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The cancer chemotherapeutic 5-fluorouracil is a potent *Fusobacterium nucleatum* inhibitor and its activity is modified by intratumoral microbiota

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SUMMARY

Fusobacterium nucleatum (*Fn*) is a dominant bacterial species in colorectal cancer (CRC) tissue that is associated with cancer progression and poorer patient prognosis. Following a small-molecule inhibitor screen of 1,846 bioactive compounds against a *Fn* CRC isolate, we find that 15% of inhibitors are antineoplastic agents including fluoropyrimidines. Validation of these findings reveals that 5-fluorouracil (5-FU), a first-line CRC chemotherapeutic, is a potent inhibitor of *Fn* CRC isolates. We also identify members of the intratumoral microbiota, including *Escherichia coli*, that are resistant to 5-FU. Further, CRC *E. coli* isolates can modify 5-FU and relieve 5-FU toxicity toward otherwise-sensitive *Fn* and human CRC epithelial cells. Lastly, we demonstrate that *ex vivo* patient CRC tumor microbiota undergo community disruption after 5-FU exposure and have the potential to deplete 5-FU levels, reducing local drug efficacy. Together, these observations argue for further investigation into the role of the CRC intratumoral microbiota in patient response to chemotherapy.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

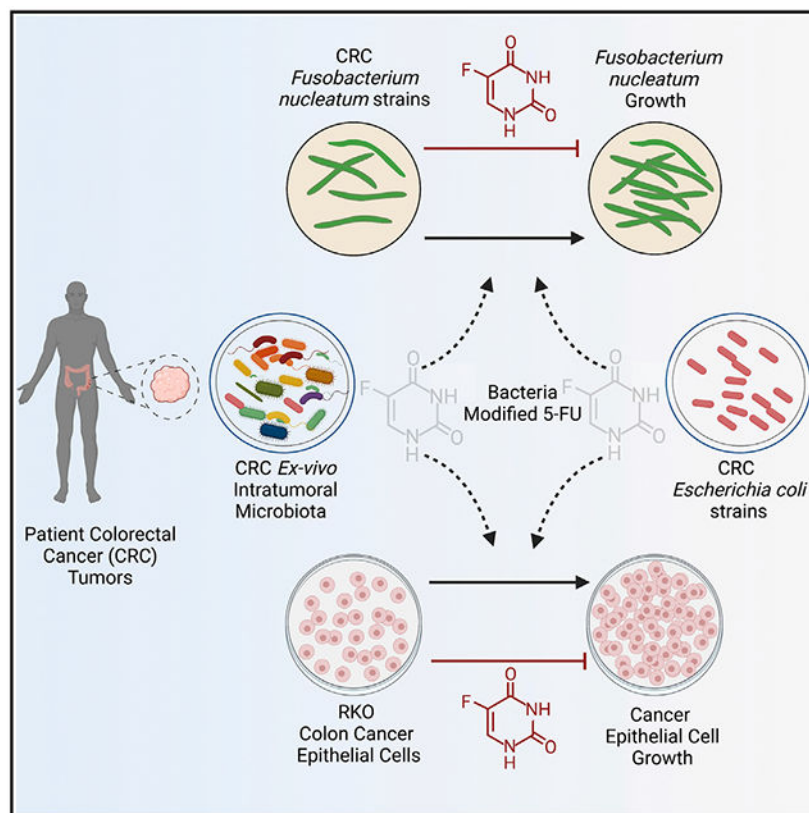
K.D.L., S.B., and C.D.J. designed the study. K.D.L., M.Z.-R., A.B., A.G.K., and S.B. performed the experiments. K.D.L., M.Z.-R., S.S.M., and S.B. analyzed data. K.D.L., C.D.J., and S.B. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.111625>.

DECLARATION OF INTERESTS

S.B. is an inventor on US patent application no. PCT/US2018/042,966, submitted by the Broad Institute and DFCI, that covers targeting of *Fusobacterium* for treatment of CRC. K.D.L. is currently an employee at NanoString Technologies, Inc. A.B. is currently an employee at Bristol-Myers Squibb.



In brief

LaCourse et al. show that *Fusobacterium nucleatum*, a bacterium associated with colorectal cancer, is inhibited by the chemotherapeutic 5-fluorouracil, suggesting that 5-fluorouracil efficacy may in part be due to its antimicrobial activity. Specific intratumoral microbiota members modify 5-fluorouracil into a nontoxic form, rescuing *Fusobacterium nucleatum* and cancer epithelial cell growth.

INTRODUCTION

Colorectal cancer (CRC) tumor cells exist within a complex microenvironment that include intimate associations with certain members of the bacterial microbiota.¹ Worldwide, genomic analyses consistently reveal enrichment of the bacterial species *Fusobacterium nucleatum* (*Fn*) in human colon cancers relative to noncancerous colon tissues.²⁻⁸ Several studies over the last decade have revealed that *Fn* contributes to tumorigenesis and accelerated cancer cell growth.⁹⁻¹⁴ A high burden of *Fn* in the tumors of patients with CRC is associated with resistance to chemotherapy, disease recurrence, metastasis, and poorer survival.^{6,7,14-18} The benefit of chemotherapy is limited for patients with CRC,¹⁹ especially in late-stage disease, highlighting the clinical need for more effective treatments to combat this disease. Our prior research demonstrated that treatment of *Fn*-positive human CRC xenografts in mice with the antibiotic metronidazole significantly reduced tumor growth and cancer cell proliferation,¹⁸ suggesting that targeting *Fn* could be a therapeutic approach

for a subset of patients with CRC. Owing to the broad-spectrum antimicrobial activity of metronidazole, which can also target beneficial members of the gut microbiota, an initial focus of this work was to identify *Fn* inhibitors with narrow spectrum activity via a small-molecule screen of 1,846 bioactive compounds.

Surprisingly, our screen identified 5-fluorouracil (5-FU), the primary chemotherapeutic used to treat patients with CRC, as a potent inhibitor of *Fn* CRC strain growth. By exploring 5-FU toxicity toward other dominant CRC-associated bacterial species that routinely co-occur with *Fn*, we show that *Escherichia coli*, *Bacteroides fragilis*, *Bifidobacterium breve*, and *Parvimonas micra* isolates from CRC tumors are resistant to physiologically relevant concentrations of 5-FU. Analysis of *ex vivo* CRC microbiota in the presence of 5-FU demonstrates that bacterial community members can resist 5-FU toxicity and potentially reduce drug bioavailability, thereby protecting both CRC tumor cells and sensitive bacterial strains. These data suggest that the interplay between the intratumoral microbiota and chemotherapeutics could inform the design of individual treatment regimes for patients with CRC.

RESULTS

Bioactive compound library screening for inhibitors of *Fn*

Initially, we sought to determine which classes of drugs could inhibit *Fn* growth. In a pilot assay, we screened a random selection of 1,846 small molecules (32 μ M) from the Broad Institute's "Bioactive Compound" library for their ability to inhibit the growth of a human CRC isolate *Fn* subsp. *animalis* (*Fna*) SB010¹⁸ (Figure 1A; Table S1). We identified 34 inhibitory compounds of *Fn* growth in broth culture (Figures 1B and S1A). Approximately half of those identified (56%) are known antimicrobial compounds. Interestingly, 15% of inhibitors are classified as antineoplastic agents that act upon either thymidylate synthase, estrogen receptors, or topoisomerase II (Figure 1C). Among these, the mainstay CRC chemotherapeutic drug tegafur and its active metabolite 5-FU were identified.

