

In vivo effects of chemotherapy on oncogenic pathways in colorectal cancer

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Abstract

Patients with advanced colorectal cancer often are treated with systemic cytotoxic therapy using fluorouracil (5-FU), oxaliplatin, irinotecan, and FOLFOX or FOLFIRI combination protocols. Additionally, signaling pathways that are active in colorectal cancer can be therapeutically targeted. Herein, we examined whether chemotherapy impacts on WNT, MAPK and NOTCH signaling pathways in xenograft models of colon cancer. Furthermore, we tested whether combining chemotherapy with MAPK and NOTCH inhibition has superior therapeutic effects. We show that colon cancer cells with high WNT, MAPK and NOTCH activity are variably affected but generally persist in xenograft tumors under different chemotherapeutic regimens, indicating limited effects of cytotoxic therapy on oncogenic signaling pathways. Although these results provided a rationale to additionally target pathway activity, we found no significant increase in therapy response when combining MAPK and NOTCH inhibition with fluorouracil chemotherapy. We attribute this finding to a decrease in tumor cell proliferation upon MAPK and NOTCH inhibition, resulting in reduced effectiveness of cytotoxic treatment. Therapeutic benefits of combining chemotherapy with targeting of oncogenic signaling pathways must therefore be critically evaluated for patients with colorectal cancer.

KEYWORDS

chemotherapy, colon and colorectal cancer, MAPK, NOTCH, WNT

1 | INTRODUCTION

Colorectal cancer is a common malignancy, ranking third in incidence among men and women.¹ Although local disease may be cured by surgical resection, advanced and metastatic disease can be treated with chemotherapy, either in adjuvant or palliative settings, or for conversion to achieve surgical resectability.² For chemotherapy, different cytotoxic agents including 5-FU, oxaliplatin, and irinotecan

are available that can be used alone but are frequently combined in treatment regimens of FOLFOX or FOLFIRI.³ Such treatments prolong patient survival with metastatic disease to median durations of almost 2 years.⁴ Nevertheless, chemotherapy is not a curative treatment approach, and most patients with advanced colorectal cancer will eventually die of the disease.⁵

In addition to chemotherapeutic options, signaling pathways may be attractive and specific therapeutic targets. WNT and

Abbreviations: EGFR, epidermal growth factor receptor; 5-FU, fluorouracil; FOLFIRI, fluorouracil + irinotecan; FOLFOX, fluorouracil + oxaliplatin; NICD, Notch 1 intracellular domain.

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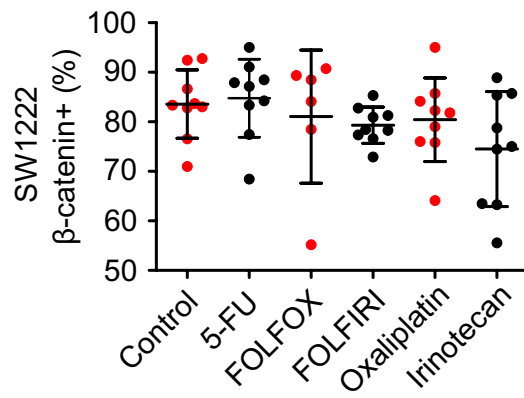
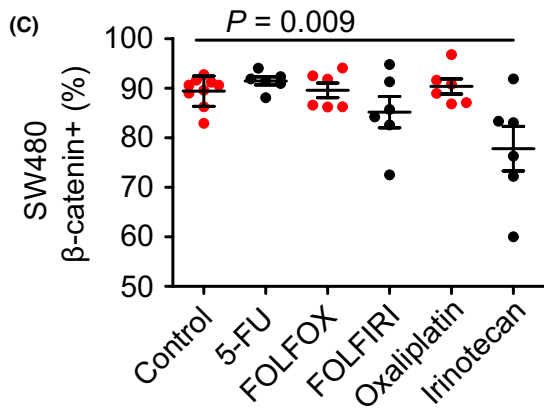
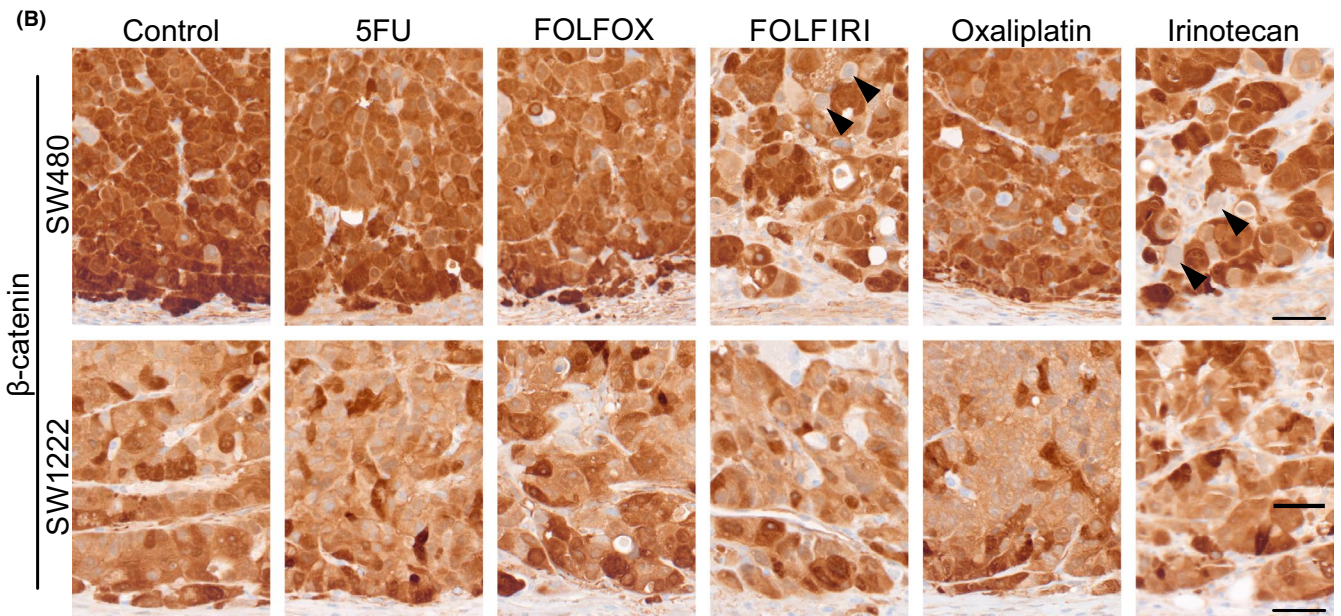
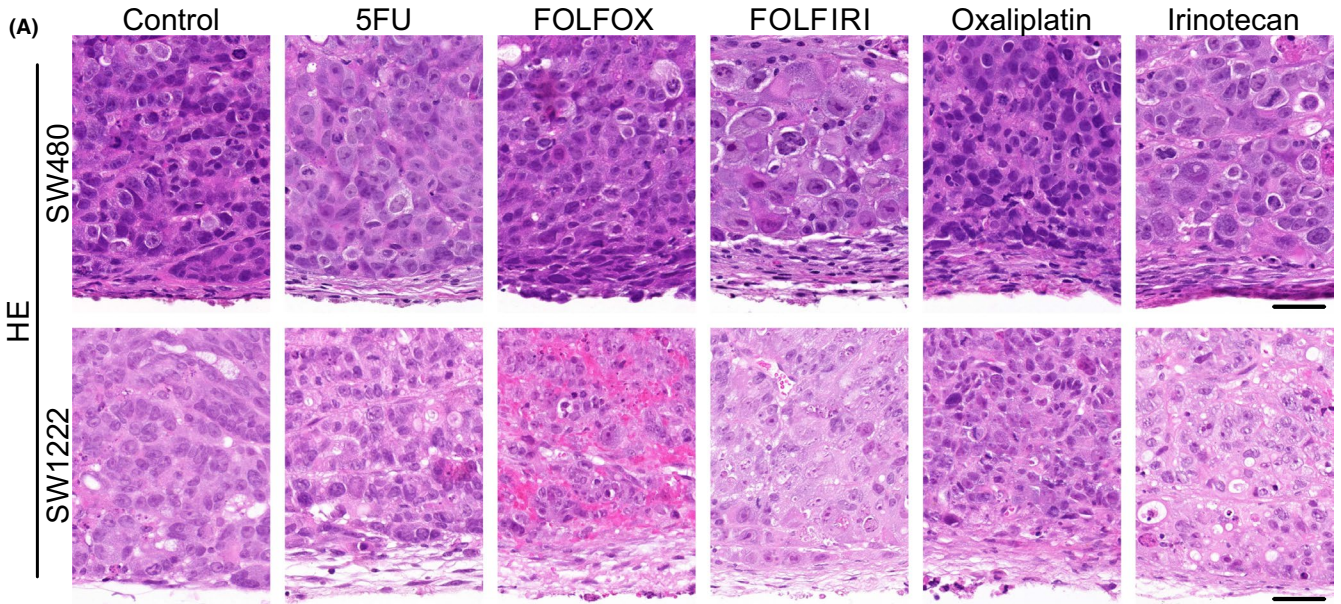


