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A Novel Riboswitch-Binding Flavin Analog that Protects Mice Against *Clostridium difficile* Infection without Inhibiting Cecal Flora

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Novel mechanisms of action and new chemical scaffolds are needed to rejuvenate antibacterial drug discovery, and riboswitch regulators of bacterial gene expression are a promising class of targets for the discovery of new leads. Herein, we report the characterization of 5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2H,5H)-dione (5FDQD)—an analog of riboflavin that was designed to bind riboswitches that naturally recognize the essential coenzyme flavin mononucleotide (FMN) and regulate FMN and riboflavin homeostasis. *In vitro*, 5FDQD and FMN bind to and trigger the function of an FMN riboswitch with equipotent activity. MIC and time-kill studies demonstrated that 5FDQD has potent and rapidly bactericidal activity against *Clostridium difficile*. In C57BL/6 mice 5FDQD completely prevented the onset of lethal antibiotic-induced *C. difficile* infection (CDI). Against a panel of bacteria representative of healthy bowel flora, the antibacterial selectivity of 5FDQD was superior to currently marketed CDI therapeutics, with very little activity against representative strains from the *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Actinomyces*, and *Prevotella* genera. Accordingly, a single oral dose of 5FDQD caused less alteration of culturable cecal flora in mice than the comparators. Collectively, these data suggest that 5FDQD or closely related analogs could potentially provide a high rate of CDI cure with a low likelihood of infection recurrence. Future studies will seek to assess the role of FMN riboswitch binding to the mechanism of 5FDQD antibacterial action. In aggregate, our results indicate that riboswitch-binding antibacterial compounds can be discovered and optimized to exhibit activity profiles that merit preclinical and clinical development as potential antibacterial therapeutic agents.

Antimicrobial Agents and Chemotherapy

Despite nearly 85 years of successful antibacterial chemotherapy, bacterial infections still pose a serious threat to human health. Continually emerging drug-resistant bacteria make existing agents less effective, and a paucity of new agents and decreased development effort from the pharmaceutical industry provide little hope of replenishing the arsenal (1, 2). Complications and mortality due to bacterial infections continue to increase, already reaching epidemic proportions in some areas of the world. If humans are to regain the upper hand in fighting bacterial infections, then innovation, investment, and new antibacterial agents will be needed. Although some of the barriers to the discovery and approval of new compounds are economic or policy related, there is also a desperate need for new targets and new mechanisms of action. A renewed effort to expand our knowledge of bacterial physiology and to translate discoveries into the clinic will be needed to address these challenges and to reinvigorate antibiotic development pipelines.

Recent advances in our understanding of how bacteria maintain physiological homeostasis revealed a promising class of potential antibiotics targets called riboswitches—non-coding mRNAs that form a structured receptor (or aptamer) which can directly bind to a specific small-molecule ligand or ion and thereby regulate gene expression (3-5). Ligand binding to a riboswitch aptamer stabilizes a conformationally distinct architecture in the mRNA that modulates the expression of the adjacent coding region(s) (4-8). To date, more than 35 riboswitch classes have been discovered and characterized (7). Three of these riboswitch classes have been revealed as important cellular targets of antibacterial small molecules whose mechanism of action had not been previously defined (9-13). More recently, several publications have demonstrated that novel small molecules can be rationally identified and optimized that bind to selected riboswitch aptamers with affinity comparable to the cognate ligand (14-21).

In some cases, synthetic or natural riboswitch ligand analogs have demonstrated potent antibacterial activity (12-15, 20, 22). For example, the phosphorylated form of roseoflavin (RoF, **Fig. 1A**), a naturally produced analog of riboflavin that inhibits the growth of Gram positive bacteria (23, 24), directly binds to riboswitches that recognize the essential coenzyme flavin mononucleotide, FMN (12, 13, 22). Accordingly, the presence of RoF in growth media represses

the expression of FMN riboswitch-regulated riboflavin biosynthesis and transport genes and inhibits bacterial growth (12, 22). The fact that RoF antibacterial action is partly due to FMN riboswitch binding underscores the potential for developing riboswitch-targeting antibiotics.

Concurrent with these initial validation studies with RoF, we established a separate research and development program that identified and progressively improved a class of novel flavin analogs that bind to FMN riboswitches *in vitro* with potency equal to FMN (P.D.G. Coish et al., 20 Jan 2011, PCT/US2010/001876; P.D.G. Coish et al., P.D.G. Coish et al., 13 Oct 2011, PCT/US2011/000617; 12 Aug 2012, PCT/US2012/024507). A subset of these compounds were found to exhibit potent and highly selective antibacterial activity against *Clostridium difficile*. These findings suggest that a novel class of antibiotics could be developed that target FMN riboswitches to provide a useful alternative to currently-approved therapies for *C. difficile* intestinal infections (CDI).

