectal cancer xenografts. Either HT-29, Colo-205, or HCT-116 cells were inoculated in the right flank of NIH-III mice (5×10^6 per mouse). Mice were treated daily with vemurafenib (50 mg/kg p.o.), cetuximab (5 mg/kg i.p.), pyrvinium pamoate (1 mg/kg i.p.), or axitinib (10 mg/kg p.o.) either alone or in the combinations indicated. Data were analyzed by a two-way ANOVA with $P < 0.05$ indicating significant difference. Ctrl: control; Vem: vemurafenib; Cetux: cetuximab. HT-29 model with combinations of vemurafenib/cetuximab and vemurafenib/ pyrvinium (**A**), Colo-205 model with combinations of vemurafenib/cetuximab and vemurafenib/ pyrvinium (**B**), HT-29 model with combination of vemurafenib/ axitinib (**C**), Colo-205 model with combination of vemurafenib/axitinib (**D**), HCT-116 model with combination of vemurafenib/axitinib (**E**), Syngeneic CT-26 model with combination of vemurafenib/axitinib (**F**).

VEGF/VEGFR signaling is essential for tumor growth, mainly via regulating the interaction between tumor cells and tumor stroma (13). The potential for cotargeting VEGF signaling in colorectal cancer is well recognized, and the anti-VEGFA antibody bevacizumab added to combination chemotherapy regimens is part of standard-of-care protocols (14, 15). Small-molecule VEGFR receptor inhibitors offer potential benefits over a mAb targeting VEGFA, as it can potentially more broadly target angiogenic pathways. A range of studies have been undertaken to investigate potential therapeutic utility in colorectal cancer of small-molecule agents targeting VEGFR signaling (10).

