Modeling Adaptive Resistance of KRAS Mutant Colorectal Cancer to MAPK Pathway Inhibitors with a Three-Dimensional Tumor Model

Pradip Shahi Thakuri, Astha Lamichhane, Sunil Singh, Megha Gupta, Gary D. Luker, and Hossein Tavana*

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ABSTRACT: Single-agent drug treatment of KRAS^{mut} colorectal cancers is often ineffective because the activation of compensatory signaling pathways leads to drug resistance. To mimic cyclic chemotherapy treatments of patients, we showed that intermittent treatments of 3D tumor spheroids of KRAS^{mut} colorectal cancer cells with inhibitors of mitogen-activated protein kinase (MAPK) signaling pathway temporarily suppressed growth of spheroids. However, the efficacy of successive single-agent treatments was significantly reduced. Molecular analysis showed compensatory activation of PI3K/AKT and STAT kinases and EGFR family proteins. To overcome the adaptation of cancer cells to MAPK pathway inhibitors, we treated tumor spheroids with a combination of MEK and EGFR inhibitors. This approach significantly blocked signaling of MAPK and PI3K/AKT pathways and prevented the growth of spheroids, but it was not effective against STAT signaling. Although the combination treatment blocked the matrix invasion of DLD1 cells, additional treatments with STAT inhibitors were necessary to prevent invasiveness of HCT116 cells. Overall, our drug resistance model elucidated the mechanisms of treatment-induced growth and invasiveness of cancer cells and allowed design-driven testing and identifying of effective treatments to suppress these phenotypes.

KEYWORDS: three-dimensional tumor model, colorectal cancer, drug resistance, cyclic treatment regimen, combination treatments

Although promising therapeutics have been developed for colorectal cancers, KRAS^{mut} disease, which accounts for 35–45% of malignant colorectal cancers, continues to have poor prognosis and limited therapy options.^{1,2} Due to the difficulty of therapeutic targeting of KRAS, a treatment strategy extensively evaluated in preclinical tests and pursued clinically has been targeting downstream effectors of RAS using molecular inhibitors of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways. However, cancer cells often adapt to these compounds by activating alternative oncogenic signaling. For example, colorectal cancer cells may develop resistance to single-agent inhibition of the MAPK pathway by activating the PI3K/AKT or JAK/STAT pathway or by activating certain receptor tyrosine kinases (RTKs).^{3,4} KRAS^{mut} cells can also impair the efficacy of RTK inhibitors by bypassing RTKs-driven signaling cascades.⁵ This suggests the need for combination treatment strategies to block the drug resistance of cancer cells. However, dose-limiting toxicities remain a major concern with combination treatments. For example, blocking extensive cross-talk between MAPK and PI3K pathways by combining inhibitors of these pathways significantly inhibited tumor growth but also resulted in excessive toxicity in cancer patients.⁶ Dose reduction is a potential approach to overcome toxicity. Alternatively, several

other combination treatment strategies are being sought. Co-targeting MEK and CDK4/6,^{7,8} MEK and histone deacetylase (HDAC),⁹ and MEK and upstream type-1 insulin-like growth factor receptor (IGF1R) were effective in preclinical models of KRAS^{mut} colorectal cancers.¹⁰

A technological challenge is identifying specific combinations that suppress resistance to single-agent treatments. Preclinical models are important tools to identify resistance mechanisms and design and test the efficacy of novel treatments. Monolayer (2D) cultures of cancer cells are convenient to screen large arrays of drug combinations but lack structural and biological complexities of solid tumors and are incompatible with long-term cultures to reproduce adaptation of cancer cells to drugs.^{11,12} Animal models are extensively used to study the intrinsic and adaptive drug resistance of cancer cells, but the high cost and difficulty of identifying molecular mechanisms of drug resistance in animals and testing arrays of drug combinations remain major drawbacks.¹³

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Three-dimensional (3D) tumor models have shown the potential to bridge this gap between 2D cultures and animal models.^{11,12,14} We have previously shown that 3D tumor spheroids reproduce key biological properties of solid tumors and treatment outcomes.^{15,16} Recently, we demonstrated the utility of this approach to identify the mechanisms of adaptive resistance of colorectal cancer cells to MAPK pathway inhibitors in a clinically relevant regimen.¹⁷ Using a rational design approach, we screened combinatorial arrays of molecular inhibitors of MAPK and PI3K pathways to identify low-dose, synergistic combinations that suppress resistance in long-term cultures.¹⁸ Here, we used this established model to demonstrate that targeting MAPK pathway at different levels, i.e., RAF, MEK, or ERK, suppresses the growth of KRAS^{mut} colorectal tumor spheroids by downregulating p-ERK1/2 signaling. However, the effect was transient and molecular analysis showed that colorectal cancer cells in drug-treated spheroids quickly adapted to the inhibitors and activated AKT, STAT, and EGFR signaling. We identified combinations of MAPK and RTK inhibitors to inhibit MAPK and PI3K pathways cross-talk and synergistically block growth of tumor spheroids. Nevertheless, the treatments were ineffective against STAT signaling that promoted the matrix invasion of cancer cells, emphasizing the utility of our model to capture multiple processes in the tumor microenvironment and the importance of simultaneously targeting them to inhibit tumor growth and invasion. We demonstrated that inhibition of STAT signaling was critical against the invasiveness of cancer cells. Altogether, our approach allows a mechanistic understanding of drug resistance of cancer cells and design-driven testing of different combinations to identify synergistic drugs that block drug resistance and other malignant processes in tumors.