In a 2013 threat assessment report, the Centers for Disease Control and Prevention estimated that CDI causes 14,000 deaths and $1B in excess medical costs each year in the United States (25). The current standard-of-care treatments for CDI, metronidazole or vancomycin, are usually effective, but the infection recurs in approximately 25% of patients, with subsequent recurrences even more likely (26-28). The broad spectrum antibacterial activities of metronidazole and vancomycin are thought to contribute to recurrence by preventing the regrowth of healthy intestinal flora (29-33). A newer treatment, fidaxomicin, has a narrower spectrum of activity than vancomycin or metronidazole (34-36) and causes less alteration to the intestinal flora of CDI patients (32, 33). Consistent with this narrower spectrum of activity, multiple clinical trials have demonstrated a lower rate of CDI recurrence after treatment with fidaxomicin than after vancomycin or metronidazole treatment (37, 38). As a result, CDI therapies currently in development primarily include narrow spectrum antibiotics or intestinal flora replacements (39-41).

Herein we report the synthesis and characterization of 5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2H,5H)-dione (5FDQD, **Fig. 1A**), an analog of FMN that was identified via medicinal chemistry optimization of FMN riboswitch-binding ligands. 5FDQD is rapidly bactericidal against *C. difficile in vitro* and can cure mice of laboratory-induced CDI

nearly as effectively as fidaxomicin. Among a collection of bacteria representative of natural intestinal microflora, 5FDQD has a narrower spectrum than fidaxomicin and vancomycin. Moreover, administration of a single dose of 5FDQD to healthy mice was less disruptive of culturable cecal flora than were fidaxomicin or vancomycin.

MATERIALS AND METHODS

Chemicals, oligonucleotides, and bacterial strains. 5FDQD was resynthesized using a method similar to that published in patent literature (P.D.G. Coish et al., 20 Jan 2011, PCT/US2010/001876; P.D.G. Coish et al., P.D.G. Coish et al., 13 Oct 2011, PCT/US2011/000617; 12 Aug 2012, PCT/US2012/024507). The detailed synthetic procedures and spectral characterization are described in the Supplementary Material. Except as specifically noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Except as specifically noted, all growth media were purchased from BD Diagnostic Systems (Sparks, MD) and prepared in accordance with the manufacturer's instructions, and blood supplements were purchased from Cleveland Scientific (Bath, OH).

In-line probing assays. The 165 *ribD* RNA used for in-line probing assays was prepared by *in vitro* transcription using a template generated from genomic DNA from *Bacillus subtilis* strain 168 (American Type Culture Collection, Manassas, VA). RNA transcripts were dephosphorylated, 5′ ^{32}P-labeled, and subsequently subjected to in-line probing using protocols similar to those described previously (42). The K_D for each ligand was derived by quantifying the amount of RNA cleaved at each nucleotide position over a range of ligand concentrations. For each region where modification was observed (A, B, and C in **Fig. 2b**), the fraction of RNA cleaved at each ligand concentration was calculated by assuming that the maximal extent of cleavage is observed in the absence of ligand and the minimal cleavage is observed in the presence of the highest ligand concentration. The apparent K_D was determined by using GraphPad Prism 6 software to fit the plot of the fraction cleaved, x versus the ligand concentration, [L], to the following equation: $x = K_D ([L] + K_D)^{-1}$.

***In vitro* RNA transcription termination assays.** Single-round RNA transcription termination assays were conducted following protocols adapted from a previously described method (43).

The DNA template covered the region –382 to +13 (relative to the start of translation) of the *B. subtilis ribD* gene and was generated by PCR from genomic DNA from the 168 strain of *B. subtilis.* To initiate transcription and form halted complexes, each sample was incubated at 37°C for 10 min and contained 1 pmole DNA template, 0.17 mM ApA dinucleotide (TriLink Biotechnologies, San Diego, CA), 2.5 µM each of ATP, GTP, and UTP, plus 2 µCi 5′-[α-^{32}P]-UTP, and 0.4 U *E. coli* RNA polymerase holoenzyme (Epicenter, Madison, WI) in 10 µl of 80 mM Tris-HCl (pH 8.0 at 23°C), 20 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.01 mg/mL bovine serum albumin (BSA, New England Biolabs, Ipswich, MA). Halted complexes were restarted by the simultaneous addition of a mixture of all four NTPs that yield a final concentration of 50 µM, 0.2 mg/mL heparin to prevent re-initiation, and varying concentrations of ligand as indicated to yield a final volume of 12.5 µl in a buffer containing 150 mM Tris-HCl (pH 8.0 at 23°C), 20 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.01 mg/mL BSA. Reactions were incubated for an additional 20 min at 37°C, and the products were separated by denaturing 10% polyacrylamide gel electrophoresis (PAGE) followed by quantitation of the fraction terminated at each ligand concentration by using a phosphorimager (Storm 860, GE Healthcare Life Sciences, Pittsburgh, PA).