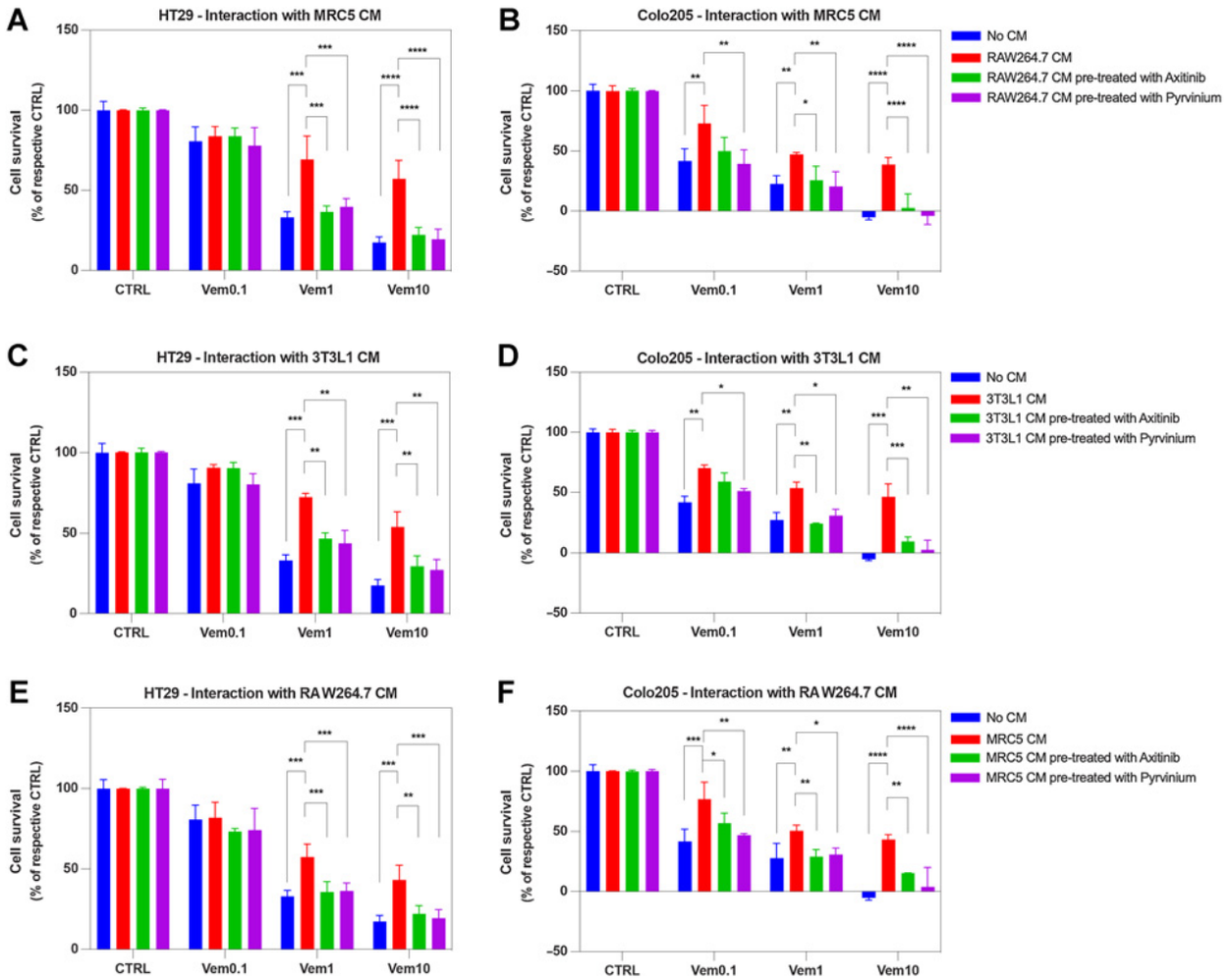

Figure 5.

Axitinib and pyrvinium disrupted the support from stromal cells for BRAF-mutant colorectal cancer cells and thus restored sensitivity of cancer cells to BRAF targeting therapies. Colorectal cancer cells were seeded in 96-well plates (5,000 cells/well) and treated 24 hours later with vemurafenib. Blue bars: treatment was done without any conditioned media from stromal cells. Red bars: treatment was done in the presence of 25% conditioned medium of MRC5 (**A** and **B**), 3T3L1 (**C** and **D**), or RAW264.7 (**E** and **F**). Green bars: treatment was done in the presence of 25% conditioned medium of axitinib-pretreated MRC5 (**A** and **B**), 3T3L1 (**C** and **D**), or RAW264.7 (**E** and **F**). Purple bars: treatment was done in the presence of 25% conditioned medium of pyrvinium-pretreated MRC5 (**A** and **B**), 3T3L1 (**C** and **D**), or RAW264.7 (**E** and **F**).

Overall, these have failed to show significant increased efficacy of the small-molecule inhibitors as single agents. For example, axitinib was not as effective as bevacizumab in combinations with chemotherapy due to increased side effects (47, 48). Although the effects of these combinations were not analyzed separately in BRAF-mutant colorectal cancer and lower doses of axitinib were not explored, results such as this may have dampened interest in using axitinib in combination therapies in colorectal cancer. Nonetheless, there is also evidence that single-agent targeting of VEGFR signaling with either regorafenib (49) or axitinib (50) can achieve improvement in clinical outcomes in patients who become resistant to first-line therapies. Again, how this relates to BRAF-mutant colorectal cancer is not fully understood. In our hands, axitinib's effects on growth of colorectal cancer models *in vitro* was restricted to BRAF-mutant lines. However, axitinib had some effect on growth of all tumor models *in vivo*. This is likely to be because in the tumor microenvironment, VEGFRs act in both tumor cells and other cells such as vascular endothelium, while in cultured cells, only the autocrine mechanism directly on tumor cells is seen. However, in our studies, vemurafenib attenuated tumor growth only in xenografts of BRAF-mutant tumor models, and it acted additively or synergistically with the effects of axitinib. The studies in cultured cells presented here show that this is likely achieved by effects on both the colorectal cancer cells themselves and tumor microenvironment cells, as we see vemurafenib and axitinib combination have additive effects on secretion of survival and angiogenic factors in cell types typical of those found in the tumor microenvironment and also in the BRAF-mutant colorectal cancer cells.