FIGURE 1 Effects of short-term chemotherapy on tumor cell morphology and WNT signaling in colon cancer. A, H&E stained sections of SW480 and SW1222 colon cancer xenografts that were treated for 10 days with different chemotherapeutics or with vehicle (control), as indicated. B, Representative immunostainings for β -catenin of treated colon cancer xenografts. Arrowheads indicate individual tumor cells without nuclear β -catenin accumulation in fluorouracil + irinotecan (FOLFIRI) and irinotecan-treated SW480 xenografts. C, Quantification of tumor cells with expression of nuclear β -catenin in xenografts after 10 d of indicated treatments. Error bars indicate mean \pm SD. *P*-values are *t* test results. *n* \geq 3. Scale bars, 50 μ m. FOLFOX, fluorouracil + oxaliplatin; 5-FU, fluorouracil

MAPK signaling are typically active in colorectal cancer and drive tumor progression.⁶ Although targeting WNT appears to be difficult,⁷ MAPK signaling can be inhibited by antibodies against EGFR or by MEK inhibitors that block signal transduction and thus may slow tumor progression.⁸ Combining such treatment with chemotherapy may increase response rates even further,^{9,10} although, based on other data, the superiority of such combinations remains a matter of debate.¹¹ Nevertheless, current guidelines recommend adding anti-EGFR-directed therapy to FOLFOX or FOLFIRI protocols for colorectal cancers without activating RAS mutations.²

Besides WNT and MAPK signaling, activation of NOTCH signaling has been observed in colorectal cancer. Although in treatment trials, NOTCH repression by γ -secretase inhibitors has so far been disappointing,^{12,13} we recently showed that dual inhibition of MAPK and NOTCH signaling by MEK and γ -secretase inhibitors strongly repressed colon cancer growth.¹⁴ However, it remains to be determined whether combined MAPK and NOTCH repression may add benefit to established chemotherapeutic regimens. Furthermore, it is generally unknown to what extent chemotherapeutic treatments may impact on signaling pathway activities in colorectal cancer and thus modulate their targetability.

To address these problems, we herein gauged the impact of cytotoxic chemotherapy on WNT, MAPK and NOTCH activity in colorectal cancer *in vivo*. Furthermore, we assessed whether combining dual MAPK and NOTCH inhibition with chemotherapy may be a promising treatment strategy for more effective management of patients with colorectal cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture

We obtained SW1222 colon cancer cells from the Ludwig Institute for Cancer Research (New York, NY, USA) and SW480 colon cancer cells from ATCC. SW1222 has a deletion of 113 bp in exon 1 of TP53, causing loss of p53 protein expression, a truncating mutation in APC(E1306*), and an activating mutation in KRAS(A146V).^{15,16} SW480 carries mutated p53(R273H/P309S), truncated APC(Q1338*), and also has an activating mutation in KRAS(G12V). Both cell lines were derived from colorectal adenocarcinomas, are microsatellite stable and tumorigenic in NOD/SCID mice.¹⁴ Cell line identity was verified by short-tandem repeat profiling, and both cell lines tested negative for *Mycoplasma* contamination. Cell lines were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Biochrom, Berlin, Germany).