***In vitro* antibacterial activity**. MIC analyses were conducted by Micromyx, LLC (Kalamazoo, MI) in accordance with standard microdilution methods for aerobic (44) and anaerobic bacteria (45). All procedures involving anaerobic bacteria were conducted within a Bactron 300 or Bactron II anaerobic chamber (Sheldon Manufacturing, Cornelius, OR) using pre-reduced media. MICs for anaerobic bacteria were determined in supplemented Brucella broth, comprising BBL Brucella broth, 5 µg/mL hemin, 10 µg/mL vitamin K, and 5% lysed horse blood. MICs for aerobic bacteria were determined in Mueller-Hinton II broth, and a supplement of 3% lysed horse blood was included for assays with *Streptococcus pneumoniae*. Stock solutions of fidaxomicin (Ontario Chemicals, Guelf, ON) and 5FDQD in dimethylsulfoxide (DMSO) or vancomycin in water were used to prepare dilution plates such that the final concentration of DMSO in the assay did not exceed 5%. Time-kill assays for bactericidal activity were conducted at 35 °C in supplemented Brucella broth. A suspension of *C. difficile* strain VPI 10463 (ATCC 43255) was prepared from day-old colonies on supplemented Brucella agar (BBL Brucella agar, 5 µg/mL hemin, 10 µg/mL

vitamin K, and 5% lysed sheep blood). 10-fold dilutions of the suspension spanning 10^2 to 10^5 were prepared and allowed to grow overnight in broth at 35°C. On the following morning, a culture with a measurable OD_{600} less than 0.4 was diluted by 40 fold into 12 mL of fresh broth that had been pre-warmed to 35°C. 2 mL aliquots of this culture were immediately mixed with 100 µL of DMSO or a solution of 5FDQD, fidaxomicin, or vancomycin sufficient to give the final concentrations indicated in **Fig. 2**. Serial dilutions of the starter culture into pre-reduced phosphate-buffered saline (PBS) were plated onto brain heart infusion agar supplemented with 0.5% yeast extract and 0.1% L-cysteine (BHIS) to measure the density at time zero and to confirm that the starting inoculum was greater than 5 x 10^5 CFU/mL (46). Serial dilutions of each test culture were prepared in PBS and plated onto BHIS agar at the indicated time points, and colonies were counted after 24 h and used to calculate the culture densities in CFU/mL. The lower limit of detection using this method was 200 CFU/mL.

Preparation of *C. difficile* spores for infecting mice. *C. difficile* spores for the mouse infection model were prepared at Micromyx (Kalamazoo, MI). A frozen culture of *C. difficile* VPI 10463 was inoculated into 4 L of pre-reduced sporulation medium comprising 80 g/L Trypticase™ Peptone, 5 g/L Proteose Peptone no. 3, 1 g/L ammonium sulfate, and 1.5 g/L Tris adjusted to a final pH of 7.4. The culture was incubated in an anaerobic chamber at 35°C for 5 days, centrifuged, resuspended in 50% ethanol, and incubated at room temperature to eliminate any remaining vegetative bacterial cells. The spores were then washed twice in sterile PBS and enumerated by dilution plating onto supplemented Brucella agar with 0.1% sodium taurocholate. Following quantitation, the spores were diluted to a concentration of 2 x 10^6 spores/mL and stored at -80°C.

Mouse model of CDI. CDI efficacy studies were conducted by Aragen Biosciences (Morgan Hill, CA) and were reviewed and approved by Aragen's Institutional Animal Care and Use Committee (IACUC). Female C57BL/6 mice weighing 18-21 g were ordered from Harlan Laboratories (Indianapolis, IN), housed five per cage in a dedicated room, and allowed to acclimatize for at least three days. Animals were fed Teklad Global Rodent Diet 2018-R (Harlan Laboratories, Indianapolis, IN) throughout the study. For the seven days following acclimatization (days -10 through -4), mice were provided an antibiotic cocktail in their