To validate and expand upon these initial findings, we monitored *Fn* growth in the presence of 24 chemotherapeutics using 8-point dose-response curves ranging from 0.23 to 30 μ M. We found that 5-FU and its prodrug tegafur are potent inhibitors of *Fn* growth (Figures 1D, S1C, and S1D; Table S2). Capecitabine, another prodrug of 5-FU, was found to have no impact on *Fn* growth *in vitro* under these conditions. The difference between 5-FU prodrug inhibition of *Fn* growth *in vitro* may be due to the enzymatic pathways required for prodrug activation. For example, tegafur is converted to 5-hydroxytegafur and then 5-FU in the presence of a cytochrome p450, a family of enzymes that are ubiquitous in prokaryotes and eukaryotes.²⁰ However, conversion of the prodrug capecitabine to 5-FU is more complex and dependent on multiple eukaryotic enzymes located in the mammalian liver²¹ (Figure S1E). In clinical settings, patients with metastatic CRC are treated with 5-FU stabilized with folinic acid in combination with either oxaliplatin or SN-38, the active metabolite of irinotecan (FOLFOX or FOLFIRI, respectively).²² We found that these compounds did not significantly impact *Fn* growth *in vitro* (Figure 1D). These initial data suggest that standard chemotherapy for CRC could concomitantly inhibit *Fn* growth at the tumor site.

5-FU is a potent inhibitor of *Fn* CRC clinical isolates

Fn is one of the most dominant CRC-associated bacterial species;^{2,3} however, sensitivity of CRC *Fn* clinical isolates to 5-FU, the primary chemotherapeutic used to treat CRC, has not been previously explored. We therefore evaluated the half-maximal inhibitory concentration (IC50) of 5-FU in 14 strains of *Fn*, comprising isolates from CRC tumor tissue (n = 11), the oral cavity (n = 2), and inflamed irritable bowel disease tissue (n = 1). IC50 values were determined from 8-point dose-response curves generated after 48 h of incubation with 5-FU, a time frame chosen to reflect a typical 2-day continuous infusion of 5-FU within a clinic setting. We defined sensitivity as IC50 values lower than the concentration of 5-FU found in patient sera (2.5–10 μ M)^{23–25} and resistance as IC50 values higher than this range. These analyses revealed that 5-FU is a potent inhibitor of the majority of *Fn* strains tested with IC50 values ranging from 0.14 to 4.3 μ M (Figures 2A and S2A–S2J). Genomic variability within *Fn* is extensive, and currently each strain can be assigned into one of four subspecies: subsp. *nucleatum* (*Fnn*), *animalis* (*Fna*), *polymorphum* (*Fnp*), or *vincentii/fusifforme* (*Fnv*).^{26,27} *Fna* is the most prevalent subspecies found in CRC in epidemiological studies,^{28,29} and we therefore sampled *Fna* at a higher frequency (10/14 isolates tested). We confirmed that 5-FU is a potent growth inhibitor of CRC *Fn* isolates representing all four subspecies, suggesting that 5-FU sensitivity is a core feature of the *Fn* species (Figure 2B). These findings indicate that the growth of *Fn* is inhibited by 5-FU exposure at physiologically relevant levels.

A CRC isolate of *E. coli* modifies 5-FU and abrogates toxicity toward *Fn* and CRC epithelial cells

Post-chemotherapeutic treatment, studies have found *Fn* persists in distant site CRC metastases,¹⁸ and a high load of *Fn* in primary CRC tissue is positively correlated with increased risk of disease re-occurrence.^{14,16} Furthermore, in patients with locally advanced rectal cancer, *Fn* positivity after fluoropyrimidine-based neoadjuvant chemotherapy significantly increases the risk of relapse.¹⁶ This suggests that in a subset of patients, *Fn* can endure chemotherapy despite 5-FU toxicity, and we hypothesize *Fn* survival impacts disease progression in patients harboring these strains. We hypothesized that co-occurring bacterial species in CRC tumors might protect *Fn* from 5-FU through sequestration or modification of the compound, lowering drug toxicity toward nearby cells in colonized microniches.

5-FU is a uracil analog that acts as a pan-antimetabolite and inhibits the activity of thymidylate synthases, enzymes that are highly conserved between humans and bacteria.³⁰ To test whether 5-FU acts as a broad antimicrobial toward bacterial species commonly isolated from CRC tissues, we measured 5-FU sensitivity of CRC tumor isolates of *B. fragilis* (SB210), *E. coli* (SB209), *B. breve* (SB213), and *P. micra* (SB214) using 13-point dose-response curves. Of note, we chose *B. breve* as one of our initial organisms because this genus is associated with a positive patient response in some cancer therapies.³¹ All species tested were resistant to physiologically relevant concentrations of 5-FU (2.5–10 μ M) (Figure 3A). Therefore, we reasoned that these strains may harbor mechanisms capable of detoxifying 5-FU. We monitored the concentration of extracellular 5-FU in the presence of these four bacterial strains over 48 h using liquid chromatography-mass spectrometry (LC-MS) and observed 5-FU depletion from extracellular media specifically in the presence

of the CRC *E. coli* strain SB209 within 24 h (Figure 3B). Local depletion of 5-FU has the potential to relieve toxicity on nearby sensitive bacterial species. To test if bacterially mediated 5-FU depletion influences *Fna* viability, we cultured *Fna* SB010 in conditioned media with 4 μ M 5-FU (~8 times the IC50 of this strain) that was previously exposed to *E. coli* or *B. fragilis* strains. Only the *E. coli* modified 5-FU rescued *Fna* SB010 growth. *B. fragilis*, which did not deplete 5-FU from media, failed to abrogate 5-FU's toxicity (Figure 3C). Additionally, we assessed a colibactin positive (*pks*+) strain of *E. coli*, owing to their prevalence in up to 55% of tissues from patients with CRC,^{32,33} and similarly observed rescue of *Fna* SB010 growth (Figure 3C). Addition of fresh 5-FU to these bacterial supernatants led to inhibition of *Fna* SB010, suggesting that *Fna* protection against 5-FU was mediated directly by *E. coli* cells and not due to an unknown protective component present in the supernatant (Figure 3C). Furthermore, inoculation of *E. coli* SB209 and *Fna* SB010 together at T0 of 5-FU exposure protects *Fna* SB010 from 5-FU toxicity (74% viability after 48 h; $p = 0.0002$) (Figure S3A). LC-MS confirmed that 5-FU disappearance occurs within 8 to 24 h in multispecies co-cultures of *Fna* SB010, *E. coli* SB209, and *B. fragilis* SB210 (Figure S3B). To gain further insight into how *E. coli* interacts with 5-FU, we performed RNA sequencing (RNA-seq) on *E. coli* SB209 exposed to 0, 5, and 40 μ M 5-FU for 8 h. In the presence of 5-FU, there was a significant increase in genes responsible for uracil metabolism to dihydrouracil (*preT* and *preA*) and pseudouridine (*psuG* and *psuK*), and down-regulation of genes involved in uracil to dTTP synthesis (Figures S3C and S3D; Table S3). The *preTA* operon in *E. coli* shares homology with human dihydropyrimidine dehydrogenase and is demonstrated to detoxify 5-FU to dihydrofluorouracil.³⁴

These findings led us to question if *E. coli* modification of 5-FU could alter its chemotherapeutic efficacy toward CRC cells. To evaluate this, we cultured a 5-FU sensitive (IC50: 5 μ M)³⁵ human CRC epithelial cell line (RKO) with *E. coli*-exposed 5-FU (5 μ M) and monitored cell growth over 72 h. Remarkably, prior exposure of 5-FU to *E. coli* (CRC strain SB209 and *pks*+) strains) completely abrogated 5-FU toxicity against human CRC epithelial cells (Figure 3D). In total, these results suggest that bacteria-mediated depletion of 5-FU reduces drug efficacy against neighboring bacteria cells and human CRC tumor epithelial cells.