To date, there has been very limited exploration of the combination of BRAF/MEK pathway inhibitor with VEGF inhibitors. A combination trial of vemurafenib with the MEK inhibitor cobimetinib and anti-VEGFA antibody bevacizumab was trialed in metastatic melanoma (51). This triple therapy incurred toxicity, which may be linked to the drug dosages chosen. While vemurafenib was used at the standard therapeutic dosing level, cobimetinib was used at 60 mg, a

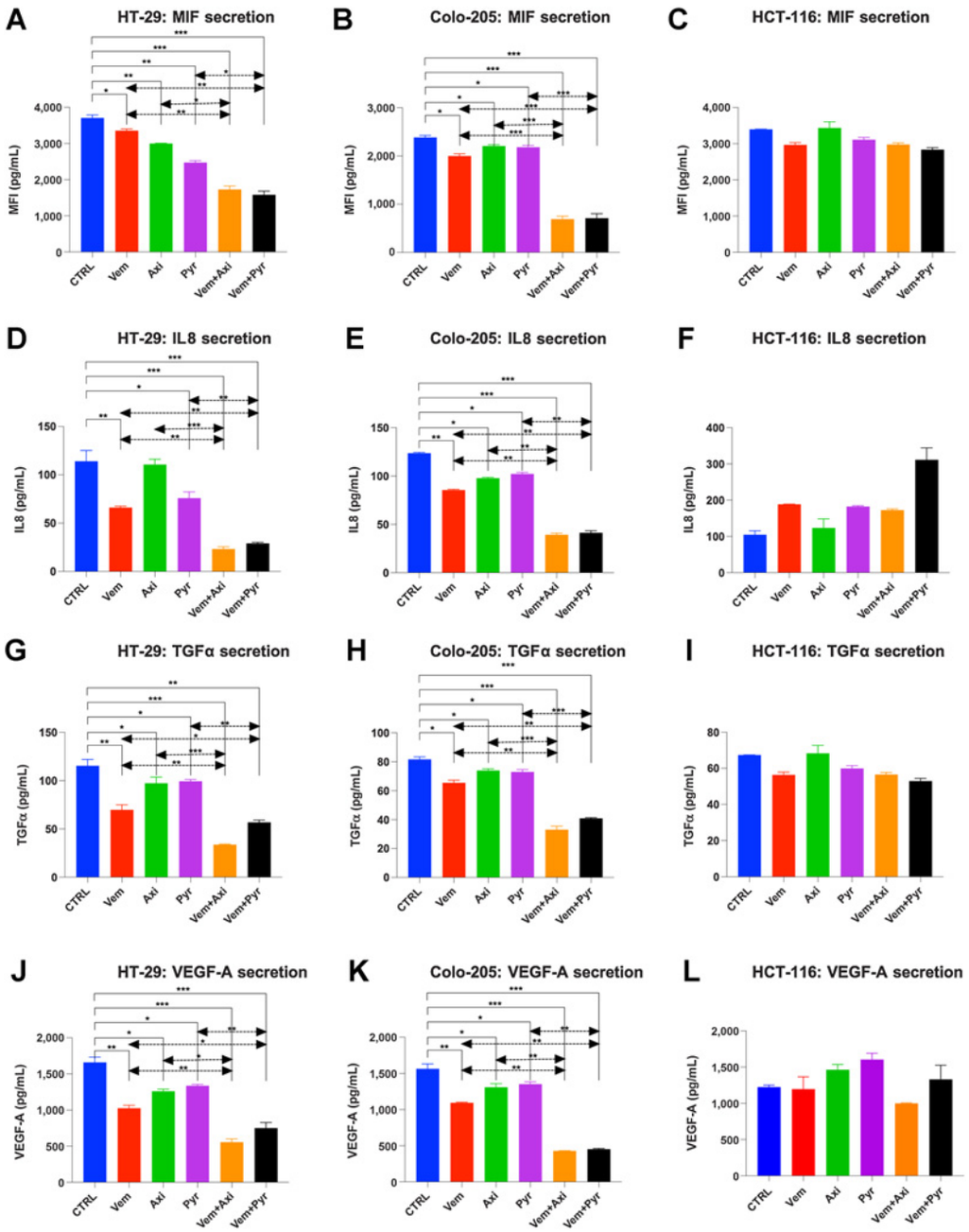

Figure 6.