2.2 | Tumor xenografts and *in vivo* treatments

Mouse experiments were reviewed and authorized by the district government of Upper Bavaria. We used NOD/SCID mice (NOD.CB17-Prkdc^{scid}; The Jackson Laboratory, Bar Harbor, ME, USA) that lack mature B- and T-cell lineages and complement activity,¹⁷ and allow growth of xenografted human tumors. Mice were accommodated in pathogen-free micro-isolator cages on wood-shred bedding, water and food available *ad libitum*, and a 12:12 hour light-dark cycle. Welfare was monitored using a score-based system including weight, fur, habitus, motion, eyes and others. Only fully healthy animals were included for further experimentation. For xenotransplantation, SW1222 or SW480 colon cancer cells were suspended in a mixture of 50 μ L PBS and 50 μ L growth factor-depleted Matrigel (Corning, New York, NY, USA), and *s.c.* injected into gender- and age-matched male or female 6-8-week-old mice. Tumor size was measured using calipers. When tumors reached volumes of 100 mm³, mice were randomly assigned to control or treatment groups, with at least three animals in each group. Investigators were not blinded to group allocations and there were no dropouts. When tumor diameters reached 1.5 cm, mice were killed by cervical dislocation, xenograft tumors were removed, formalin fixed and paraffin embedded for histology, and subjected to further analysis.

2.3 | Chemotherapy and inhibitor treatment

Irinotecan was obtained from Pfizer (Berlin, Germany), selumetinib (AZD) from Selleck Chemicals (Munich, Germany), and dibenzazepine (DBZ) from Axon Medchem (Groningen, the Netherlands). All other chemicals were obtained from Sigma (St Louis, MO, USA). Cytotoxic agents were dissolved in sterile 0.9% NaCl in H₂O, and given as monotherapies or in combinations. AZD was dissolved in 0.5% Methocel (Sigma) and 0.1% Tween 80. DBZ was dissolved in 0.1% DMSO, 0.5% Methocel and 0.1% Tween 80. For short-term chemotherapy, 0.3 mg 5-FU and 0.4 mg leucovorin were given daily by *i.p.* injection, and 0.2 mg oxaliplatin or 1 mg irinotecan was given *i.p.* on day 1 or on days 1 and 6, for 3- and 10-day treatment regimens, respectively. For FOLFOX and FOLFIRI combinations, we used the same concentrations as for monotherapies. For long-term therapy, mice were treated with 0.3 mg 5-FU and 0.4 mg leucovorin *i.p.* 5 days per week, and/or with 1.25 mg AZD *per os* and 0.35 mg DBZ *i.p.* twice per week. Control groups were treated with vehicle only. Treatments were carried out under a sterile workbench environment during mid-day, and mice were treated for up to 31 days.

2.4 | Immunohistochemistry

For immunohistochemistry, 5- μ m tissue sections of formalin-fixed and paraffin-embedded xenografts were cut and subjected to heat-induced epitope retrieval in Cell Conditioning 2 (Ventana Medical Systems, Tucson, AZ, USA) or TRS6 (Dako, Glostrup, Denmark). Sections then were incubated with prediluted mouse anti- β -catenin (Ventana Medical Systems), rabbit anti-cleaved Notch1 (NICD; 1:100; Cell Signaling Technology, Danvers, MA, USA), mouse anti-FRA1 (1:50; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-Ki67 (1:150; Dako), rabbit anti-cleaved Caspase-3 (1:100; Cell Signaling Technology), or mouse anti-thymidylate synthase (1:100; Santa Cruz Biotechnology) primary antibodies. Staining then was visualized with UltraView or OptiView DAB detection kits on BenchMark Ultra autostainers (Ventana Medical Systems). Nuclei were counterstained with hematoxylin. Slides then were scanned on Panoramic DESK II (3DHISTECH, Budapest, Hungary), and frequencies of tumor cells expressing respective markers were quantified using ImageJ software.

2.5 | Statistical analyses and data availability

Sample sizes were based on preliminary data and previous experience. To analyze differences between groups, we used two-tailed Student's *t* test, and data are mean \pm SD if not indicated otherwise. The Kaplan-Meier method was used to show differences in tumor survival, for which tumor diameters of 1.5 cm were defined as endpoints. *P*-values for survival statistics were calculated with the log-rank test. Differences were regarded statistically significant when *P* < .05. Significant *P*-values are indicated within figures. Biological replicates indicate single animals and are given as *n*-values. Statistics were calculated with Excel (Microsoft) or GraphPad Prism (GraphPad software). Data and materials are available from the corresponding author upon reasonable request.

3 | RESULTS

3.1 | Limited effects of chemotherapy on signaling pathway activity in colon cancer

To determine the effects of chemotherapy on signaling pathway activity, we examined SW1222 and SW480 colon cancer xenografts that we treated for 3 and 10 days with 5-FU, oxaliplatin, irinotecan, or the combinations of 5-FU and oxaliplatin (FOLFOX) or 5-FU and irinotecan (FOLFIRI). H&E-stained tissue sections showed that all xenografts had retained substantial amounts of