MATERIALS AND METHODS

Cell Culture and Spheroid Formation. Two colorectal cancer cell lines, HCT116 and DLD1, were purchased from ATCC. McCoy's 5A and RPMI-1640 (ATCC) media were used to culture HCT116 and DLD1 cells, respectively. Media were supplemented with 10% fetal bovine serum (FBS, Sigma), 1% streptomycin/penicillin (Life Technologies), and 1% glutamine (Life Technologies). Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. Cells were dissociated using 0.25% trypsin (Life Technologies) from 80 to 90% confluent monolayer cultures. Trypsin was neutralized using the complete growth media. Each cell suspension was centrifuged down at 1000 rpm for 5 min, and after removing the supernatant, cells were suspended in 1 mL of culture medium and counted using a hemocytometer prior to spheroid formation. Spheroids were formed with 1.5×10^4 cells using our aqueous two-phase system (ATPS) microtechnology in ULA 384-well plates (Corning).^{19–21} We have previously shown that the size of colorectal tumor spheroids made with this approach is within ~5% of an average diameter across a microwell plate.^{22,23}

Dose-Dependent Screening of Molecular Inhibitors against Tumor Spheroids. Twelve therapeutic compounds were purchased from Selleckchem: trametinib, PD0325901, selumetinib, dabrafenib, sorafenib, AZ628, GDC0994, SCH772984, ulixertinib, neratinib, sapitinib, and lapatinib. All compounds were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were stored in –80 °C. All compounds were tested in a dose-dependent manner against spheroids of HCT116 and DLD1 cells. All compounds were prepared in a

concentration range of 2×10^{-3} – 2×10^1 μ M, except for SCH772984 that was prepared in a concentration range of 1×10^{-3} – 2×10^0 μ M. The drug solutions were prepared twice the final concentrations for testing against tumor spheroids. The media volume in the microwells was measured, and an equal volume of the drug solutions was added. This addition reduced the drug concentrations in half to the desired concentrations. Vehicle control spheroids were grown in cell culture medium. A total of 14 replicates was used for both control (nontreated) and drug-treated spheroids. After 4 days of drug treatment, PrestoBlue (Life Technologies) was added to wells. After 4 h of incubation, the fluorescence signal was measured with a plate reader (Synergy H1M, BioTek Instruments). The fluorescence signal from each treatment was normalized to that of the vehicle control to determine percent viability of drug-treated spheroids. GraphPad Prism 5 was used to fit a four-parameter sigmoidal dose–response curve to the viability data. Area under the curve (AUC) and concentration of each compound that reduces proliferation of cells by 50% (GI₅₀) were computed from the respective dose–response curve.

Cyclic Treatments of Spheroids with Inhibitors and Recovery from Treatments. Trametinib (MEK inhibitor), SCH772984 (ERK1/2 inhibitor), and AZ628 (pan-Raf inhibitor) were used at their GI₅₀ concentrations to cyclically treat spheroids of HCT116 and DLD1 cells: 10 nM trametinib, 150 nM SCH772984, and 1 μ M of AZ628 with HCT116 spheroids and 35 nM trametinib, 700 nM SCH772984, and 10 μ M AZ628 with DLD1 spheroids. Each experiment included four cycles of treatment separated by three cycles of recovery. The treatments were designated as T1, T2, T3, and T4, whereas the recovery periods were designated as R1, R2, and R3. Each treatment and recovery phase lasted 4 days, resulting in a total treatment time of 28 days. Each treatment phase included drug addition to the spheroids at the beginning only. At the end of each treatment phase, drug solutions were thoroughly removed from the wells and spheroids were rinsed with PBS. Then, culture medium was added to allow the spheroids recover from the treatments. The same concentration of each drug was used throughout the treatment periods. At the end of each treatment and recovery cycle, the size of spheroids was measured. To quantify the resistance of HCT116 spheroids to each inhibitor, a growth rate metric (k_c) was defined as the difference in the size of spheroids after and before each treatment. We have previously shown that reduction in the size of spheroids treated with molecular inhibitors strongly correlates with cell viability of spheroids.²²

Western Blotting. Western blot analysis with spheroids was performed using our established protocol with spheroids.^{17,22} Primary antibodies were purchased from Cell Signaling Technology against the following: phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), phospho-Akt (Ser473), Akt (pan) (C67E7), phospho-STAT1 (Tyr701), phospho-SAT6 (Tyr641), and β -actin (13E5). Solutions of primary antibodies were prepared at concentrations recommended by the manufacturer. Membranes were incubated overnight at 4 °C with primary antibody solutions. After repeated washing, membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h, followed by repeated washing. Detection was carried out using an ECL chemiluminescence detection kit (GE Healthcare) with a FluorChem E imaging system (ProteinSimple).