CRC patient-derived *ex vivo* tumor microbiota interact with 5-FU

Based on our findings that certain members of the microbiota can detoxify 5-FU, we next sought to determine if 5-FU exposure affects the CRC microbiota community structure by facilitating expansion of 5-FU-resistant bacterial species. To generate *ex vivo* CRC communities, treatment-naïve CRC tissue ($n = 6$ patients) was manually homogenized in bacterial broth and passaged through a 16G needle until homogeneous. Human cells and debris were pelleted by gentle centrifugation at $300 \times g$, leaving the tissue-associated microbiota in suspension. The remaining bacterial cells were incubated either in broth only or in the presence of a high dose of 5-FU (30 μ M) under anaerobic conditions for 48 h. The resulting community structure was then determined through metagenomic sequencing (Figure 4A; Table S4). Bacterial presence in an aliquot of these patient tumors was examined prior to dissociation via RNAscope fluorescence *in situ* hybridization (FISH) (Figures 4B and S4A-S4F). Metagenomic analyses revealed that, indeed, exposure to 5-FU alters the

relative abundance of community members, allowing for the expansion of 5-FU-resistant bacterial species (patients CRC_01, CRC_04, and CRC_06).

However, some patient community structures appeared relatively stable, which suggests resilience of these populations toward 5-FU toxicity (patients CRC_02, CRC_03, and CRC_05) (Figure 4C; Table S4). To determine if any of these *ex vivo* CRC communities can impact 5-FU availability, we assessed 5-FU levels in the presence of these patient's *ex vivo* microbiota over 48 h. We observed rapid 5-FU depletion in 50% of patient *ex vivo* CRC communities (Figure 4D), with the tumor microbiota from patient CRC_06 (Figure 4D) being the most efficient at removing 5-FU from media. To delineate the species responsible for 5-FU modification in patient CRC_06, we cultured isolates of the three most abundant species within the community (*B. fragilis*, *B. thetaiotaomicron*, and an *E. coli*) and again assessed 5-FU levels over time via LC-MS. In agreement with our previous observation, a strain of *E. coli* was responsible for 5-FU disappearance (Figure 4E). Further analysis of the metagenomic data found that the patient microbiome communities that demonstrated removal of 5-FU (CRC_01, CRC_04, and CRC_06), which we refer to as the “modifier” group (Figure S4H; Table S5), had a significant increase in the relative abundance of *preT* and *preA* transcripts in the presence of 5-FU (Figure S4G; $p = 0.03$).

Additionally, random forest analysis of the top 20 features/species in the metagenomics data identified *E. coli* as the classifier for the “modifier” group following 5-FU exposure and, overall, had the highest mean decrease accuracy value supporting the importance of *E. coli* as a classifier in the model (Figure S4G). Furthermore, linear discriminant analysis effect size (LEfSe) analysis identified *E. coli* as being significantly associated with the “modifier” group following 5-FU exposure (Figure S4J). The association of *E. coli* with the “modifier” group was further supported by classical univariate statistical analysis ($p < 0.05$; Figure S4K).

Together, these results offer support to the hypothesis that in a subset of patients with CRC, the intratumoral microbiota harbor the ability to deplete 5-FU, which could lower chemotherapeutic efficacy within colonized microniches. Importantly, 5-FU reduction would protect both 5-FU sensitive tumor-supportive bacteria such as *Fn* and resident cancer cells from the drug's toxicity. These findings support the benefits of taking the tumor-associated microbiota into consideration when stratifying patients into risk categories for 5-FU resistance, especially in the setting of neoadjuvant chemotherapy prior to tumor resection.

DISCUSSION

Bacterial members of the intratumoral microbiota are metabolically active in CRC and, along with malignant cells, interact with the antimetabolite chemotherapeutic 5-FU. Our analyses reveal that interactions between the CRC tumor microbiota and 5-FU are highly complex, and bacterial community members appear to fit into three distinct categories in relation to the drug: highly sensitive (e.g., *Fn*), resistant (e.g., *B. fragilis*, *B. breve*, and *P. micra*), and resistant and depleting (e.g., *E. coli*). Analysis of community composition in *ex vivo* CRC microbiota after exposure to 5-FU demonstrates that there is considerable loss of species diversity in a subset of communities of patients with CRC, suggesting that

in addition to *Fn*, other bacterial species may be sensitive to 5-FU, which has also been noted in recent a pre-print,³⁴ albeit not including CRC isolates. Conversely, bacterial species present in CRC tumors have the potential to internalize and detoxify 5-FU, likely through dedicated nucleoside import and pyrimidine scavenging pathways.³⁶⁻⁴⁰

Fn is highly enriched in CRC tissue, and multiple studies indicate that *Fn* is pathologically and clinically associated with cancer recurrence and patient outcomes.^{14,16,18,41} We found that 5-FU has potent antibacterial activity against CRC tumor isolates of *Fn* (n = 11), with an average IC50 in the nanomolar range (720 nM), indicating that 5-FU-based chemotherapeutic treatment could inhibit the growth of this oncomicrobe within patient tumors. As a chemotherapeutic, 5-FU provides the greatest efficacy in CRC compared with other cancer types,¹⁹ raising the intriguing possibility that 5-FU treatment efficacy could in part be due to its unanticipated role as a potent antimicrobial agent against dominant members of the intratumoral microbiota, including *Fn*.

The presence of *Fn* is also associated with cancer recurrence after treatment. A study by Yu et al.¹⁴ found that *Fusobacterium* was consistently enriched and had higher bacterial loads in recurrent CRC tissues compared with nonrecurrent CRC tissues across multiple patient cohorts. In a congruent study, Serena et al.¹⁶ detected *Fn* in 58% of treatment-naïve tumor biopsies from patients (n = 143) with locally advanced rectal cancer and discovered that *Fn* persists in 26% of tumors treated with fluoropyrimidine-based neoadjuvant chemoradiotherapy (nCRT).¹⁶ *Fn* positivity in tissue after nCRT, but not its baseline status in tissue, was significantly correlated with a 7.5-fold increased risk of relapse in patients.¹⁶ This work supports both studies; we expect that the subset of patients where *Fn* survives 5-FU toxicity likely contain an intratumoral bacterial community capable of metabolizing 5-FU, protecting both cancer and sensitive bacterial cells such as *Fn* and promoting CRC recurrence. To expand upon this, prospective studies that investigate tumor microbiomes of biopsies pre-chemotherapy, and resections post-chemotherapy, in addition to patient treatment response are warranted.

We identify multiple strains of *E. coli* that lead to 95% depletion of available 5-FU as early as 24 h post-exposure (Figures 3B, 4E, and S3B). *E. coli*-exposed 5-FU no longer inhibits CRC epithelial cell growth, and this treatment is protective of *Fn* isolated from tumors. These results complement research from two recent studies that reported that 5-FU efficacy was altered in *Caenorhabditis elegans* that were fed genetically different strains of *E. coli*.^{42,43} In humans, 5-FU is detoxified through metabolic conversion to dihydrofluorouracil (DHFU) by the protein dihydropyrimidine dehydrogenase (DPD).^{44,45} Prior research has demonstrated that *E. coli* harbors a functional homolog of human DPD within an operon called *preTA* that can convert uracil into 5,6-dihydrouracil *in vivo*.⁴⁶ Additionally, it was demonstrated that bacterial homologs of PreTA, including those in *E. coli*, can metabolize 5-FU into nontoxic DHFU *in vitro*.³⁴ Since *pks+* *E. coli* is prevalent in tumor tissue from patients with CRC,^{47,48} it has the potential to contribute to the local depletion of 5-FU and repression of its toxicity on otherwise-sensitive cells in the tumor. This work is consistent with a growing body of literature supporting that microbiota composition has a dramatic impact (both directly and indirectly) on the chemotherapeutic efficacy of many drugs in

a wide variety of cancers.^{1,49} Collectively, these findings support that the composition of microbiota might inform the design of patient treatment regimens in CRC.

Our *in vitro* work suggests that microbiota-associated modification of 5-FU might contribute to patient 5-FU chemoresistance. In humans, changes in expression of thymidylate synthase, nucleoside importers, and multidrug efflux pumps contribute to chemoresistance to 5-FU in patients.⁵⁰⁻⁵² Given that these classes of proteins also exist in many bacterial species,^{30,37,52} it is possible that repeat exposure selects for mutations in these pathways leading to 5-FU tolerance.⁵³ Clinical studies that assess the co-occurrence of intratumoral *Fn* and 5-FU-modifying microbiota, such as *E. coli*, along with patient response to 5-FU-based chemotherapeutics are warranted. A more detailed understanding of the mechanisms underlying bacterial-mediated 5-FU chemoresistance could aid in stratifying patients for combinatorial treatment with chemotherapeutics and targeted antimicrobials; informed by microbial profiling of CRC biopsies.