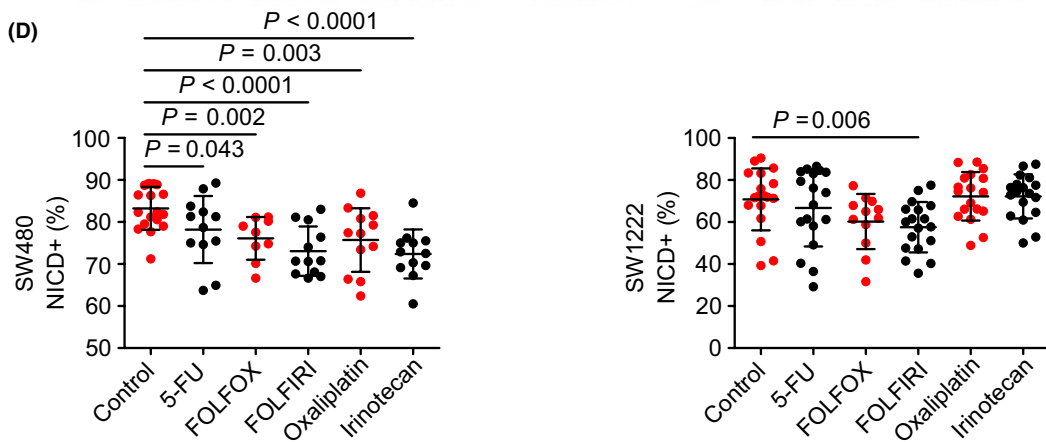
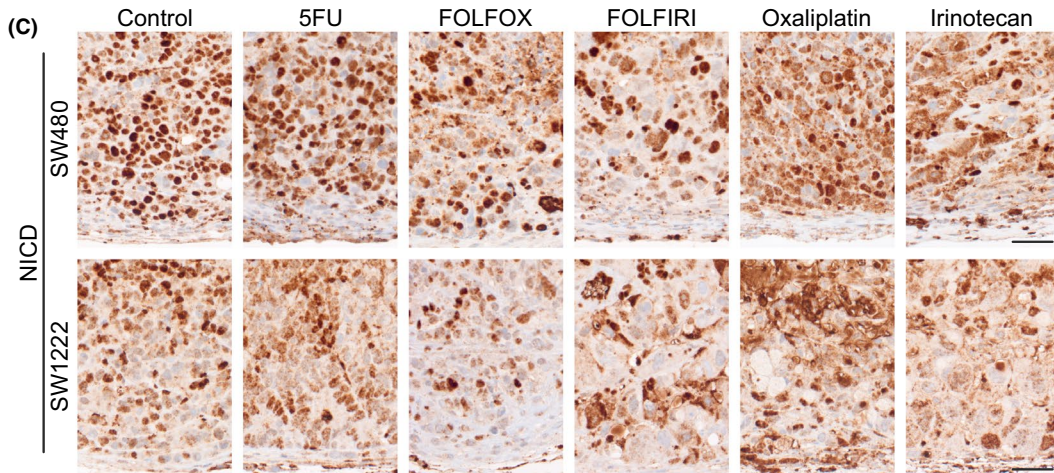
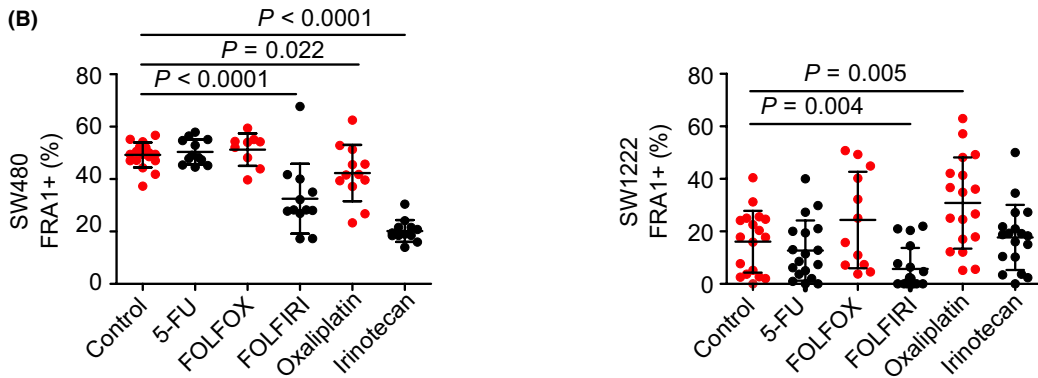
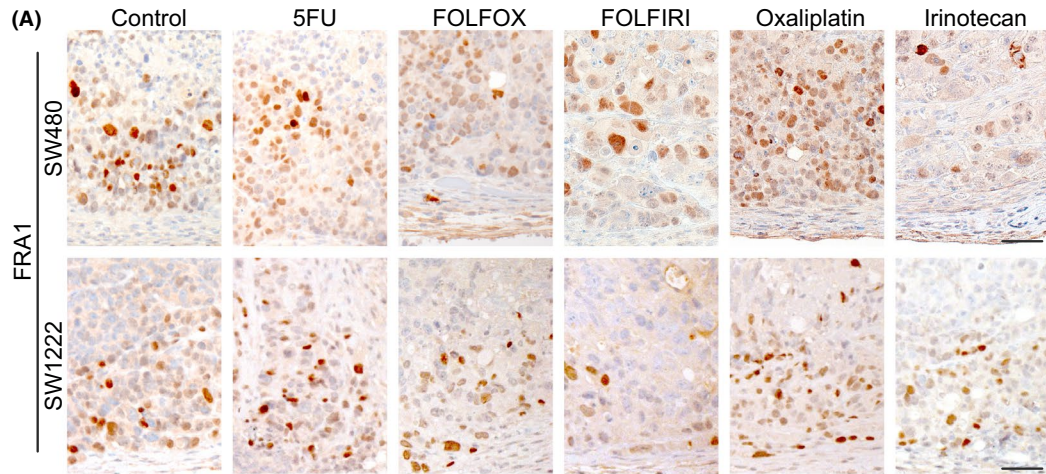
vital tumor tissue (Figure 1A). Morphologically, irinotecan, either alone or in combination with 5-FU, showed the strongest cytopathic effects with swollen tumor cells, giant and irregular nuclei, and pale cytoplasm, whereas the other treatments did not cause overt morphological changes upon this short-term treatment (Figure 1A).

We then examined these xenograft tumors for nuclear β -catenin, which indicates WNT signaling activity.¹⁸ Non-treated SW480 and SW1222 colon cancer xenografts showed accumulation of nuclear β -catenin in tumor cells that tended to be more pronounced at the tumor edge (Figure 1B). Interestingly, in SW480 xenografts, treatment with irinotecan or FOLFIRI caused a loss of nuclear β -catenin staining in some tumor cells, and this effect was significant after 3 days for both treatments and after 10 days for irinotecan treated xenografts only (Figures 1B,C and S1A). The other treatments did not significantly impact on nuclear β -catenin in SW480 tumors. Furthermore, in SW1222 xenografts, none of the treatments had significant effects on the frequency of nuclear β -catenin-positive tumor cells (Figures 1B,C and S1A).