autoclaved drinking water that included kanamycin (0.5 mg/mL), gentamicin (0.044 mg/mL), colistin (1062.5 U/mL), metronidazole (0.269 mg/mL), ciprofloxacin (0.156 mg/mL), ampicillin (0.1 mg/mL) and vancomycin (0.056 mg/mL). Mice were then returned to normal autoclaved drinking water for three days (days -3 through -1), followed by a single oral dose of 10 mg/kg clindamycin in a volume of 0.5 mL. Immediately following clindamycin dosing, animals were randomized and distributed into new cages of five animals such that the average weight across the groups of five animals was similar. On the following day (day 0), an inoculum of 2,000 spores of *C. difficile* VPI 10463 (ATCC 43255) in 0.5 mL of sterile PBS was orally administered to each mouse. Within one hour of inoculation, infected mice were administered the treatments indicated in **Fig. 3** by oral gavage twice a day (with approximately 10 hours elapsing between AM and PM treatments) for five days, with ten animals per treatment group. All test agents were dosed as a suspension in 0.5% aqueous methylcellulose. Body weights were measured three times a week prior to *C. difficile* infection and daily following infection. Animals were examined in the morning and evening for eight consecutive days following spore inoculation for clinical signs of morbidity, evidence of diarrhea, and mortality. At the end of the study, surviving animals were humanely euthanized.

Pharmacokinetic analysis. The Yale University IACUC reviewed and approved all animal experimental protocols for pharmacokinetic analysis (protocol number 20114-11652). Female C57BL/6 mice weighing 18-21 g were ordered from Harlan Laboratories, housed five per cage, and allowed to acclimatize for fourteen days. Animals were fed Teklad Global Rodent Diet 2018-R throughout the study. After acclimatization, 24 mice each received a single oral dose of 0.2 mL of a 1 mg/mL suspension of 5FDQD in aqueous 0.5% methylcellulose for a total dose of 10 mg/kg. At 1, 2, 4, 6, 8, and 24 h after dosing, three mice per time point were anesthetized with isoflurane and exsanguinated via cardiac puncture with a pre-heparinized syringe, and the collected blood was transferred to lithium heparinized tubes and held on ice until further processing. After exsanguination the mice were humanely euthanized by cervical dislocation, the ceca were immediately removed, and the cecal contents were collected into pre-weighed tubes and frozen at -80°C until extraction. The previously collected blood samples were

centrifuged at 4°C for 10 min at 6,000 RPM, and the plasma supernatant was collected into a separate tube and frozen at -80°C until extraction.

Sample extraction and bioanalyses were conducted by Northeast Bioanalytical (Hamden, CT). Cecal content samples were suspended in 2.0 mL of extraction/precipitation solution that contained 50% acetonitrile, 50% methanol, and 0.1% formic acid. The resulting suspensions were placed on a shaker table for 20-30 min, followed by centrifugation. Thawed plasma samples were combined with three volumes of extraction/precipitation solution, mixed, and allowed to warm to room temperature. A 100 µl aliquot of the cleared cecal extraction supernatant or plasma solution was transferred into a labeled autosampler vial and combined with 100 µl of an internal standard compound and 200 µl of extraction/precipitation solution. Samples were then capped, vortexed, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Test samples were injected onto a Phenomenex Synergi Polar-RP column (150 x 4.6 mm) with a corresponding guard column. The column temperature was maintained at 50°C. The analytes were separated using a variable composition of eluent A (acetonitrile) and eluent B (HPLC grade water with 0.1% formic acid). The initial composition of 40% eluent A and 60% eluent B was maintained at a flow rate of 1,000 µL/min for 1 min, at which point the composition of eluent A was increased to 95% over 0.5 min and held at 95% for 4 min at a flow rate of 1,300 µL/min. The composition of eluent A was then decreased to 40% at a flow rate of 1,100 µL/min and held at 40% for 0.5 min, at which point the flow rate was reduced to 1,000 µL/min and re-equilibrated before injecting another sample. Analyte detection was performed by a Sciex API-4000 tandem mass spectrometer fitted with a Turbo Ion Spray source operating in positive ion mode. Samples were quantitated against a calibration standard curve constructed from a stock solution of a known concentration of 5FDQD and using a linear regression of theoretical concentration of calibrators versus the response ratio (where the response ratio = analyte peak area/labeled internal standard peak area). The regression used a $1/x^2$ weighting factor in the following equation $y = mx + b$. An internal standard that is a structural analog of 5FDQD was included in all samples. The lower and upper limits of

quantitation for 5FDQD in plasma and cecal contents were 12 ng/mL and 5,000 ng/mL, respectively.