Limitations of the study

All *ex vivo* CRC microbiota were cultured under strict anaerobic conditions, which may lead to loss of microbial isolates that prefer microaerophilic or aerobic conditions for growth. Growth of the *ex vivo* CRC microbiota in rich broth for 48 h following isolation from tissue resulted in the enrichment of fast-growing bacterial taxa that may ultimately outcompete more fastidious taxa, such as *Fusobacterium*, and lower species diversity in these communities. In this study, we have only assessed microbes in liquid media and have not assessed the impact of bacterial biofilms on chemotherapeutic sensitivity or resistance.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Susan Bullman (sbullman@fredhutch.org).

Materials availability—This study did not generate new unique reagents.

Data and code availability

- Metagenomic sequencing and RNA-seq data have been deposited in the NIH Sequence Read Archive (SRA) and are publicly available as of the date of publication (PRJN817195 and PRJNA859520 respectfully). The BioProject numbers are listed in the key resources table. Microscopy, mass spectrometry, and cell viability measurements reported in this paper will be shared by the lead contact upon reasonable request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects and sample collection—The six patient specimens included in this study were collected under Fred Hutchinson Cancer Center IRB protocol RG1006974. All six patients were treatment naive, and tissue was collected during tumor resection. We did not have access to information on patient’s preoperative treatment or antibiotic use prior to resection. Tissue was cryopreserved and stored at –80°C until use, multiple aliquots were available per patient. Tumor pieces used for RNAscope imaging were formalin fixed and paraffin embedded.

Patient	Gender	Age	Tumor	Geographical location	Specimen storage	Treatment
CRC_01	Female	67	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive
CRC_02	Male	20	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive
CRC_03	Female	46	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive
CRC_04	Male	39	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive
CRC_05	Female	67	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive
CRC_06	Female	77	Colon Adenocarcinoma	Washington, USA	Cryopreserved	Naive

Microbial strains—All bacteria were grown from cryostocks on fastidious anaerobe agar plates (FAA; Neogen) supplemented with 10% defibrinated horse blood (DHB; HemoStat Laboratories). For *Fusobacterium* culture FAA+10% DHB plates included josamycin (3 µg/mL), vancomycin (4 µg/mL), and norfloxacin (1 µg/mL) for selective culturing (JVN). For liquid-based assays, bacteria were incubated in either tryptic soy broth (TSB; Sigma Aldrich), brain heart infusion broth (BHI; Research Products International), or fastidious anaerobe broth (FAB; Neogen). All strains were stored at –80°C in TSB supplemented with 40% (v/v) glycerol. All bacterial culturing occurred under anerobic conditions in an anaerobic chamber (Anaerobe Systems AS-580; 5%H2, 5%CO2, 90% N2) or sealed box with an AnaeroGen gas pack (Oxoid) and incubated at 37°C. *Fn* isolates, *P. micra*, *B. fragilis*, and *B. breve* were cultured for 2 days on FAA +10% DHB prior to assay inoculation. *E. coli* was cultured for 1 day on FAA +10% DHB prior to assay inoculation. List of strains used in this study can be found in key resources table.

Cell lines—Human colon cancer epithelial RKO cells (ATCC CRL-2577) were grown in McCoys 5A with L-glutamine (Corning) supplemented with 10% (v/v) fetal bovine serum (Sigma). Antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin; Gibco) were included in the growth medium during maintenance of cell lines and removed 24 h prior to bacterial co-culture. Cell cultures were incubated at 37°C in 5% CO2.

METHOD DETAILS

Bioactive library screen and dose-response validation

Bioactive library screen: 1,846 compounds were printed in duplicate on the bottom of 384 well plates by the Broad Therapeutics Platform (Table S1). The addition of 30 µL of liquid in each well results in 32 µM compound. Each plate had multiple neutral controls (TSB only) and inhibitory controls (5 µg/mL metronidazole in TSB).

Dose-response validation of inhibitory compounds and antineoplastic agents: 384 well plates printed with eight concentrations of each compound in duplicate (30, 15, 7.5, 3.75, 1.88, 0.94, 0.47, and 0.23 μ M). These plates included the 34 inhibitory compounds confirmed in both biological replicates of our pilot screen and an additional 21 antineoplastic agents of interest (Table S2). Each plate had multiple neutral controls (TSB only) and inhibitory controls (5 μ g/mL metronidazole in TSB).

Plate inoculation and viability determination: Each well was inoculated with 30 μ L of 2×10^6 colony forming units (CFU) per mL of *Fna* SB010 resuspending in TSB. Plates were incubated anaerobically at 37°C for 48 h. Cells were lysed via the addition of 10 μ L BacTiter Glo (Promega) and ATP levels measured through luminescence detection on a PerkinElmer Envision. Spectrometry parameters: read time = 0.1 s, plate height = 0 cm.

Data analysis: The inhibitor hit cut-off was set at three standard deviations from the mean of the neutral control (untreated bacterial cells: *Fna* plus SB010 TSB only).

Bacterial dose-response curves—5-fluorouracil (5-FU; >99% purity; Tokyo Chemical Industry) stocks were stored as 5 mg/mL aliquots in dimethyl sulfoxide (DMSO) at 4°C for up to one month. Dose-response plates were set up through the dilution of 5 mg/mL 5-FU stock in BHI +0.1% DMSO to the desired upper concentration, then serially diluted 2-fold in BHI +0.1% DMSO until the desired lower concentration was reached. The lowest 5-FU concentration used was 0.0375 μ M and the highest concentrations reached 4.8 to 38.4 μ M 5-FU for 8 pt and 11 pt dose-response curves respectively. The control for positive inhibition (0% expected growth) for *Fn* isolates, *B. fragilis*, and *B. breve* was 5 μ g/mL metronidazole +0.1% DMSO in BHI. The positive inhibition control for *P. micra* and *E. coli* was 30 μ g/mL gentamycin in BHI +0.1% DMSO. The negative inhibition control (100% growth) was the base BHI +0.1% DMSO media. Every plate tested included additional no bacteria controls wells of 5-FU, metronidazole, gentamycin, and broth alone to monitor for any sources of external bacterial contamination. Each well was inoculated with 5 μ L of 1×10^8 CFU/mL of *Fn* and *P. micra* (final: 5×10^4 CFU/well/100 μ L) or 5 μ L of 2×10^7 CFU/mL *B. fragilis*, *B. breve*, and *E. coli* (final: 1×10^4 CFU/well/100 μ L). Of note, lower inoculating CFU's were used for *B. fragilis*, *B. breve*, and *E. coli* compared to the slower growers *Fn* and *P. micra*. Plates were incubated in a 2.5L sealed box at 37°C for 48 h (hours) under anaerobic conditions using an AnaeroGen gas pack. Bacterial viability was determined through ATP level measurements using a BacTiter Glo microbial cell viability assay (Promega). Cells were lysed through addition of 33 μ L BacTiter Glo and ATP levels were measured through luminescence quantification on a spectrophotometer (BioTek Synergy H4) using the following parameters: Gain: 135, Integration Time: 1 s, Read Height: 4.5 mm).

Bacterial co-culture with 5-FU and the generation of “modified” 5-FU supernatants

For *Fn* protection or mass spectrometry assays: 4 μ M 5-FU was incubated with 5×10^8 CFU/mL bacteria in a total volume of 1 mL of broth (TSB for *Fn* protection assays and BHI for mass spectrometry) for 48 h at 37°C. For patient-derived ex-*vivo* CRC communities, initial communities were diluted 1/50 in 4 μ M 5-FU in 1 mL of FAB for 48 h. Broth media

+400 μ M 5-FU (100x) was incubated for 48 h at 37°C in order to generate the “fresh” 5-FU control.