Phospho-RTK Array. A human phospho-RTK dot blot array (ARY001B; R&D Systems) was used to simultaneously

Figure 1. Inhibition of MAPK pathway in colorectal tumor spheroids. Dose–response curves of (a) HCT116 and (b) DLD1 spheroids treated with MAPK pathway inhibitors. Each data point is an average of 14 replicates. Ranking of the efficacy of the inhibitors according to the area under the curve (AUC) analysis for spheroids of (c) HCT116 and (d) DLD1 cells. (e) GI₅₀ values of MAPK inhibitors against HCT116 and DLD1 spheroids. The symbol ‘-’ indicates a GI₅₀ value could not be obtained.

detect relative phosphorylation levels of 49 RTKs. An equal amount of cell lysate (300 μ g) from each experimental sample was used. The array was visualized using a FluorChem E Imaging system. All arrays of each experiment were exposed simultaneously. An adequate exposure time was selected to capture differences in activities of receptors between vehicle control and treatment groups. A pixel density module in ImageJ (NIH) was used to quantify phosphorylated levels of the proteins. The pixel density of the background signal was subtracted from the average of the measured signal of a pair of dots for each protein on the array. The phosphorylated level of each protein in a treated group was determined by normalizing it with the pixel density value in the respective vehicle control group.

Combination Treatments of Spheroids. Seven concentration pairs of trametinib and neratinib were used. Solutions of 4 $\times$ concentrations of GI₅₀ from each compound were prepared. From these solutions, 20 μ L of trametinib solution and 20 μ L of neratinib solution were added to each well containing a spheroid in 40 μ L of cell culture medium to dilute each compound four folds. In addition, single-agent treatments

with trametinib and neratinib were performed to compare with the combination treatment. For single-agent treatments, solutions of 2 $\times$ concentrations of GI₅₀ from each compound were prepared, and 40 μ L of one of the solutions was added to a microwell containing a spheroid in 40 μ L of cell culture medium. Vehicle control spheroids were maintained in the cell culture medium. Treatments were done for 4 days and viability of cells was quantified using a PrestoBlue assay. The fraction of cells affected by each treatment was calculated as (1 – viability). A synergy analysis was performed for combination treatments using the Chou and Talalay method,²⁴ which generated seven combination indices (CI) for the seven combined concentrations.

Long-Term Cyclic Combination Treatments of Spheroids. Of the trametinib/neratinib pairs, 35 nM/200 nM and 10 nM/500 nM concentrations were used against DLD1 and HCT116 spheroids, respectively. In parallel, spheroids were treated with trametinib or neratinib only. The long-term effectiveness of the treatments was assessed by comparing the growth rates of spheroids (k_c) under combination and single-agent treatments during T1–T4. k_c values for control, single-

Figure 2. Modeling drug resistance of colorectal cancer spheroids to cyclic treatments with MAPK pathway inhibitors. Growth kinetics of (a) HCT116 and (c) DLD1 spheroids cyclically treated with GI₅₀ concentrations of MAPKi (trametinib, SCH772984, AZ628) for four treatment cycles T1, T2, T3, and T4 with recovery intervals R1, R2, and R3. Each data point in the line graphs is an average of 32 replicates. Growth rates of (b) HCT116 and (d) DLD1 spheroids during four cycles of treatments with the MAPKi. * denotes statistically significant differences in the growth rates between treatment rounds. Error bars represent standard errors.

agent treatments and combination treatments were calculated by subtracting the volume of spheroids at the end of the 28 day treatments and the volume of the spheroids at the beginning of experiments.

Invasion Assay. Of the trametinib/neratinib pairs, 35 nM/200 nM and 10 nM/500 nM concentrations were used against DLD1 and HCT116 spheroids, respectively. In parallel experiments, spheroids were treated with trametinib or neratinib only. After 4 days, spheroids were recovered and suspended in a 4 mg/mL solution of type I rat tail collagen (Corning). Collagen gelation depends on pH and temperature. A stock solution of collagen with a concentration of 8.56 mg/mL dissolved in 0.02 N acetic acid was diluted to the desired concentration using the manufacturer's protocol. For example, to prepare 1 mL of 4 mg/mL collagen solution, 467 μ L of stock collagen solution was mixed with 100 μ L 10 $\times$ DMEM medium (Sigma) and 422 μ L of sterile distilled water, and then the pH was adjusted to a neutral level using 11 μ L of 1 N NaOH solution. All the reagents were kept on ice during collagen preparation to maintain the temperature at 4 $^{\circ}$ C and prevent premature gelation of collagen. Incubation at 37 $^{\circ}$ C for 30 min resulted in gelation of collagen. Invasion of cells from spheroids into the matrix was captured using an Axio Observer microscope (Zeiss) with a 10 $\times$ objective on days 3 and 5. Cell invasion was quantified by normalizing the invasion pixel area with the spheroid pixel area. In a separate experiment, HCT116 spheroids were pretreated for 4 days with 10 nM/500 nM trametinib/neratinib combination, recovered, embedded in a 4 mg/mL collagen solution, and treated with 10 μ M of

STAT6 (AS1517499) or STAT1 (fludarabine) inhibitors. In parallel, collagen-embedded spheroids were maintained in drug-free medium to evaluate the inhibitory effects of STAT inhibitors on matrix invasion of cancer cells.