Cecal flora analysis. The Yale University IACUC reviewed and approved all animal experimental protocols for cecal flora analysis (protocol number 20114-11652). A total of 28 female C57BL/6 mice weighing 18-21 g were ordered from Harlan Laboratories, housed four per cage, and allowed to acclimatize for seven days. Animals were fed Teklad Global Rodent Diet 2018-R throughout the study. After acclimatization, mice were randomized into groups of four mice per cage for each treatment group. All four mice in a given treatment group received an oral dose of 0.2 mL of aqueous 0.5% methylcellulose (vehicle control) or 0.2 mL of an aqueous 0.5% methylcellulose suspension of 5FDQD, fidaxomicin, or vancomycin in aqueous 0.5% methylcellulose such that the total doses administered were 10 or 50 mg/kg 5FDQD, 3 or 50 mg/kg fidaxomicin, or 50 mg/kg vancomycin. 24 h after dosing, mice were humanely euthanized by cervical dislocation, the ceca were immediately removed, and the cecal contents were collected into preweighed tubes, weighed, and transferred immediately into an anaerobic chamber such that cecal contents were never exposed to ambient atmosphere for greater than 10 min. Within the anaerobic chamber, the cecal contents were resuspended in 1 mL of pre-reduced 0.05% yeast extract per 100 mg of contents and homogenized by vortex mixing. A series of 10-fold serial dilutions ranging from 10^1 to 10^7 were prepared in 0.05% yeast extract, and 50 µL aliquots of each dilutions were plated in parallel onto agar media selective for each bacterial taxa, including Bacteroides bile esculin agar (47), *Bifidobacterium*-selective agar (48), *Lactobacillus*-selective agar (49), Enterococcosel, MacConkey agar, and mannitol salt agar. To enumerate *Clostridium* spores, an aliquot of the 10^1 and 10^2 dilutions for each animal was mixed with an equal volume of ethanol and incubated for 1 h to kill vegetative cells. 50 µL of each dilution was then plated onto egg yolk agar (Anaerobe Systems, Morgan Hill, CA). After growth at 35°C for the number of days appropriate to each medium and bacterial type, colonies were counted, categorized according to visible morphology and were used to calculate the density of each taxa in the starting cecal contents in units of log CFU/mL. The reported densities represent an average of all countable dilution plates from four mice per treatment group. Each

colony type was presumptively identified to the genus or species level based on visible appearance, Gram stain, and microscopic appearance. Where necessary, catalase, indole, and oxidase tests and antimicrobial disc susceptibility testing were used to confirm the presumptive identifications (47). *Clostridium spiroforme* was presumptively identified by colony morphology on egg yolk agar, Gram stain, ability to form spores, a unique helical shape viewable under a microscope, and antimicrobial susceptibility pattern including rifampicin resistance (50, 51). The relative density of vegetative *C. spiroforme* was enumerated in parallel by dilution plating of selected cecal content samples onto brucella agar that contained 5% defibrinated sheep blood and 50 µg/mL rifampicin.

Results

The FMN analog 5FDQD is bound by FMN aptamers and modulates riboswitch activity. An in-line probing assay (42) was used to assess the binding of 5FDQD to the *ribD* FMN riboswitch aptamer from *B. subtilis* (**Fig. 1B**) and to determine an apparent dissociation constant (K_D) for the binding interaction. This assay exploits the fact that the internucleotide linkages in unstructured regions of an RNA will usually undergo phosphoester cleavage more rapidly than those in structured regions (52). The heightened flexibility of unstructured regions usually permits more frequent access to a conformation necessary for spontaneous cleavage by an internal RNA phosphoester transfer reaction. As the secondary and tertiary structures of the RNA change upon binding to a ligand, the reactivity of some of the internucleotide linkages will change, resulting in a different pattern of degradation products. The differences in RNA degradation products and the location of structural changes can be detected and quantified via denaturing PAGE.

Using the in-line probing assay, 5FDQD and FMN induce nearly identical structural changes in the *B. subtilis ribD* FMN aptamer (**Fig. 1C**, regions 1 through 6). The only observable difference was that 5FDQD binding increases spontaneous cleavage of the internucleotide linkage between G33 and G34, whereas FMN decreases reactivity at this same position (**Fig. 1C**, arrowhead). The three-dimensional x-ray structure model of a *Fusobacterium nucleatum* FMN riboswitch aptamer complexed with FMN predicts an interaction between G34 and a hydroxyl

group of the ribityl side-chain of FMN (53). This same interaction cannot be formed in a complex with 5FDQD because the analog lacks hydroxyl groups, which likely explains this single difference in reactivity as revealed by in-line probing. Therefore, the natural FMN ligand and the FMN analog 5FDQD are most likely recognized by the same binding pocket and induce nearly identical structural changes in the aptamer.