For use in mammalian cell culture assays: 20 μ M 5-FU was incubated with 5×10^8 CFU/mL bacteria in a total volume of 1 mL McCoy's 5A + L-glutamine + 10% FBS for 48 h. Cells were then pelleted at 7000 $\times$ g for 3 min and resulting supernatants filtered through a 0.2 μ m filter. Media alone with 2 mM 5-FU (100x) was incubated for 48 h at 37°C in order to generate the “fresh” 5-FU control.

Supernatant generation: Cells were then pelleted at 7000 $\times$ g for 3 min and resulting supernatants filtered through a 0.2 μ m PVDF filter (Millipore). The no bacteria “fresh” 5-FU controls were also filtered in this way to account for any potential loss of 5-FU by filtration. For the “fresh” 5-FU conditions, bacterial supernatants (500 μ L) that had grown in broth alone were spiked with the 100X “fresh” 5-FU control (5 μ L).

***Fn* “modified” 5-FU protection assays:** *Supernatant protection:* 5×10^6 CFU/mL *Fna* SB010 was grown in *B. fragilis*, *E. coli* SB209, or *E. coli pks* + supernatants incubated in TSB alone, TSB +4 μ M 5-FU (modified), or TSB alone supplemented with 4 μ M 5-FU after bacterial filtration (fresh). *Fn* was grown for 48 h at 37°C under anaerobic conditions, and bacterial viability was measured through ATP determination with BacTiter Glo described in detail in the “bacterial dose-response curves” methods section. 100% growth was set to *Fna* SB010 viability in TSB alone, or in each bacterial supernatant along without the addition of 5-FU.

Direct co-culture protection: 5×10^6 CFU/mL *Fna* SB010 was incubated with 4 μ M 5-FU alone or with the addition of 1×10^6 CFU/mL *E. coli* SB209 for 0, 24, and 48 h at 37°C under anaerobic conditions. As a control, *Fna* SB010 was also incubated in media alone or with 1×10^6 CFU/mL *E. coli* SB209 alone without the addition of 5-FU. Bacterial viability was monitored at 0, 24, and 48 h by 10-fold serial dilutions and plating for CFU. *Fna* SB010 colonies were isolated through plating on FAA +10% DHB +30 μ g/mL gentamycin and incubating under anaerobic conditions. *E. coli* SB209 colonies were isolated through plating on FAA +10% DHB and incubating under aerobic conditions.

CRC epithelial cell “modified” 5-FU protection assays

Supernatant protection: RKO cells were seeded into a clear bottom white-96 well plate at a density of 5000 cells/well (100 μ L total volume) and incubated for 16 h at 37°C to allow for surface attachment. Filtered bacterial supernatants 48 h incubation with media alone (McCoy's 5A), 20 μ M 5-FU (modified), or media alone supplemented with 20 μ M 5-FU after bacterial filtration (fresh) were added to RKO cells cell culture (Passage 4, 50% confluency) media in a 1:4 dilution (33.33 μ L) for an anticipated final concentration of 5 μ M 5-FU (the reported IC₅₀ for RKO cells). RKO cells were then incubated for 72 h at 37°C in an IncuCyte S3 live imaging incubator (Sartorius and Essun BioScience). Each well was imaged in five locations with a 10X objective every two hours for a total of 72 h (Camera: Basler Ace 1920-155 μ m CMOS). RKO cell growth over time was determined by calculating

the % confluence of each well after training the IncuCyte Live Cell-Analysis software on RKO cell parameters and filtering out debris (volumes <150 μm^3).

RNAscope-based fluorescent *in situ* hybridization—Paraffin embedded sections (5 μm were deparaffinized and hybridized using RNAscope Multiplex Fluorescent Assay V2 (Advanced Cell Diagnostics, Inc.) in accordance with the manufacturer's protocol. The EB-16s-rRNA probe targeting the 16s rRNA of all bacteria. Whole slide scanning was performed on a Vectra Polaris multispectral imaging system (Akoya Biosciences Inc.) at 40x (25 $\mu\text{m}/\text{pixel}$). Sample and corresponding negative control probe slides were scanned under the same exposure conditions.

5-FU analysis through mass spectrometry—5-FU from bacterially modified media samples was analyzed by liquid-chromatography with tandem quadrupole mass spectrometry (LC-MS/MS QQQ) using a Waters Xevo-XS with a Waters Acquity I-Class UPLC. Samples were mixed 1:1 with 0.4 μM of a heavy, stable isotope of 5-FU in $\text{d}_2\text{H}_2\text{O}$ (98 atom % ^{15}N , 99 atom % ^{13}C ; empirical formula: $^{13}\text{CC}_3\text{H}_3\text{F}_3^{15}\text{N}_2\text{O}_2$; Millipore Sigma) that acted as an internal standard for 5-FU quantitation. Chromatographic separation was achieved using a Thermo Hypercarb column (2.1 $\times$ 50 mm, 3 μm) at room temperature. The mobile phase consisted of solution A: 100 mM NH_4OAc in H_2O , pH 9.5 using NH_4OH , and solution B: 0.1% NH_4OH in acetonitrile. A flow rate of 0.3 mL/min was used with the following gradient elution profile: 100% A from 0-6 min, 50% A 50% B from 6-7.1 min, 100% A from 7.1-10 min. 50 μL of sample was injected for LC-MS/MS QQQ analysis. This assay used multiple reaction monitoring (MRM) in electrospray negative ionization, and the ions measured for the quantitation were: 5-Fluorouracil: 129.03->42.16 & 129.03->129.0 and C13-5-Fluorouracil: 132.1->60.1 & 132.1->44.1. The retention time for 5-fluorouracil in the column and gradient was 2.65 min. Quantitative analysis for 5-FU was performed using an isotope dilution method with a 7 pt calibration curve (4-fold dilutions starting at 20 μM) run in duplicate with a linear regression coefficient of $R^2 = 0.9998$. The level of quantitation achieved was 0.078 μM and spanned to 20 μM .

RNA sequencing—An overnight culture of *E. coli* SB209 in BHI broth was diluted to OD 0.5 (5×10^8 CFU) and incubated with 0, 5, or 40 μM 5-FU for 8 h at 37°C under anaerobic conditions. Cells were pelleted at 8,000 rpm for 3 min and washed once in 1xPBS to remove residual 5-FU and DMSO. Cells were pelleted, washed once in RNAlater (Thermo Fisher), pelleted again and all supernatant removed prior to storage at -80°C. RNA was extracted using the RNeasy Extraction Kit (Qiagen) for Illumina Stranded RNA library preparation with RiboZero Plus rRNA depletion. RNA library was sequenced to a minimum read count of 12M paired-end reads.

Metagenomic analysis methods—Metagenomic sequencing reads were analyzed using the *geneshot* analysis workflow.⁵⁴ All eggNOG database cluster of orthologous groups (COG) tags for PreT and PreA were used to identify and quantify the relative abundance of *preT* and *preA* reads per patient sample. For 5-FU modifier patient (CRC_01, CRC_04, CRC_06) and non-modifier patient (CRC_02, CRC_03, CRC_05) groups, the relative

abundances of *preT* and *preA* reads in 5-FU treated versus no treatment controls were compared using a paired Wilcoxon rank-sum test (RStudio).