RESULTS

Screening of Inhibitors of MAPK Pathway. HCT116 and DLD1 cells have KRAS and PIK3CA mutations that activate oncogenic MAPK and PI3K/AKT pathways.²⁵ We previously demonstrated that single-agent inhibition of MAPK pathway more effectively blocked the growth of colorectal tumor spheroids than using a PI3K/AKT pathway inhibitor,^{17,18} suggesting dependency of the cells on MAPK signaling to proliferate. This prompted us to screen several molecular inhibitors that target various levels of MAPK pathway, i.e., RAF, MEK, and ERK. We screened nine different MAPK inhibitors (MAPKi) against HCT116 and DLD1 spheroids. The inhibitors dose-dependently reduced cell viability of spheroids but showed significant differences in effectiveness (Figure 1a,b). Our AUC analysis of dose-responses resulted in the lowest AUC values of 0.41 and 0.45 with trametinib treatments of HCT116 and DLD1 spheroids, respectively (Figure 1c,d). The other two MEK inhibitors, PD0325901 and selumetinib, resulted in the second and third lowest AUC values of 0.45 and 0.60 for HCT116 spheroids and 0.51 and 0.75 for DLD1 spheroids. This indicates that the cancer cells were more sensitive to inhibition of MEK than its upstream or downstream kinases. Additionally, the ERKi were more effective than the RAFi (Figure 1c,d). On the basis of

Figure 3. Activity levels of AKT, STAT, and RTKs in MAPKi-treated colorectal tumor spheroids. Phosphorylated and total levels of AKT and quantified values of p-AKT/t-AKT in (a) HCT116 and (b) DLD1 spheroids at the end of T1 phase. Phosphorylated levels of STAT1 and STAT6 and quantified value of p-STAT1/β-actin and p-STAT6/β-actin in (c) HCT116 and (d) DLD1 spheroids at the end of T1 phase. Phospho-RTKs in (e) HCT116 spheroids under no treatment (control) or treatment with 10 nM trametinib, 150 nM SCH772984, and 1×10^3 nM AZ628, and (f) DLD1 spheroids under no treatment (control) or treatment with 35 nM trametinib, 700 nM SCH772984, and 1×10^3 nM AZ628. The bar graphs show pixel density of each phospho-protein from treatments normalized with the respective vehicle control. Error bars represent standard errors. Each Western blot experiment was repeated twice. * denotes $p < 0.05$ when comparing each treatment and the vehicle control.

these results, we selected trametinib, SCH772984, and AZ628 as the most effective MEKi, ERKi, and RAFi, respectively, against both cell lines. The GI_{50} values of the compounds with both cells are shown in Figure 1e. Thus, we used single-agent

treatment of colorectal tumor spheroids with these three inhibitors to model adaptive resistance to MAPKi.

Long-Term Cyclic Treatments of Spheroids with MAPK Pathway Inhibitors. To demonstrate treatment-

induced resistance of colorectal cancer cells to MAPKi, we evaluated responses of HCT116 and DLD1 spheroids to long-term cyclic treatments with the respective GI_{50} concentration of each compound. Trametinib, SCH772984, and AZ628 potentially inhibited growth of HCT116 cells during the first treatment round (T1) and decreased the size of spheroids by 1.80, 1.42, and 1.67 fold, respectively (Figure 2a). However, after the first recovery (R1), the inhibitors were significantly less effective, and despite treatments, the size of spheroids increased. At the end of T4, HCT116 spheroids treated with trametinib, SCH772984, and AZ628 were, respectively, 3.17-, 2.91-, and 1.90-fold larger than those at the end of T1. Furthermore, we used a growth rate metric (k_c) to quantify the effects of treatments (Figure 2b). k_c for HCT116 spheroids from T1 to T4 significantly increased in the range of -0.0061 to 0.0021 mm^3/day for trametinib treatment, -0.0040 to 0.0052 mm^3/day for SCH772984 treatment, and -0.0050 to 0.0027 mm^3/day for AZ628 treatment.

To establish that this adaptive resistance was not specific to a cell line, we repeated the cyclic treatments with DLD1 spheroids. The size of DLD1 spheroids at the end of T1 decreased by 1.84-, 1.41-, and 1.71-fold with trametinib, SCH772984, and AZ628 treatments, respectively (Figure 2c), but cyclic treatments with the MAPKi led to resistance. At the end of T4, DLD1 spheroids treated with trametinib, SCH772984, and AZ628 were respectively 2.46-, 2.80-, and 2.71-fold larger than those after T1. The k_c values of DLD1 spheroids from T1 to T4 significantly increased from -0.0054 to 0.0048 mm^3/day for trametinib treatment, from -0.0033 to 0.0029 mm^3/day for SCH772984 treatment, and from -0.0049 to 0.0058 mm^3/day for AZ628 treatment (Figure 2d). The significant decrease in the efficacy of the MAPKi indicates that colorectal cancer cells develop adaptive responses to inhibition of signaling (RAF, MEK, and ERK) in this pathway. Importantly, our drug resistance model reliably emulated several *in vivo* studies that showed reduced efficacy of MEK1/2 inhibitors during cyclic treatments of tumor xenografts.^{26,27}

Molecular Analysis of Adaptive Resistance. Next, we investigated the underlying molecular mechanisms of resistance of colorectal cancer cells to MAPKi by performing a comprehensive molecular analysis of PI3K pathway, JAK/STAT pathway, and upstream RTKs.