Next, we examined the presence of tumor cells with expression of FRA1, which labeled a subset of colon cancer cells in SW1222 and SW480 xenografts, and which indicates high MAPK signaling activity.¹⁹ After 10 days of treatment, we found that irinotecan and oxaliplatin monotherapies as well as FOLFIRI decreased FRA1-positive tumor cells in SW480 tumors (Figure 2A,B). In SW1222 xenografts, oxaliplatin monotherapy increased and FOLFIRI decreased the frequency of FRA1-positive tumor cells, whereas other treatments did not significantly affect FRA1 expression (Figure 2A,B). However, when analyzing tumors after only 3 days of treatment, the frequency of FRA1-positive tumor cells slightly increased upon FOLFIRI therapy and decreased upon FOLFOX therapy in SW480 xenografts, when compared to non-treated control tumors (Figure S1B). Moreover, treating SW1222 xenografts for 3 days with 5-FU monotherapy significantly increased the frequency of FRA1-positive tumor cells (Figure S1B).

To test for the effects of chemotherapy on NOTCH signaling, we then examined xenograft tumors for accumulation of NICD. Non-treated SW480 and SW1222 xenografts showed high frequencies of NICD-positive tumor cells, indicating widespread NOTCH activity (Figure 2C). Interestingly, in SW480 xenografts, after 10 days, all treatments resulted in a significant reduction of NICD-positive tumor cells (Figure 2C,D). Similar effects could already be observed after 3 days of treatment, except for oxaliplatin monotherapy (Figure S1C). However, in SW1222 xenografts, only FOLFIRI after 10 days and irinotecan monotherapy after 3 days caused significant reductions of NICD-positive tumor cells (Figures 2C,D and S1C).

FIGURE 2 Analysis of MAPK and NOTCH signaling activity in colon cancer after short-term chemotherapy. A, Representative immunostainings for FRA1 and (B) quantification of FRA1-positive tumor cells in colon cancer xenografts after 10 d of indicated chemotherapeutic or vehicle (control) treatments. C, Representative immunostainings for Notch 1 intracellular domain (NICD) and (D) quantification of NICD-positive tumor cells in colon cancer xenografts after 10 d of indicated chemotherapeutic or vehicle (control) treatments. Error bars indicate mean \pm SD. *P*-values are *t* test results. *n* \geq 3. Scale bars, 50 μ m. FOLFIRI, fluorouracil + irinotecan; FOLFOX, fluorouracil + oxaliplatin; 5-FU, fluorouracil



Collectively, these findings indicated that chemotherapeutic regimens may variably impact on the frequency of tumor cells with high WNT and MAPK activity, whereas tumor cells with high NOTCH activity are often reduced. Nevertheless, tumor cell subpopulations with different pathway activities generally persisted upon chemotherapy.

3.2 | Combining MAPK and NOTCH-targeted treatment with 5-FU chemotherapy

We previously showed that combined inhibition of MAPK and NOTCH signaling slowed tumor growth in colon cancer xenografts.¹⁴ In order to compare these therapeutic effects with chemotherapy, we treated SW480 and SW1222 xenografts for longer terms of up to 31 days with 5-FU chemotherapy or, for MAPK and NOTCH inhibition, with AZD and DBZ (Figure 3A). Furthermore, we tested for additive effects of chemo- and inhibitor therapy. Non-treated xenografts served as controls. All treatment protocols significantly slowed growth of SW480 and SW1222 colon cancer xenografts (Figure 3B) and prolonged survival (Figure S2). In SW480 tumors, therapy with 5-FU or with AZD and DBZ was similarly effective in reducing tumor growth. In SW1222, combined AZD and DBZ was slightly more effective than 5-FU monotherapy, but this difference was non-significant (Figure 3B). Unexpectedly, when we tested the combination of 5-FU treatment with AZD and DBZ, we found that this did not significantly improve therapy effects (Figure 3B). However, in SW1222 xenografts, the combination nearly caused full arrest of tumor growth.

To further assess treatment effects, we analyzed xenografts that had been treated with 5-FU, AZD and DBZ, or their combination, for tumor necrosis. All tumors, including non-treated controls, showed large areas of tumor necrosis (Figure 3C). Measuring relative necrotic areas showed that in SW480 xenografts, 5-FU alone or the combination of 5-FU with AZD and DBZ significantly increased necrotic areas, and thus reduced the amount of vital tumor tissue, when compared to non-treated xenografts (Figure 3D). In SW1222 xenografts, all treatments significantly increased tumor necrosis, with similar effects of 5-FU, AZD and DBZ, and their combination (Figure 3D). Importantly, however, the combination of 5-FU with AZD and DBZ did not further increase tumor necrosis when compared to either therapy alone.

Taken together, these data showed that therapeutic effects of 5-FU or MAPK and NOTCH inhibition on tumor growth were similar. Combining both treatments did not significantly improve therapy response.

3.3 | Impact of 5-FU, MAPK and NOTCH inhibition on proliferation and apoptosis

To better understand the observed therapeutic effects on colon cancer xenografts, we analyzed proliferation and apoptosis rates in treated tumors. In SW480 colon cancer xenografts, 5-FU monotherapy slightly reduced proliferation as assessed by Ki-67 immunostaining when compared to non-treated control tumors (Figure 4A,B). AZD and DBZ treatment reduced Ki-67 proliferation rates more strongly in these tumors (Figure 4A,B). However, combining both treatments had only slightly more repressive effects on proliferation than AZD and DBZ alone. Similar, but overall, stronger repressive effects of these treatments were observed in SW1222 colon cancer xenografts, where AZD and DBZ in combination with 5-FU reduced the frequency of Ki-67-positive tumor cells by more than 50% (Figure 4A,B).