RNA-ligand binding curves were established by evaluating the relative change in cleavage at regions 1 through 6 as a function of compound concentration (**Fig. 1D**). The concentrations of ligands needed to half-maximally modulate RNA aptamer structure, representing K_D values, were 7.5 nM and 6.4 nM for 5FDQD and FMN, respectively. Thus, 5FDQD and FMN bind to the *B. subtilis ribD* FMN aptamer *in vitro* with equivalent potency. Moreover, the binding curves suggest that both compounds bind a single saturable site with 1-to-1 stoichiometry.

An *in vitro* transcription termination assay was performed to determine whether 5FDQD can induce FMN riboswitch function (**Fig. 1E,F**). Using a double-stranded DNA template that included the *B. subtilis ribD* endogenous promoter, FMN riboswitch region, and the first 13 nucleotides of the open reading frame, the concentration at which FMN or 5FDQD induce half-maximal transcription termination (T_{50}) was determined. The T_{50} values for 5FDQD and FMN were 0.5 µM and 0.6 µM, respectively, indicating that 5FDQD can induce riboswitch function *in vitro*, again with the same potency as FMN.

5FDQD exhibits robust and selective antibacterial activity. Against a panel of 21 strains of *C. difficile*, including four hypervirulent North American pulsed-field gel electrophoresis type 1 (NAP1) ribotype 027 strains (54, 55) and 14 isolates from hospital patients, the MIC_{50} and MIC_{90} of 5FDQD were determined to be 1 µg/mL by broth microdilution methods (**Table 1**), compared to MIC_{50} and MIC_{90} values of 0.06 and 0.12 µg/mL for fidaxomicin. Although less potent than fidaxomicin, 5FDQD shows equivalent potency to published values for both vancomycin and metronidazole (56). Notably, there was less variability among the MIC values for 5FDQD than for fidaxomicin among the isolates tested.

The bactericidal activity of 5FDQD, fidaxomicin, and vancomycin were compared via a time-kill assay with *C. difficile* VPI 10463 (ATCC 43255, **Fig. 2**). At a concentration 4- or 8-fold higher

than the MIC, 5FDQD reduced the viable density of *C. difficile* by 99.9% to 1.7 x 10^3 and 8.0 x 10^3 CFU/mL, respectively, within 4 h. Within 6 h, incubations containing these concentrations of 5FDQD reduced viable cells below the 200 CFU/mL limit of quantitation. Similar bactericidal activity was observed for 5FDQD against *C. difficile* strain 630 (data not shown). In contrast, at a concentration 4-fold above their MIC values fidaxomicin and vancomycin only reduced the viable density by 6.3- and 64-fold, respectively, which is consistent with their previously reported activity (57). Based on these data, 5FDQD appears more rapidly bactericidal than these well-established CDI therapeutic compounds.

To assess the selectivity of 5FDQD antibacterial activity, we measured MIC values against a diverse panel of anaerobic and aerobic bacteria that included many common intestinal flora species (**Table 1**). Neither 5FDQD nor fidaxomicin showed appreciable activity against *B. fragilis* group species, whereas vancomycin and metronidazole are known to inhibit the *B. fragilis* group at pharmacologically relevant concentrations (35, 39, 58). Likewise, little to no activity was observed for 5FDQD and fidaxomicin against other anaerobic Gram negative rods, which are usually susceptible to metronidazole but not vancomycin. Interestingly, 5FDQD showed no appreciable activity against the *Bifidobacterium*, *Lactobacillus*, or *Actinomyces* species tested, whereas fidaxomicin inhibited these strains as potently as it inhibits *C. difficile*. The activity of fidaxomicin, vancomycin, and metronidazole against these species has been well documented elsewhere (35, 58). Based on these data, the selectivity of 5FDQD with respect to anaerobic intestinal flora appears to be at least as good as, or superior to, currently marketed CDI therapeutic compounds. 5FDQD also exhibited very little activity against representative aerobic pathogens, with the exception of activity against some strains of *Enterococcus faecalis*.

***In vivo* efficacy.** An antibiotic-induced mouse CDI model was used to evaluate the *in vivo* efficacy of 5FDQD, fidaxomicin, and vancomycin. In accordance with published protocols (59), CDI was established in C57BL/6 mice by administering an antibiotic cocktail via their drinking water to disrupt intestinal flora and an oral dose of *C. difficile* VPI 10463 spores. If the infected mice are not subsequently treated with an effective antibiotic, they exhibit severe diarrhea, lethargy, weight loss, failure to groom, and 40-60% mortality within 48 h (59, 60).