PI3K/AKT Kinases. Cross-talk between MAPK and PI3K pathways is common in many cancers.^{28–30} We previously showed that MEK1/2 inhibition of BRAF^{mut} HT-29 spheroids leads to activation of the PI3K pathway.¹⁷ Thus, we asked whether targeting MAPK pathway at different levels in KRAS^{mut} colorectal cancer cells still generates this effect. Quantifying AKT kinase activity following MAPKi treatments of HCT116 and DLD1 spheroids showed significant AKT phosphorylation only after 4 days of treatment with MEKi, ERKi, and RAFi (Figure 3a,b). Trametinib, SCH772984, and AZ628 treatments respectively increased p-AKT levels in HCT116 cells by 1.95-, 2.33-, and 2.19-fold (Figure 3a), and in DLD1 cells by 11-, 9-, and 10-fold (Figure 3b). This established that suppressing MAPK pathway at various levels induces feedback activation of PI3K pathway in these cells.

STAT Kinases. JAK/STAT pathway activation is a potential mechanism of resistance to MEKi.³ This prompted us to study potential changes in the JAK/STAT signaling pathway following MAPKi treatments of HCT116 and DLD1 spheroids. Our Western blot analysis showed activation of

STAT proteins. Trametinib, SCH772984, and AZ628 treatments respectively increased p-STAT1 level by 5.14-, 4.14-, and 3.45-fold and p-STAT6 by 3.07-, 2.75-, and 4.25-fold in HCT116 cells (Figure 3c) and p-STAT6 level by 2.33-, 1.1-, and 2.2-fold in DLD1 cells (Figure 3d).

Receptor Tyrosine Kinases (RTKs). Feedback activation of AKT and STAT kinases suggests a potential role for these pathways in colorectal cancer cells to survive MAPK pathway inhibition. It has been reported that suppressing the RAF/MEK/ERK cascade may activate upstream RTKs in colorectal cancers,^{3,4,31} which in turn can activate PI3K/AKT and JAK/STAT pathways. To determine which RTKs may be activated following MAPK pathway inhibition, we used a phospho-RTK dot blot array to quantify relative phosphorylation levels of 49 different RTKs in colorectal cancer spheroids. The blot arrays for vehicle control and MAPKi-treated HCT116 and DLD1 spheroids are shown in Figure 3e,f. We quantified phosphorylation levels of each protein from a treatment group with respect to the respective vehicle control group. This analysis showed significant upregulated activities of epidermal growth factor receptors EGFR, HER2, and HER3 following MAPKi treatments. All three EGFR proteins showed greater activities in HCT116 cells following inhibition of RAF, MEK, or ERK. Trametinib, SCH772984, and AZ628 treatments of HCT116 spheroids significantly increased phosphorylated EGFR by 3.01-, 4.13-, and 4.11-fold, HER2 by 1.70-, 1.60-, and 1.70-fold, and HER3 by 1.29-, 1.06-, 1.16-fold, respectively. In DLD1 spheroids treated with trametinib, SCH772984, and AZ628, phosphorylated EGFR increased by 1.11-, 1.23-, and 1.14-fold and phosphorylated HER3 increased by 1.39-, 1.35-, and 1.19-fold, respectively. Activity of HER2 increased only after trametinib treatment of DLD1 spheroids by 1.27-fold. SCH772984 and AZ628 did not alter HER2 activity in DLD1 cells. Significant upregulation of other RTKs such as c-MET following MEK inhibition has been shown to activate STAT3 signaling to promote tumors growth and MEKi resistance in colorectal cancer.³ However, we did not observe c-MET activity following MAPK pathway inhibition.

Short-Term Combination Treatments with MAPKi and EGFRi. Single-agent inhibition of the MAPK pathway upregulated activities of AKT and STAT kinases and the upstream receptors EGFR, HER2, and HER3. These RTKs often initiate signaling through multiple pathways including PI3K/AKT and JAK/STAT.^{32,33} Although AKT was activated in both colorectal cancer cell lines following MAPK pathway inhibition, activation of STAT kinases was cell-line-dependent. Therefore, we asked whether targeting upstream RTKs along with MAPK pathway would be effective against feedback activation of these signaling pathways to overcome adaptive resistance to MAPKi. We showed that colorectal cancer cells were more sensitive to the MEKi than either RAFi or ERKi (Figure 1). Additionally, resistance to the MEKi occurred slower than that to the RAFi or ERKi (Figure 2). This suggested combining trametinib (MEKi) with an EGFRi to block growth of colorectal tumor spheroids. First, we tested three EGFRi against HCT116 and DLD1 spheroids (Figure S1). Neratinib and sapatinib, but not lapatinib, dose-dependently reduced viability of DLD1 spheroids. With HCT116 spheroids, except for neratinib that showed a minimal effect, the other two EGFRi were ineffective. On the basis of these results, we selected neratinib as the EGFRi for combination treatments.