Ex-vivo CRC tumor microbiota generation—Frozen patient tumor tissue was thawed at 37°C and tissue was transferred to a sterile Petri dish and minced with scalpels. Tissue was then transferred to a 5 mL conical tube and covered with 2.5 mL of FAB and vortexed for 30 s. Tissue was then manually ground against the bottom of the conical tube with a 5 mL serological pipette. Tissue was then passed through a 16-gauge needle until there was a uniform suspension, followed by an 18-gauge needle. Cells were centrifuged at 300xg for 4 min, and the top 2 mL of supernatant was moved into a 2 mL eppitube. The eukaryotic cell pellet and remaining 500 µL was cryopreserved for downstream microbial culturing. From this 2 mL supernatant that contained the ex-vivo tissue microbiota, 1 mL was removed and centrifuged at 7,000xg for 3 min, and the pellet stored at -20°C as marker of the initial microbial population prior to growth in broth. The remaining 1 mL of supernatant was divided into two tubes (500 µL each) and supplemented to 500 µL of FAB. One tube was supplemented with 50 µL of 600 µM 5-FU (final 30 µM). Both tubes were transported to an anaerobic chamber, opened to allow gas exchange, the sealed and incubated for 48 h at 37°C. After 48 h, 100 µL was removed, diluted 1:5 in cryoprotectant and stored at -80°C for downstream microbial culturing (eg. 5-FU disappearance assays). The remaining 900 µL was centrifuged at 7,000xg for 3 min, the supernatant removed, and the pellets stored at -20°C for future 16S rRNA sequencing and metagenomic analysis.

Shotgun metagenomic sequencing—The samples were processed and analyzed with the ZymoBIOMICS® Shotgun Metagenomic Sequencing Service for Microbiome Analysis (Zymo Research, Irvine, CA).

DNA extraction: One of three DNA extraction kits was used depending on the sample type and sample volume. The ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) was used to extract DNA using an automated platform.

Shotgun metagenomic library preparation: Genomic DNA samples were profiled with shotgun metagenomic sequencing. Sequencing libraries were prepared with either the KAPA™ HyperPlus Library Preparation Kit (Kapa Biosystems, Wilmington, MA) with up to 100 ng DNA input following the manufacturer's protocol using internal single-index 8 bp barcodes with TruSeq® adapters (Illumina, San Diego, CA) or the Nextera® DNA Flex Library Prep Kit (Illumina, San Diego, CA) with up to 100 ng DNA input following the manufacturer's protocol using internal dual-index 8 bp barcodes with Nextera® adapters (Illumina, San Diego, CA). All libraries were quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and then pooled in equal abundance. The final pool was quantified using qPCR.

Sequencing: The final library was sequenced on either the Illumina HiSeq® or the Illumina NovaSeq®.

Bioinformatics analysis: Raw sequence reads were trimmed to remove low quality fractions and adapters with Trimmomatic-0.33⁵⁵; quality trimming by sliding window

with 6 bp window size and a quality cutoff of 20 and reads with size lower than 70 bp were removed. Antimicrobial resistance and virulence factor gene identification was performed with the DIAMOND sequence aligner.⁵⁶ Microbial composition was profiled with Centrifuge⁵⁷ using bacterial, viral, fungal, mouse, and human genome datasets. Strain-level abundance information was extracted from the Centrifuge outputs and further analyzed: (1) to perform alpha- and beta-diversity analyses; (2) to create microbial composition barplots with QIIME⁵⁸; (3) to create taxa abundance heatmaps with hierarchical clustering (based on Bray-Curtis dissimilarity). MicrobiomeAnalyst⁵⁹ was used to perform: (i) the random forest analysis including the top 20 features with the number of training trees grown to 500 with $n = 7$ predictors, (ii) dot plot of LEfSe analysis⁶⁰ to identify features enriched in each group and (iii) classical univariate statistical comparisons between groups with Mann-Whitney/Kruskal-Wallis analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Most of the statistical tests were performed in GraphPad Prism 7.0 with $\alpha = 0.05$; $p < 0.05$ is indicated by asterisks with the following key: * <0.05 , ** <0.01 , *** <0.001 . The metagenomic sequencing statistics were performed with R. Statistical information for each experiment can be found within the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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Highlights

- 5-Fluorouracil (5-FU) inhibits growth of the oncomicrobe *Fusobacterium nucleatum*
- CRC strains of *Escherichia coli* rapidly modify 5-FU into a nontoxic product
- Bacterially modified 5-FU no longer inhibits cancer or *F. nucleatum* cell growth
- 50% of patient-derived *ex vivo* CRC microbial communities can reduce 5-FU levels

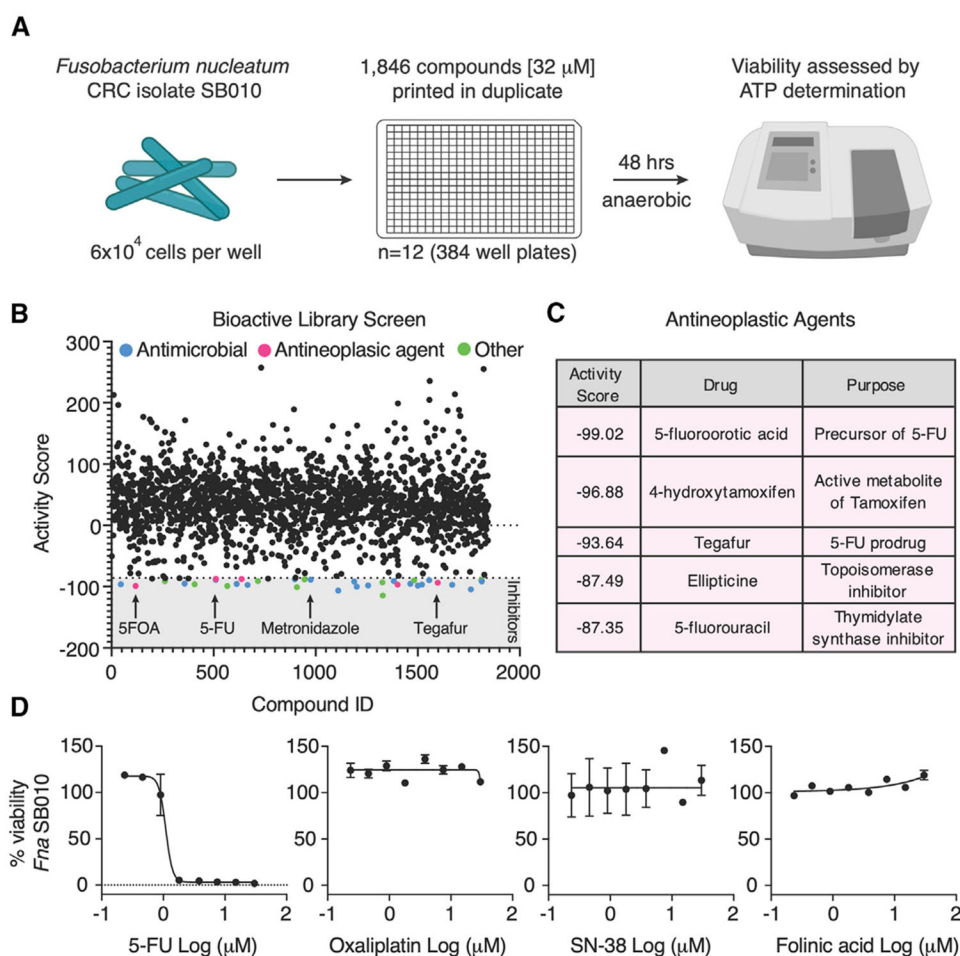


Figure 1. A bioactive library screen identifies several antineoplastic agents as *Fn* growth inhibitors

(A) Schematic describing the small-molecule screen workflow to determine *Fn* drug sensitivity.

(B) Activity scores for $n = 1,846$ compounds on *Fn* viability, determined through ATP measurements. Inhibitors are compounds that are three standard deviations away from the neutral control (media alone).

(C) A list of the antineoplastic agents found to be *Fn* inhibitors arranged by their respective activity scores.

(D) Eight-point dose-response curves of *Fn* viability after 48 h exposure to 0.25–32 μ M of the active metabolites for various chemotherapeutics used to treat metastatic colon cancer. The connecting line is a nonlinear regression of the log(inhibitor) versus response with a variable slope (four parameters). Error bars represent standard deviation (SD), $n = 3$ replicates.