Using this model, the efficacy of 5FDQD was compared to that of fidaxomicin and vancomycin, with all test agents suspended in aqueous 0.5% methylcellulose and administered orally twice each day for five days, except vancomycin, which was administered once a day for five days. Mice that received only the 0.5% methylcellulose vehicle orally developed signs of severe CDI within three days of inoculation, and 40% died by day 6 (vehicle, **Fig. 3A**). The average weight of surviving vehicle-treated mice relative to their weight at the time of inoculation decreased by up to 17% during the study (**Fig. 3B**).

By contrast, treatment with 10 mg/kg 5FDQD prevented any mortality or weight loss, with the average weight increasing by 6% by day 8. Furthermore, although symptoms of mild diarrhea developed in one mouse, the symptoms resolved by day 3 of dosing. The activity of 10 mg/kg 5FDQD is comparable to the activity of both 3 mg/kg fidaxomicin and 50 mg/kg vancomycin. Symptoms of mild diarrhea were observed in two of the fidaxomicin-treated mice and one vancomycin-treated mouse, but the symptoms resolved by day 4. These data indicate that 5FDQD prevented the onset of severe CDI in mice as effectively as fidaxomicin and vancomycin.

Pharmocokinetics of 5FDQD are advantageous for treating intestinal tract infections. To assess the pharmacokinetic distribution of 5FDQD at an efficacious dose, mice were administered a single 10 mg/kg oral dose of 5FDQD, and the concentration of the compound in the cecal contents and plasma was measured at specific time points after dosing. Levels of 5FDQD in the cecal contents were 192, 204, 174, 86, 82, and 0.3 µg/g at 1, 2, 4, 6, 8, and 24 h after dosing, respectively. This rapid transit into the lower intestinal tract followed by a sustained concentration over several hours is comparable to the distribution observed after a single dose of oral fidaxomcin (61). Levels of 5FDQD in the plasma were 23 ng/mL at 1 h and below the limit of quantitation (12 ng/mL) at the remaining times. These very low plasma levels would constitute a potential safety advantage for a drug intended to treat an intestinal tract infection such as caused by *C. difficile*.

Cecal flora is only modestly affected by 5FDQD. Since the disruption of endogenous intestinal flora is closely linked with both initial *C. difficile* infection and with recurrence rates (40, 62), we evaluated the extent to which 5FDQD, fidaxomicin, or vancomycin altered the culturable cecal flora in mice. A single oral dose of each compound or of the vehicle alone was administered to uninfected, healthy C57BL/6 mice. After 24 h, the mice were euthanized, the visible morphology of the ceca were recorded, and the densities of representative bacterial taxa within the cecal contents were determined by plating onto agar media selective for *B. fragilis*, bifidobacteria, lactobacilli, enterococci, enterobacteriaceae, or staphylococci. In addition, the density of *Clostridium* spores in the cecal contents was determined by incubating an aliquot of the cecal contents with ethanol for 1 h followed by dilution plating onto egg yolk agar (63). The identity of each countable colony type on each media type subsequently was confirmed to the genera or species level (see MATERIALS AND METHODS).

Ceca from mice treated with the aqueous 0.5% methylcellulose vehicle were visually indistinguishable from ceca isolated from untreated mice. The cecal contents from vehicle-treated mice had a high density of culturable *B. fragilis*, bifidobacteria, and lactobacilli (2.1 x 10^7, 9.3 x 10^8, and 2.0 x 10^7 CFU/g, respectively) and a lower density of *Clostridium* spores, enterococci, enterobacteriaceae, and staphylococci (6.8 x 10^4, 7 x 10^4, 4.6 x 10^3, and 3.0 x 10^4 CFU/g, respectively) (**Fig. 4**). This distribution was very similar to that measured for cecal contents from untreated mice (data not shown) and is consistent with previous characterizations of mouse cecal flora (64-66).

Treatment with a single 10 mg/kg or 50 mg/kg oral dose of 5FDQD did not affect the visible morphology of the ceca and had very little effect on the densities of the cultured cecal flora groups compared to the vehicle-treated mice. The only difference observed was a 5-fold increase in the density of enterobacteriaceae. Thus, even at a dose 5-fold above the efficacious dose, 5FDQD did not dramatically alter the cecal morphology or flora. Speculation on why 5FDQD is highly selective is presented in the Discussion section (see below).