Figure 4. Inhibition of MEK1/2 and EGFR in colorectal tumor spheroids. Combination treatments of (a) HCT116 and (b) DLD1 spheroids with trametinib and neratinib and the respective single-agent treatments. AUC values from combination and single-agent treatments of (c) HCT116 and (d) DLD1 spheroids. Synergy plots show combination index (CI) versus fraction of cells affected (Fa) by each concentration pair in (e) HCT116 and (f) DLD1 spheroids.

Figure 5. Molecular effects of simultaneous inhibition of MEK1/2 and EGFR in colorectal tumor spheroids. Combinations of trametinib and neratinib downregulate p-ERK1/2 and p-AKT in (a) HCT116 and (b) DLD1 spheroids but are ineffective against (c) p-STAT1 and p-STAT6 in HCT116 and (d) p-STAT6 in DLD1 cells.

Following both single-agent and combination treatments of HCT116 and DLD1 spheroids (Figure 4a,b), we computed the AUC values to quantitatively compare efficacy of the treatments. Combined use of trametinib and neratinib with HCT116 spheroids resulted in an AUC value of 0.50, which was significantly lower than those with the respective single-agent treatments (Figure 4c). Similarly, with DLD1 spheroids, the combination treatment significantly reduced the AUC value to 0.44 (Figure 4d). We computed a combination index (CI) to determine synergism of combined trametinib and

neratinib treatments. $CI < 1$ indicates synergism, and the synergy level increases as CI approaches zero.^{23,24} Except for the lowest pair of concentrations with both cell lines, all other pairs resulted in $CI < 1$, indicating synergistic effects of the two drugs to block signaling that promotes cancer cell proliferation (Figure 4e,f). Next, we studied molecular effects of simultaneous inhibition of MEK1/2 and EGFR on MAPK, PI3K/AKT, and STAT pathways. We used three pairs of concentrations with spheroids of each cell line based on the dose-response results (Figure 4a,b). The combination treat-

Figure 6. Long-term combination and single-agent treatment/recovery of colorectal tumor spheroids. Size of (a) HCT116 and (b) DLD1 spheroids under cyclic treatments with trametinib, neratinib, and their combination. Each data point in the graphs is an average of 32 replicates. Error bars represent standard errors. * on the single-agent treatments represents a statistically significant difference with the corresponding combination drug treatments ($p < 0.05$).

ments very effectively downregulated AKT and ERK activities at all three concentration pairs in HCT116 and DLD1 spheroids (Figure 5a,b). However, STAT kinases still remained active (Figure 5c,d). The results suggest that co-inhibition of AKT and ERK accounts for the synergy between trametinib and neratinib. In addition, either insufficient inhibition of EGFR by neratinib or feedback activation of other RTKs may lead to the sustained STAT signaling.

Long-Term, Cyclic Combination Treatments with MAPKi and EGFRi. We established above that combinations of trametinib and neratinib act synergistically in short-term, 4 day experiments. Next, we evaluated the effectiveness of this approach during long-term cyclic treatments on proliferative activities of colorectal cancer cells and compared it to the respective single-agent treatments. We selected concentrations of the two compounds based on the analysis of both dose–response (Figure 4) and protein activity (Figure 5). Combination of trametinib and neratinib significantly and effectively suppressed the growth of HCT116 and DLD1 spheroids over a 16 day period compared to the respective single-agent treatments (Figure 6a,b). To quantitatively compare these treatments, we calculated the growth rate of spheroids. The k_c values for trametinib, neratinib, and their combination were respectively -0.00068 , -0.00019 , and -0.00150 mm^3/day against HCT116 spheroids and -0.00014 , 0.00022 , and -0.00215 mm^3/day against DLD1 spheroids. The k_c values for the combination treatments being significantly more negative than those for the respective single-agent treatments indicates a continuous growth suppression of tumor spheroids. The results suggest that deregulated activities of MAPK and PI3K/AKT pathways, but not STAT pathway, drives proliferation of HCT116 and DLD1 cells and that simultaneous blocking of these two pathways is sufficient to inhibit cancer cell proliferation.

Drug Combination against Matrix Invasion of Cancer Cells. Although combined trametinib and neratinib suppressed growth of free-floating spheroids of HCT116 and DLD1 cells by blocking ERK and AKT activities, it was not effective against elevated activities of STAT proteins (Figure 5c,d). We hypothesized that this could promote an invasive phenotype in cancer cells despite their suppressed growth.^{34–36} To test this hypothesis, we first treated HCT116 and DLD1 spheroids with neratinib, trametinib, or their combination for 4 days and

maintained a group of spheroids untreated. Then we recovered the spheroids from the treatments and embedded them in a collagen hydrogel to examine matrix invasion of cancer cells from spheroids. To account for the size differences of drug treated and control spheroids, we quantified the invasion area relative to the spheroid area. After 5 days, vehicle control and neratinib-pretreated HCT116 spheroids showed only minimal matrix invasion. In contrast, spheroids pretreated with trametinib or the combination led to 29.4- and 28.8-fold more collagen invasion than did the vehicle control spheroids (Figure 7a,b). With DLD1 spheroids, pretreatment with neratinib and the drug combination was effective against cell invasion (Figure 7c,d).