See also Figure S1 and Tables S1 and S2.

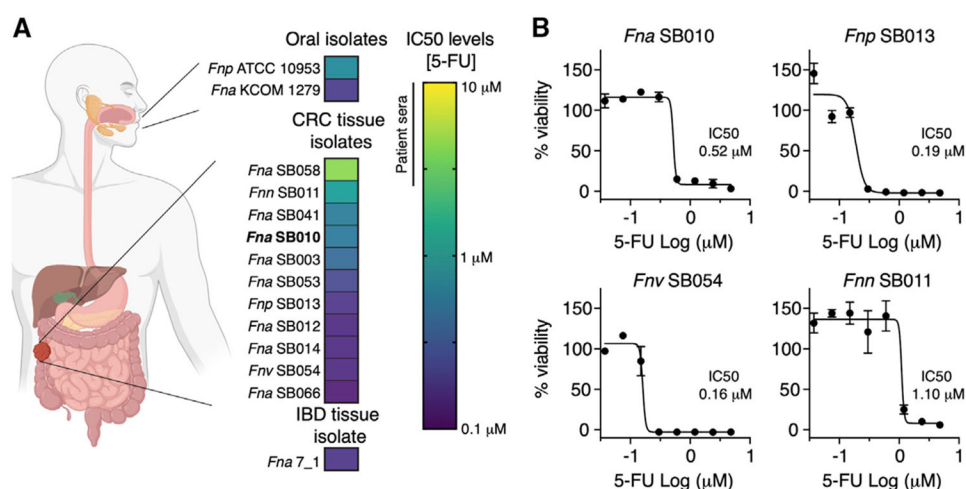


Figure 2. *Fn* isolates are sensitive to physiological concentrations of 5-FU

(A) Heatmap depicting the concentrations of 5-FU where *Fn* isolates (n = 14) are 50% viable. The clinical isolates are grouped by the sampling source. The level of 5-FU in patient sera is labeled between 2.5 and 10 μM.

(B) Eight-point dose-response curves of *Fn* viability for a single representative of each subspecies: *animalis*, *polymorphum*, *vincentii*, and *nucleatum* after exposure to 0.0375–4.8 μM 5-FU for 48 h. The connecting line is a nonlinear regression of the log(inhibitor) versus response with a variable slope (four parameters). Error bars represent SD, n = 3 biological replicates.

See also Figure S2.

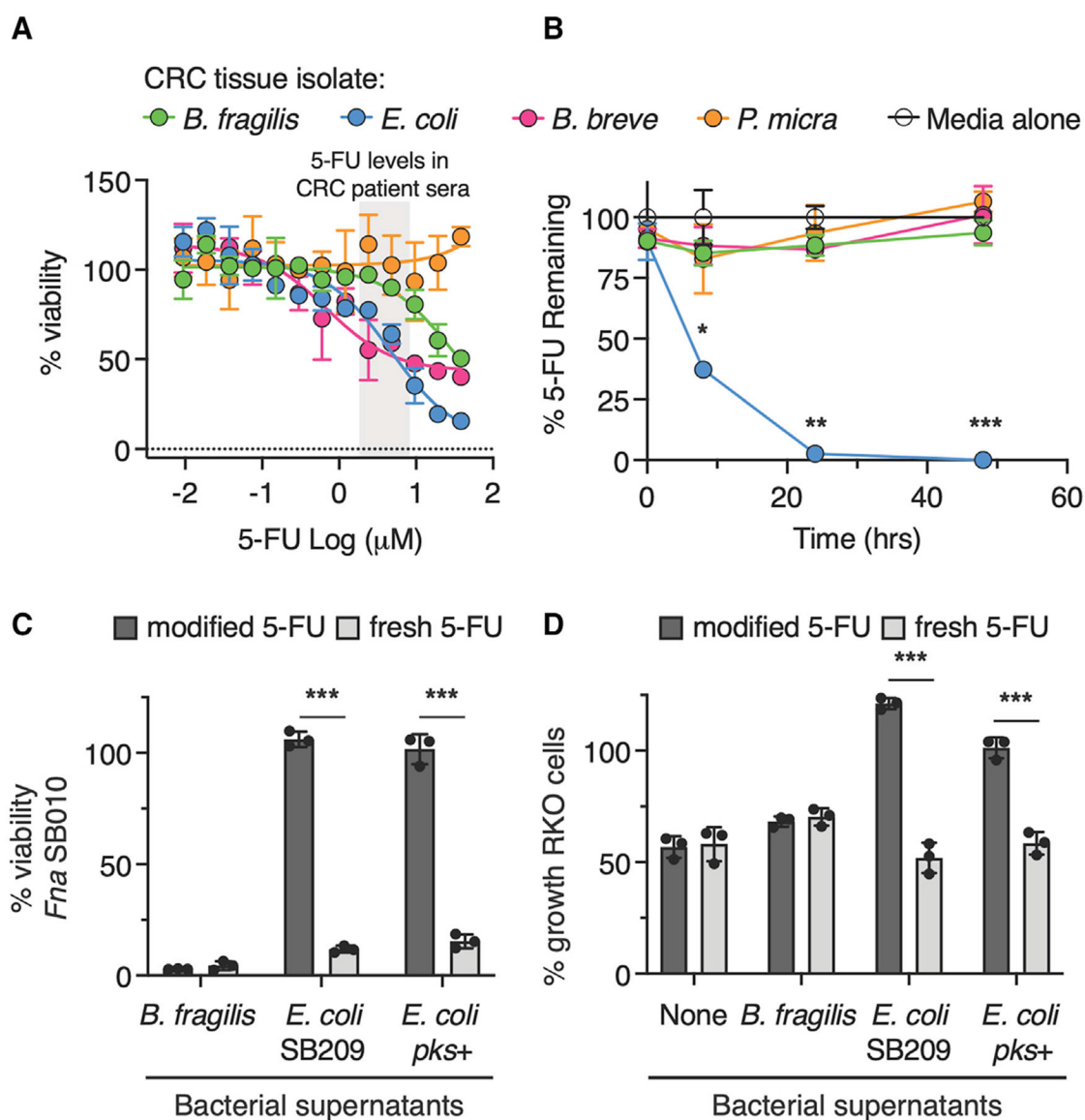


Figure 3. *E. coli* isolates deplete 5-FU and reduces drug toxicity toward *Fn* and human CRC cells

(A) Thirteen-point dose-response curves of *B. fragilis* SB210, *E. coli* SB209, *B. breve* SB213, and *P. micra* SB214 viability after exposure to 0.0023–38.4 μM 5-FU for 48 h.

The range of 5-FU in patient sera is labeled between 2.5 and 10 μM . The connecting lines are a nonlinear regression of the log(inhibitor) versus response with a variable slope (four parameters). The error bars represent SD, $n = 3$ biological replicates.

(B) Measurement of 5-FU (4 μM) disappearance in the supernatant when exposed to the indicated bacterial strains or media alone for 0, 8, 24, and 48 h 100% is set to the media-alone condition at 0 h. Error bars represent SD, $n = 3$ biological replicates.

(C) The viability of *Fna* SB010 when incubated in the conditioned supernatants of the indicated bacterial species for 48 h. The conditioned supernatants of the indicated bacterial species were incubated with 5-FU (4 μM) for 48 h (dark gray bars) or had fresh 5-FU (4 μM) added immediately prior to sterilization using a 0.2 μm filter (light gray bars). 100% is set

to the *Fn* viability in the indicated bacterial supernatants alone for 48 h. Error bars represent SD, n = 3 biological replicates.

(D) Relative growth of RKO CRC epithelial cells when incubated in conditioned supernatants of indicated bacterial species for 72 h. The conditioned supernatants were incubated with 5-FU (20 μ M) for 48 h (dark gray bars) or had fresh 5-FU (20 μ M) added immediately prior to sterilization using a 0.2 μ m filter (light gray bars). This supernatant was diluted $\frac{1}{4}$ when added to the RKO culture, resulting anticipated 5 μ M 5-FU. 0% growth is the confluency of the RKO cells in each condition at 0 h. 100% growth is the confluency of the RKO cells incubated with the indicated bacterial supernatant alone for 72 h. *p < 0.05, **p < 0.01, and ***p < 0.001 as determined by a two-sided Student's t test. Error bars represent SD, n = 3 biological replicates.