Enhanced inhibition of cytokine secretion from BRAF-mutant colorectal cancer cells by the combinations. Colorectal cancer cells were seeded in 24-well plates, 2.5×10^5 cells per well, and treated with a single compound (vemurafenib, axitinib, or pyrvinium) or combinations of vemurafenib and axitinib, or vemurafenib and pyrvinium. After 12 hours, the culture supernatant was collected and assayed for MIF, IL8, TGFα, and VEGF-A using immunobead-based assay. Data were normalized on the basis of the cell counts when culture supernatant was collected. Data were presented as mean ± SD; comparison was performed using a one-way ANOVA with *, **, *** indicate *P* value less than 0.05, 0.01, and 0.001, respectively. HT-29/MIF (**A**), Colo-205/MIF (**B**), HCT-116/MIF (**C**), HT-29/IL8 (**D**), Colo-205/IL8 (**E**), HCT-116/IL8 (**F**), HT-29/TGFα (**G**), Colo-205/TGFα (**H**), HCT-116/TGFα (**I**).

dose equating to MTD in phase I trials (52). Bevacizumab was used at a dose of 15 mg/kg, which is significantly higher than the standard dosing used in colorectal cancer clinical trials or routine treatment of 5 to 10 mg/kg (53, 54). A phase I basket trial of a combination of the MEK inhibitor trametinib with pazopanib, a multi kinase inhibitor that targets VEGFR2 and related kinases (55), failed to show efficacy (56), although colorectal cancer was poorly represented in this trial. A question that arises is what value is added by including an MEK inhibitor. Notably, in the BEACON trial, addition of an MEK inhibitor to combinations involving a mutant BRAF inhibitor does not increase

efficacy in BRAF-mutant colorectal cancer (8). This indicates mutant BRAF may be the more important target in combination therapies for BRAF-mutant colorectal cancer. It is therefore of interest that a combination of the mutant BRAF inhibitor dabrafenib and pazopanib achieved indications of efficacy in a phase I basket trial of BRAF-mutant tumors (57). In the studies described here, we show combination efficacy with axitinib, a potent and selective inhibitor of VEGFR1, VEGFR2, and VEGFR3 (26, 55), which suggests the other kinase activities targeted by pazopanib are not required for combination efficacy. While this suggests using axitinib might help reduce side-effect profiles by reduced targeting of kinases such as PDGFR, cKIT, and CSF1R as would be the case with pazopanib, axitinib in itself is known to have toxicities when used at high doses (47, 48, 55). Therefore, in our studies, we also aimed to minimize potential toxicities by using lower doses of axitinib. Preclinical studies using mouse models have shown antitumor activity using axitinib at different doses ranging from 30 to 120 mg/kg (26, 58–63), but here we have used only 10 mg/kg and still retain significant efficacy. A clinical study using conservative doses of axitinib in conjunction with a BRAF-mutant selective kinase inhibitor may thus be warranted in BRAF-mutant colorectal cancer.

In summary, we identify two combinations that have potential to add to the efficacy of BRAF-mutant kinase inhibitors in BRAF-mutant colorectal cancer. Each of these target different mechanisms to cetuximab so these combinations may be useful when resistance to encorafenib/cetuximab combination therapy develops in BRAF-mutant colorectal cancer.

Authors' Disclosures

No potential conflicts of interest were disclosed.

Authors' Contributions

K.B. Tran: Conceptualization, formal analysis, methodology, writing–review and editing. **S. Kolekar:** Formal analysis, investigation, writing–review and editing. **Q. Wang:** Formal analysis, investigation, writing–review and editing. **J.-H. Shih:** Formal analysis, investigation. **C.M. Buchanan:** Supervision, investigation, writing–review and editing. **S. Deva:** Formal analysis, writing–review and editing. **P.R. Shepherd:** Conceptualization, formal analysis, supervision, funding acquisition, validation, visualization, writing–original draft, writing–review and editing.

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Note

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