STAT Inhibition Prevents Matrix Invasion of HCT116 Cells. The trametinib/neratinib combination did not prevent matrix invasion of HCT116 cells (Figure 7a,b), nor was it effective against activation of STAT1 and/or STAT6 (Figure 5c). We asked whether these STAT proteins promote invasiveness of HCT116 cells. To explore this question, we treated HCT116 spheroids with trametinib/neratinib pairs for 4 days and then recovered and embedded them in a collagen matrix. We treated the cultures with fludarabine (Selleckchem), an inhibitor of STAT1, or AS1517499 (Selleckchem), an inhibitor of STAT6. Spheroids not treated with a STAT inhibitor readily invaded the collagen matrix (Figure 7e). Compared to the vehicle control condition, STAT6 inhibition using AS1517499 reduced the matrix invasion of the cells by 14.60-fold (Figure 7f,h), and STAT1 inhibition using fludarabine reduced the matrix invasion of cells by 4.36-fold (Figure 7g,h). This experiment validated that the STAT signaling promotes an invasive phenotype in HCT116 cells.

DISCUSSION

Our study showed that single-agent inhibition of MAPK pathway is significantly more effective than single-agent inhibition of PI3K pathway in colorectal cancer cells with KRAS and PIK3CA mutations.^{17,18,37} Although EGFR drives both MAPK and PI3K signaling pathways, inhibition of EGFR, HER2, or HER3 was either less effective than inhibition of the downstream pathways or completely ineffective (Figure S1). This indicates at best a partial response of KRAS^{mut} colorectal cancer cells to EGFRi, consistent with other studies.^{38,39} The

Figure 7. STAT signaling mediated matrix invasion of colorectal cancer cells from tumor spheroids. Spheroids of (a) HCT116 and (c) DLD1 were treated with trametinib (10 nM), neratinib (500 nM), or their combination for 4 days and then recovered and embedded in a collagen matrix for 5 days. Cell invasion has been marked with red lines in the images. (b, d) Quantified invasion area/spheroid area for vehicle control and pretreated spheroids are shown. Representative images of collagen invasion of (e) nontreated, (f) AS1517499-treated, and (g) fludarabine-treated spheroids. (h) Quantified invasion area/spheroid area for nontreated and treated spheroids. Error bars represent standard errors. $n = 4$. “ns” indicates no significant difference and $p < 0.001$ represents statistical difference at a 99.9% confidence interval. Scale bar is 300 μm . *, $p < 0.001$

effectiveness of MAPKi suggests that MAPK pathway is the primary driver of proliferation in these cells. However, blocking this pathway only generated a transient antiproliferative effect, and cells quickly activated adaptive responses to treatments with RAFi, MEKi, and ERKi (Figure 2). This is consistent with studies that showed intermittent treatment of KRAS^{mut} tumor xenografts with MEKi was not effective against tumor growth.^{26,27}

We previously showed that single-agent MEK1/2 inhibition transiently downregulates pERK and significantly shrinks the size of BRAF^{mut} colorectal tumor spheroids, but it results in feedback activation of AKT.^{17,18} In this study, we demonstrated that blocking RAF, MEK, or ERK in KRAS^{mut} colorectal cancer cells also leads to AKT activity (Figure 3). These results suggest that compensatory signaling of PI3K/AKT pathway is a common mechanism of resistance to MAPKi

in colorectal cancer. In addition, we observed significant activation of STAT kinases following MAPK pathway inhibition (Figure 3c,d). Unlike studies that showed activation of STAT3 in colorectal cancer cells following MAPKi treatments,^{3,40} our results showed phosphorylation of STAT1 and/or STAT6.

PI3K/AKT and JAK/STAT pathways have been implicated in growth, survival, and metastasis of colorectal cancers.^{36,41–45} Blocking activities of either of these pathways along with the MAPK pathway has shown promising antitumor effects in colorectal cancer.^{40,46–48} However, this approach may also cause excessive toxicity to patients, as shown with combinations of MAPK and PI3K inhibitors in several cancers including colorectal cancer.⁶ Although dose reduction is a potential strategy to mitigate toxicity,⁴⁹ feedback activation of multiple pathways in addition to the constitutively active

MAPK pathway limits the effectiveness of dual combination treatments. Considering that activation of these intracellular pathways is often driven by specific RTKs, a potential strategy to address this issue is combining RTKi and MAPKi. On the basis of this rationale, our screening for 49 phospho-RTKs showed only significant upregulation of EGFR, HER2, and HER3 in the MAPKi-treated colorectal cancer spheroids (Figure 3). Treatments with MAPKi significantly activated EGFR and HER2 in HCT116 cells and moderately activated EGFR and HER3 in DLD1 cells (Figure 3e,f). This difference is potentially due to genetic differences of these cell lines including in PIK3CA mutation. DLD1 cells carry PIK3CA exon 9 mutations (p.E54S K, p.D549N), whereas HCT116 cells carry PIK3CA exon 20 mutations (p.H1047R).²⁵ A previous study also showed this is responsible for the differential response of these cells to anti-EGFR therapy using cetuximab.⁵⁰ Due to the role of the EGFRs in activating MAPK, PI3K, and JAK/STAT pathways,^{51,52} we evaluated the efficacy of simultaneous inhibition of EGFR and MAPK pathway. Combinations of EGFRi and MEKi synergistically inhibited growth of colorectal tumor spheroids both during short-term continuous treatment and long-term cyclic treatment (Figures 4 and 6). This is consistent with the effectiveness of this strategy on growth inhibition of colorectal tumors *in vivo*.^{53–55} Considering that our molecular analysis showed suppression of ERK and AKT by the combination treatment, the results indicate that co-inhibition of MAPK and PI3K/AKT pathways by neratinib and trametinib effectively and synergistically blocks proliferation of cancer cells and growth of spheroids.