See also Figure S3 and Table S3.

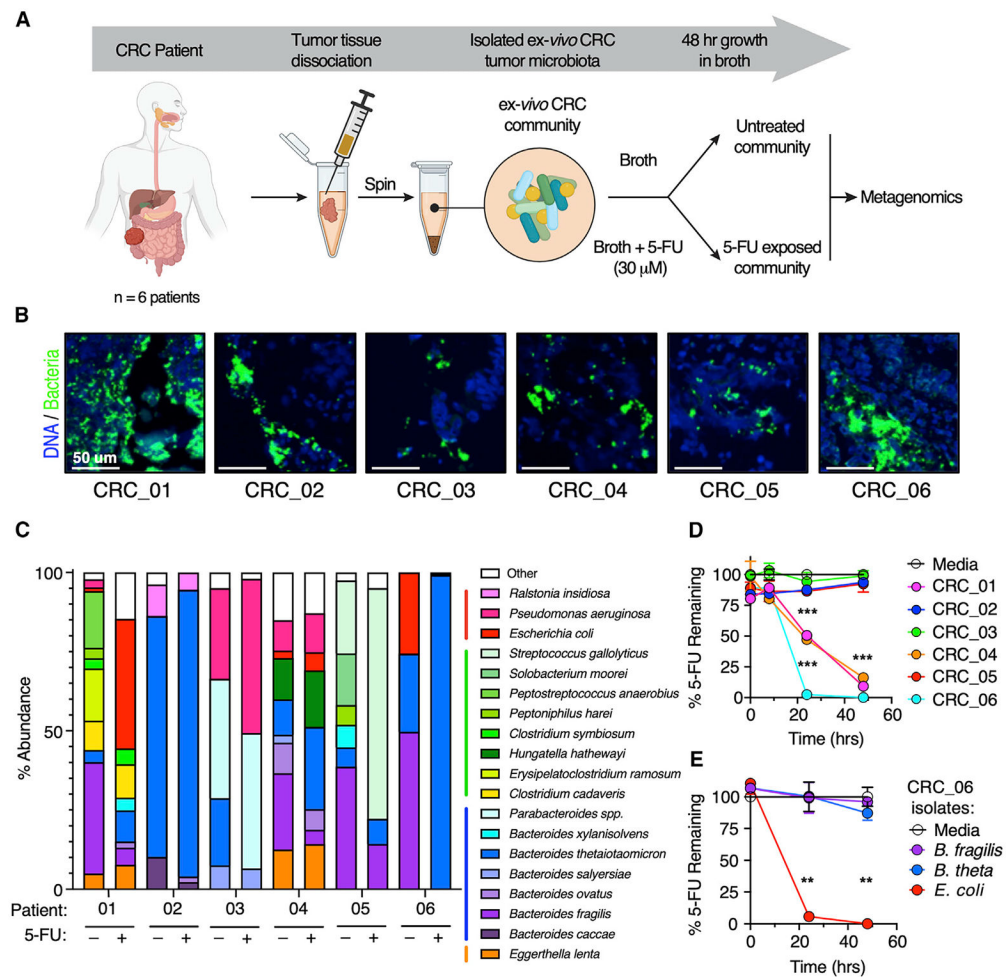


Figure 4. Patient-derived *ex vivo* CRC microbiota can deplete 5-FU and reduce chemotherapeutic toxicity

(A) Schematic depicting the workflow of patient tissue processing for bacterial community isolation and downstream 5-FU exposure for 48 h followed by metagenomic sequencing.

(B) RNAscope-based fluorescence *in situ* hybridization (FISH) of tumor tissue from patients with CRC (n = 6 patients). Color key: eubacterial 16S rRNA (green) and DNA (blue).

(C) Relative abundance of bacterial species greater than 1% in their respective tissue samples (n = 6 patients). The exposure to 5-FU is indicated for each community.

(D) Measurement of 5-FU (4 μ M) disappearance in the supernatant when exposed to the indicated *ex vivo* CRC bacterial communities or media alone for 0, 8, 24, and 48 h. Error bars represent standard deviation, n = 3 replicates.

(E) Measurement of 5-FU (4 μ M) disappearance in the supernatant when exposed to the indicated bacterial strains isolated from patient CRC_06 or media alone for 0, 24, and 48 h. **p < 0.01 and ***p < 0.001 as determined by a two-sided Student's t test. Error bars represent standard deviation, n = 3 replicates.

See also Figure S4 and Tables S4 and S5.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Fn</i> subsp. <i>animalis</i> SB003	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB010	Bullman et al., 2017 ¹⁸	Fn COCA36F3
<i>Fn</i> subsp. <i>animalis</i> SB012	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB014	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB041	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB053	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB058	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> SB066	This study	N/A
<i>Fn</i> subsp. <i>animalis</i> 7_1	Allen-Vercos Lab	Strauss et al., 2011 ⁶¹
<i>Fn</i> subsp. <i>animalis</i> KCOM1279	Korean Collection for Oral Microbiology	N/A
<i>Fn</i> subsp. <i>nucleatum</i> SB011	This study	N/A
<i>Fn</i> subsp. <i>polymorphum</i> SB013	This study	N/A
<i>Fn</i> subsp. <i>polymorphum</i> ATCC 10953	America Type Culture Collection	NCTC 10562
<i>Fn</i> subsp. <i>vincentii</i> SB054	This study	N/A
<i>Escherichia coli</i> SB209	This study	N/A
<i>Escherichia coli</i> <i>pks+</i> ATCC 25922	America Type Culture Collection	NCIB 12210
<i>Escherichia coli</i> SB215	This study	<i>E. coli</i> CRC_06
<i>Bacteroides fragilis</i> SB210	This study	N/A
<i>Bacteroides fragilis</i> SB211	This study	<i>B. fragilis</i> CRC_06
<i>Bacteroides thetaiotaomicron</i> SB212	This study	<i>B. thetaiotaomicron</i> CRC_06
<i>Bifidobacterium breve</i> SB213	This study	N/A
<i>Parvimonas micra</i> SB214	This study	<i>P. micra</i> CRC_02
Biological samples		
Colorectal cancer tumor tissue samples from treatment naive patients	Fred Hutchinson Cancer Center IRB RG1006974	N/A
Chemicals, peptides, and recombinant proteins		
Fastidious Anaerobe Agar	Neogen	Cat# NCM0014A
Fastidious Anaerobe Broth	Neogen	Cat# NCM0199A
Tryptic Soy Broth	Sigma-Aldrich	Cat# T8907
Brain Heart Infusion Broth	Research Products International	Cat# B11000
McCoys 5A	Corning	Cat# 10-050-CV
5-fluorouracil >99.0%	Tokyo Chemical Industry	Cat# F0151
Heavy 5-fluorouracil 2- ¹³ C, ¹⁵ N ₂	Millipore Sigma	Cat# 723258
Critical commercial assays		
BacTiter Glo Microbial Viability Assay	Promega	Cat# G8231
Deposited data		
Metagenomic Data	Sequence Read Archive-NCBI	BioProject PRJN817195
RNAseq Data	Sequence Read Archive-NCBI	BioProject PRJNA859520

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human Colon Carcinoma: RKO	America Type Culture Collection	CRL-2577
Software and algorithms		
Prism v9	GraphPad	https://www.graphpad.com
R	The R Foundation	https://www.r-project.org/
Other		
RNAscope Multiplex Fluorescent Reagent Kit v2	ACD Systems	323100
EB-16s-rRNA-c2	ACD Systems	ACD Systems
RNAscope 3-plex Negative Control Probe	ACD Systems	320871

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