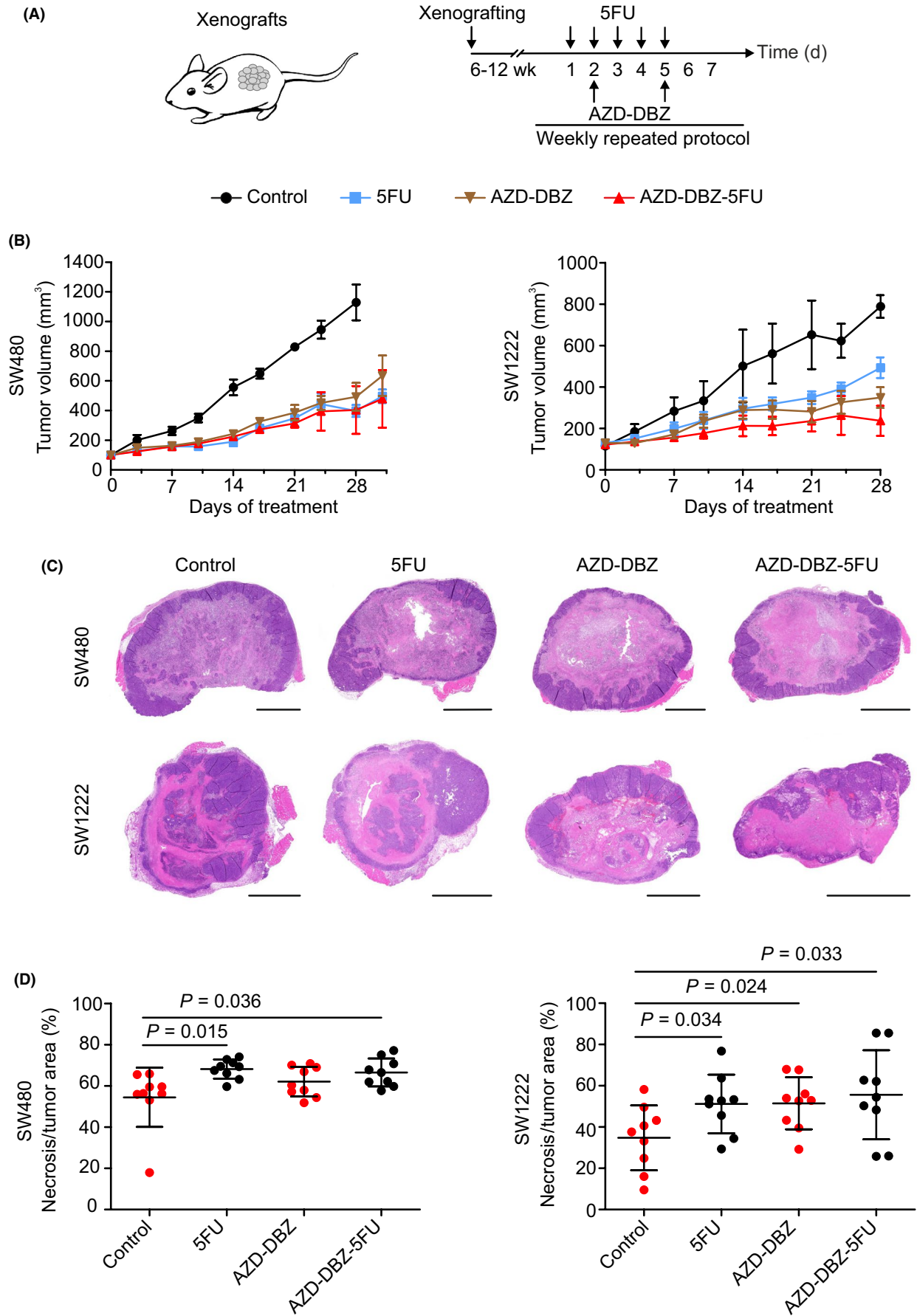
We then examined apoptosis by analyzing cleaved Caspase-3. In both SW480 and SW1222 xenografts, apoptosis rates increased upon treatment with 5-FU, AZD and DBZ, or their combination, when compared to non-treated controls (Figure 4C,D). This increase was more pronounced in SW1222 than in SW480-derived xenograft tumors. However, the combination of 5-FU with AZD and DBZ did not significantly increase apoptosis when compared to either treatment alone (Figure 4D).

Taken together, these findings indicated that MAPK and NOTCH inhibition had stronger repressive effects on colon cancer proliferation than 5-FU monotherapy, whereas apoptosis rates were similarly affected by either treatment modality or their combination.

3.4 | Effects of 5-FU, MAPK and NOTCH inhibition on thymidylate synthase expression

Resistance to therapy with 5-FU may be caused by increased expression of thymidylate synthase. In order to examine whether this mechanism may be relevant for our observations, we analyzed thymidylate synthase expression in treated and non-treated xenograft tumors. SW480 and SW1222 xenografts showed thymidylate synthase expression in 59.4% and 63.8% of the tumor cells, respectively (Figure 5A,B). As expected, 5-FU monotherapy significantly increased the fraction of thymidylate synthase-positive tumor cells to an average of 86.9% and 78.8% in SW480 and SW1222 xenografts, respectively (Figure 5A,B). In contrast, AZD and DBZ treatment substantially reduced thymidylate synthase levels in both tumor models. However, when adding 5-FU to the treatment protocol, this effect was either fully or partially reversed (Figure 5A,B). These findings suggested that MAPK and NOTCH inhibition did not

FIGURE 3 Impact of long-term fluorouracil (5-FU) chemotherapy and MAPK and NOTCH inhibition on colon cancer. A, Schema and experimental schedule for xenografting, treatment and tumor analysis. B, Long-term treatment effects of 5-FU, selumetinib (AZD) and dibenzazepine (DBZ), and their combination compared to vehicle (control) on SW480 and SW1222 colon cancer xenografts. Data are shown as growth curves and represent mean \pm SE. $n \geq 3$ for each treatment group. C, Representative H&E-stained tissue sections of xenograft tumors after long-term treatment, as indicated. Scale bars, 2 mm. D, Quantification of tumor necrosis in treated colon cancer xenografts. Error bars indicate mean \pm SD. *P* values are *t* test results. $n \geq 3$