However, this approach did not block STAT1 and/or STAT6 activities, suggesting that signaling other than EGFR activates STAT1 and/or STAT6. The STAT kinases did not appear to have a role in cancer cell proliferation, but several studies have shown a role for STAT proteins in promoting invasiveness of colorectal cancer cells.^{34–36} Our analysis showed a cell-line-dependent effect of upregulated STAT activity on cell invasion. Combination of MEKi and EGFRi suppressed matrix invasion of DLD1 spheroid. Our results suggest that inhibition of invasion is driven by a neratinib effect against the relatively high basal level activities of EGFR proteins in DLD1 cells (Figure 4f) and that combined trametinib/neratinib treatment is necessary to block both cell proliferation (Figure 6b) and invasion (Figure 7c). We also note that upregulated pSTAT6 in trametinib/neratinib-pretreated DLD1 spheroids did not affect cell proliferation or invasion, suggesting that DLD1 rely on signaling through MAPK and EGFR for proliferation and invasion. This is in contrast to a study that showed EMT and migration of HT-29 (BRAF^{mut}) and SW480 (KRAS^{mut}) colorectal cancer cells due to IL-13-mediated STAT6 upregulation,³⁵ highlighting a potential role for signaling of immune cells to drive cancer cell invasiveness. HCT116 spheroids showed minimal invasion under no treatment or pretreatment with neratinib (Figure 7a), potentially due to the low basal activities of EGFR proteins (Figure 4e). Our results suggest that pretreatment with trametinib, both as single-agent and in combination with neratinib, accounted for invasiveness of HCT116 cells potentially due to feedback activation of STAT signaling. This was supported by suppression of HCT116 cells invasiveness by inhibition of STAT1 or STAT6 (Figure 7e), suggesting that combined inhibition of MAPKi and EGFRi

followed by inhibition of STAT signaling may suppress proliferative and metastatic activities of the cells.

Overall, we established mechanisms of resistance to single-agent inhibition of the MAPK pathway in a KRAS^{mut} colorectal tumor model and demonstrated that combination treatments driven by molecular mechanism of resistance suppress growth of tumor spheroids. Additionally, we showed that despite the promising effect of combining RTKi and MAPKi against tumor spheroids growth, this approach may not be effective against invasiveness of cancer cells mediated by treatment-induced activation of STAT kinases. Future studies are necessary to further validate this observation using other KRAS^{mut} cell lines and primary cells and including stromal cells in the tumor model. Our mechanistic study using a 3D tumor model captured the complexity of events in the tumor microenvironment and identified treatments that suppress growth and invasiveness of cancer cells. Future developments of our model to consider tumor stroma, including immune cells that regulate the JAK/STAT cytokine pathway, will enable systematic studies that elucidate the role of tumor-stromal signaling on different functions of cancer cells.

CONCLUSION

We established that colorectal cancer cells in tumor spheroids under cyclic, single-agent treatments develop resistance to inhibitors of MAPK pathway by activating AKT, specific STATs, and EGFRs. Using a rational-design strategy guided by molecular analysis of drug resistance, we combined inhibitors of the constitutively active MAPK pathway and feedback-activated EGFRs to overcome the drug resistance of cancer cells and to suppress the growth of tumor spheroids by blocking both MAPK and PI3K/AKT pathways. We additionally established that treatment-induced STAT pathway activity leads to the matrix invasion of cancer cells and a strategy to block it. Our design-driven approach to determine highly synergistic drug pairs and concentrations offers a valuable tool to prioritize compounds for subsequent xenograft studies in preclinical tests. This approach is promising to significantly reduce the number of animal studies and accelerate the discovery of effective treatments for clinical use.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.0c00115>.

Responses of colorectal tumor spheroids to EGFR inhibitors (PDF)

AUTHOR INFORMATION

Corresponding Author

Hossein Tavana — Department of Biomedical Engineering, The University of Akron, Akron, Ohio 44325, United States;
orcid.org/0000-0003-3872-1869; Phone: (330) 972-6031; Email: tavana@uakron.edu

Authors

Pradip Shahi Thakuri — Department of Biomedical Engineering, The University of Akron, Akron, Ohio 44325, United States;
orcid.org/0000-0002-4899-6375

Astha Lamichhane — Department of Biomedical Engineering, The University of Akron, Akron, Ohio 44325, United States

Sunil Singh – Department of Biomedical Engineering, The University of Akron, Akron, Ohio 44325, United States

Megha Gupta – Department of Arts and Sciences, The University of Akron, Akron, Ohio 44325, United States

Gary D. Luker – Department of Radiology, Microbiology and Immunology, and Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48105, United States

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acsptsci.0c00115>

Notes

The authors declare no competing financial interest.

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