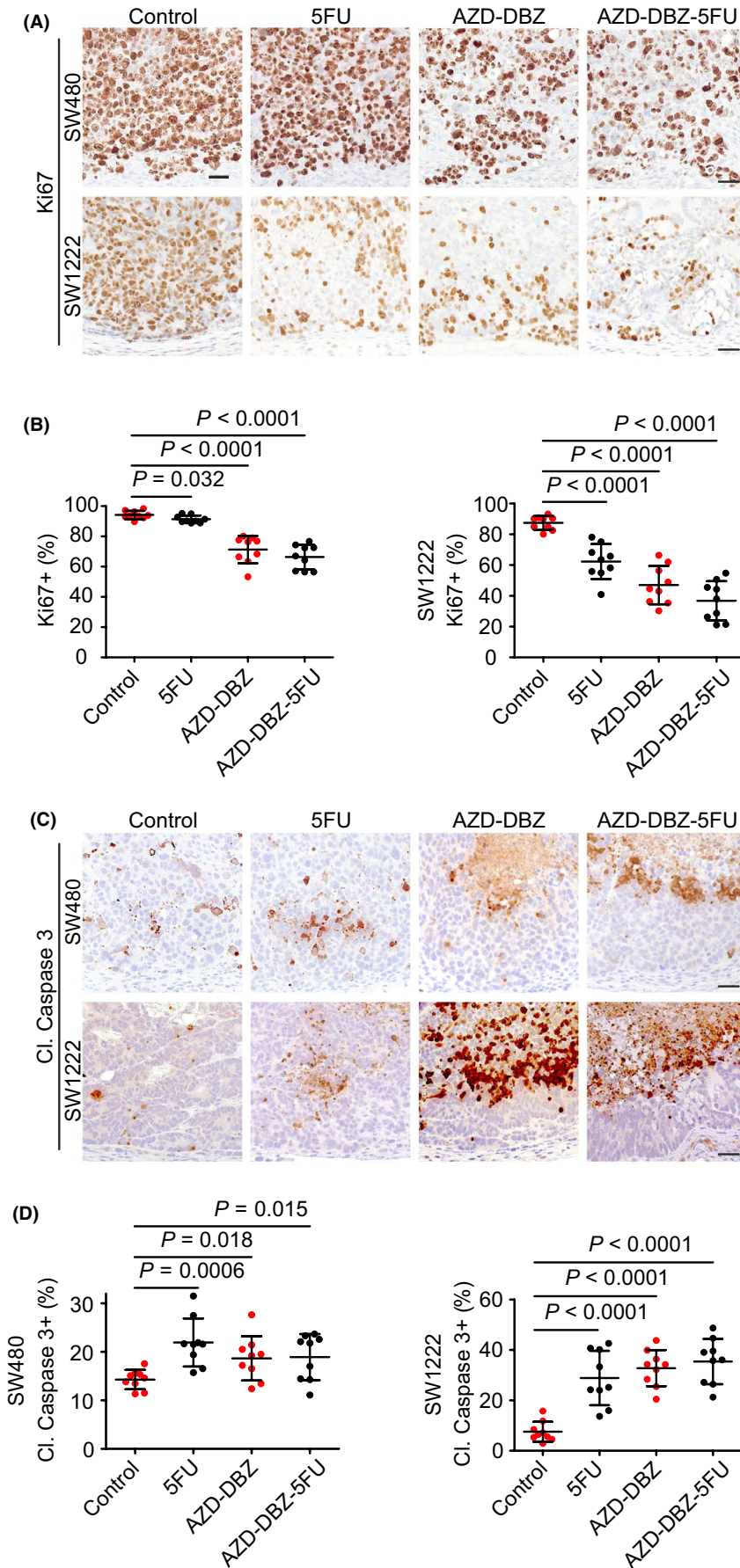
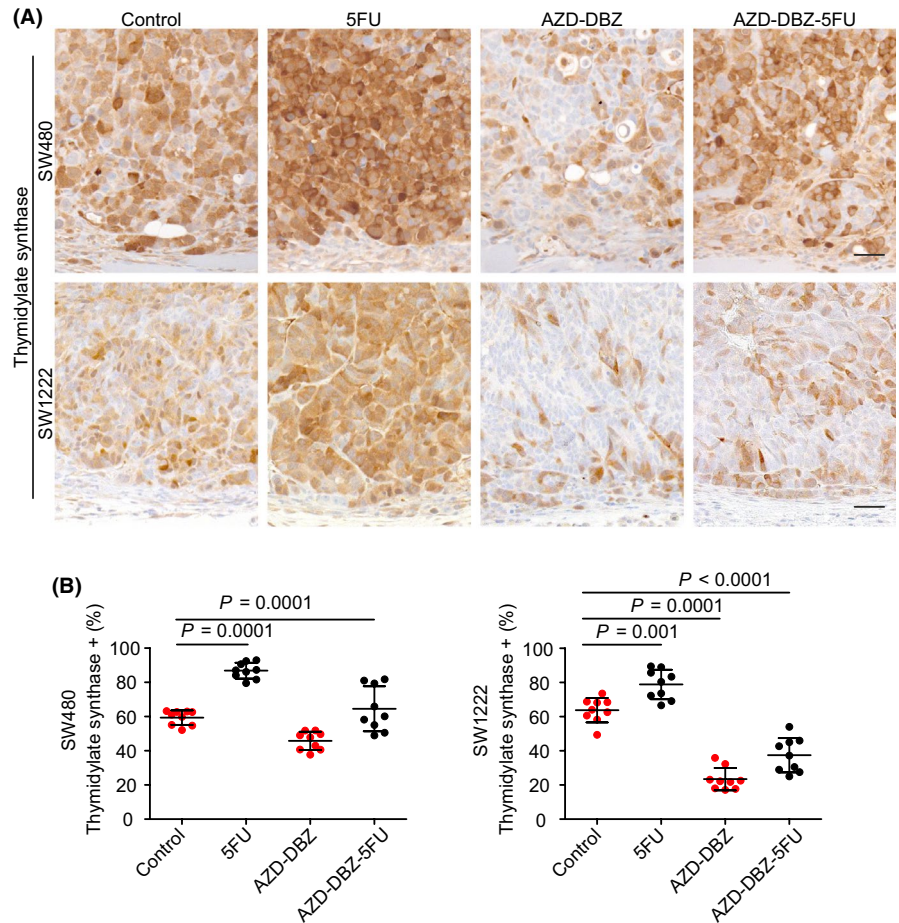


FIGURE 4 Proliferation and apoptosis in colon cancer after long-term therapy. A, Representative immunostaining for Ki-67 and (B) quantification of Ki-67-positive tumor cells in colon cancer xenografts after indicated chemotherapeutic and/or inhibitor treatments in comparison to vehicle (control). C, Representative immunostaining for cleaved (Cl.) Caspase-3 and (D) quantification of cleaved Caspase-3-positive tumor cells in colon cancer xenografts after indicated chemotherapeutic and/or inhibitor treatments, or vehicle (control) treatment. Error bars indicate mean \pm SD. *P*-values are *t* test results. $n \geq 3$. Scale bars, 50 μ m. AZD, selumetinib; DBZ, dibenzazepine; 5-FU, fluorouracil

FIGURE 5 Expression of thymidylate synthase on long-term therapy in colon cancer xenografts. A, Representative immunostaining for thymidylate synthase and (B) quantification of thymidylate synthase positive tumor cells in colon cancer xenografts after indicated chemotherapeutic and/or inhibitor treatments, or vehicle (control) treatment. Error bars indicate mean \pm SD. *P*-values are *t* test results. $n \geq 3$. Scale bars, 50 μ m. AZD, selumetinib; DBZ, dibenzazepine; 5-FU, fluorouracil



induce resistance to 5-FU chemotherapy through induction of thymidylate synthase.

4 | DISCUSSION

Herein, we investigated the effects of frequently used chemotherapeutic agents and their combinations on key signaling pathways in colon cancer. We used xenografts of SW1222 and SW480 colon cancer cell lines that both contained tumor cell subpopulations with high WNT, MAPK, and NOTCH signaling activity, and thus adequately modeled primary colon cancers.¹⁴ Our data showed that 5-FU, irinotecan and oxaliplatin, either when used alone or in typical combinations of FOLFOX and FOLFIRI, had variable and rather moderate effects on the frequency of tumor cells with high WNT and MAPK activity that we gauged through analyses of nuclear β -catenin and FRA1, respectively. Such tumor cell subpopulations have previously been linked to tumor progression and were suggested to represent putative colon cancer stem cells.^{19,20} Our observations therefore imply that these tumor cell subsets are not specifically affected or targeted by typical chemotherapeutic protocols, and may therefore be responsible for tumor recurrence and therapy resistance.²¹ However, we observed more consistent effects on tumor cells with high NOTCH activity that we

identified through staining for NICD and that were significantly reduced upon different cytotoxic treatments. As we previously demonstrated that colon cancer cells with high NOTCH signaling proliferated more actively than those with high MAPK signaling,¹⁴ we suggest this may explain why this tumor cell subset is more consistently affected by cytotoxic agents that predominantly target proliferating cells.²² Collectively, we showed that currently used chemotherapeutic regimens for patients with colorectal cancer can variably impact on tumor cell subpopulations with distinct pathway activities. Importantly, however, subpopulations with high WNT, MAPK and NOTCH signaling activity generally persisted to varying proportions within remaining vital tumor tissue.

We previously demonstrated that therapeutic inhibition of MAPK and NOTCH signaling with the MEK inhibitor AZD and the gamma secretase-inhibitor DBZ significantly slowed growth of colon cancer xenografts.¹⁴ The observation that tumor cell subpopulations with high MAPK and NOTCH activity persisted upon different chemotherapeutic regimens provided a rationale to combine chemotherapy with such inhibitor therapy. We selected 5-FU as chemotherapy for this combination as this drug had little effect on the presence of tumor cell subpopulations with high MAPK and NOTCH activity, and is also part of most chemotherapeutic regimens for colorectal cancer.² Unexpectedly, however, we observed that combining MAPK and NOTCH inhibition with

5-FU resulted in minimally improved therapy response in one of two xenograft models only, when compared to 5-FU or inhibitor treatment alone. Also, tumor damage, as reflected by necrosis and apoptosis, did not significantly increase upon combined treatment when compared to either 5-FU or inhibitor treatment. Importantly, however, we found that combined MAPK and NOTCH inhibition significantly reduced proliferation in treated tumors. This observation may explain the insignificant additive effects of combining the inhibitors with 5-FU, as reduced cell proliferation may have reduced the DNA-damaging effects of this cytotoxic agent.²³ Furthermore, because we observed that MAPK and NOTCH inhibition decreased the expression of thymidylate synthase, a known mediator of 5-FU resistance,²⁴ this also supported the idea that inhibition of MAPK and NOTCH rather reduced the effectiveness of 5-FU chemotherapy than vice versa. It therefore remains to be determined whether specific scheduling of cytotoxic drug treatments and inhibitor therapies may lead to better synergistic treatment effects. Also, because MAPK and NOTCH inhibition showed similar effects in reducing tumor growth when compared to 5-FU chemotherapy alone, such inhibitor treatment may still find consideration for tumors with resistance to 5-FU.

In conclusion, we herein demonstrate that combining chemotherapy with therapeutic targeting of signaling pathways that are active in colon cancer may not generally result in additive therapeutic effects. We suggest this may, in part, be due to reduced proliferation upon pathway inhibition and thus decreased efficacy of cytotoxic agents. However, our study has certain limitations. The data we show were derived from immune-compromised animals, which partially lack an inflammatory microenvironment and tumor-directed immune response.¹⁷ Treatment effects in human patients with colorectal cancer may thus differ significantly. Moreover, the SW1222 and SW480 xenograft models we used showed a variable response of tumor cell subpopulations to cytotoxic treatments, despite a comparable genetic background with microsatellite stability and mutations in *TP53*, *APC* and *KRAS* genes. It therefore remains to be determined to what extent our findings depend on individual tumor characteristics, keeping in mind that colon cancers are genetically heterogeneous malignancies.^{25,26} Further study in this context may then inform therapeutic trials in order to determine treatment combinations that provide benefit for patients with colorectal cancer.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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