Ceca isolated from mice treated orally with 3 mg/kg fidaxomicin appeared normal, but those isolated from mice treated orally with 50 mg/kg fidaxomicin were visibly larger than those from vehicle- or 5FDQD-treated mice, and the mass of their cecal contents was increased

by 40%. Treatment with a single 3 mg/kg oral dose of fidaxomicin also resulted in a 10-fold reduction of *B. fragilis*, a 10-fold increase of enterobacteriaceae, and a 3-fold increase in *Clostridium* spores relative to vehicle-treated mice. These changes are consistent with alterations to bowel flora observed in human patients treated with fidaxomicin (32, 33). Treatment with 50 mg/kg fidaxomicin altered the flora to a greater extent, most notably a >1,000-fold increase in enterobacteriaceae (**Fig. 4**). Although the density of *Clostridium* spores was not dramatically altered by either dose of fidaxomicin, the diversity of clostridial species was decreased as judged by colony visual appearance and microscopic examination. One colony type presumptively identified as *C. spiroforme* (51) was increased 10-fold by fidaxomicin treatment to become the predominant culturable *Clostridium* species. Overall, fidaxomicin exhibited a moderate, dose-responsive effect on both cecal morphology and culturable cecal flora populations.

Ceca from mice treated orally with 50 mg/kg vancomycin mice were significantly enlarged, had visible gas bubbles, and contained 80% more contents by weight than ceca from vehicle- or 5FDQD-treated mice. Vancomycin also profoundly altered the culturable cecal flora (**Fig. 4**). *B. fragilis*, *Clostridium* spores, and staphylococci were decreased by 10,000-, 200-, and 10-fold, respectively, and lactobacilli, enterococci, and enterobacteriaceae were increased by 100-, 32, and >30,000-fold, respectively. These changes are consistent with the large alterations of bowel flora that vancomycin causes in human patients (30, 32). Overall, vancomycin treatment dramatically alters both cecal morphology and culturable cecal flora.

Discussion

Given the urgent need for new antibiotics targets and lead compounds, we have established a research program aimed at identifying antibacterial compounds that target riboswitches. From a starting point of FMN and RoF, an intensive medicinal chemistry optimization effort yielded a series of flavin analogs that are potent FMN riboswitch ligands *in vitro* and that inhibit the growth of certain Gram positive bacteria (P.D.G. Coish et al., 20 Jan 2011, PCT/US2010/001876; P.D.G. Coish et al., P.D.G. Coish et al., 13 Oct 2011, PCT/US2011/000617; 12 Aug 2012, PCT/US2012/024507). Herein, we report the *in vitro* and *in vivo* characterization

of one of these compounds 5FDQD—a flavin analog that differs from FMN in that it has an N1-deaza flavin core and in that the ribityl phosphate moiety is replaced by an aryl-alkyl moiety.

The observation that *in vitro* 5FDQD binds to and triggers the function of an FMN riboswitch with the same potency as FMN is remarkable given the network of interactions that the aptamer appears to form with the ribose-phosphate moiety of FMN in crystallographic structure models (53, 67). Our in-line probing assay data indicate that 5FDQD binds to the riboswitch in the same region as FMN and causes nearly the same structural rearrangements, suggesting that the aptamer pocket is capable of forming equally favorable interactions with the aryl-alkyl side chain of 5FDQD. The transcription termination data confirm that these interactions are sufficient for 5FDQD to trigger riboswitch function *in vitro* with the same potency as FMN.

Replacement of the ribityl-phosphate moiety of FMN with a hydrophobic moiety to yield compounds that retain riboswitch binding function constitutes a key medicinal chemistry advance. This modification obviates the need to deliver a phosphorylated drug to the target cell or to deliver a pro-drug that the target cell needs to phosphorylate in order to achieve full potency. These differences greatly expands the diversity of possible functional analogs. By contrast, the weak riboswitch-binding potency of the natural compound RoF *in vitro* suggest that it needs to be phosphorylated to RoF-P inside cells to be active (12), and this necessity likely diminishes the range of compound variations that could serve as effective antibiotics. Thus, 5FDQD and related analogs have two key advantages that are common characteristics of some classes of drugs (68). First, molecules in this series are uncharged and have reduced polarity compared to FMN or RoF, which might favor their uptake by target cells. Second, the compounds bind their RNA targets with high affinity without the need for modification by cellular enzymes. Moreover, our results suggest that 5FDQD is a representative of a much larger class of compounds that could be created based on this scaffold and that additional compounds could be generated and tested to further optimize the function of FMN riboswitch-binding antibiotic compounds.

In its current form, 5FDQD is a potent *C. difficile* antibacterial agent with an MIC_{90} of 1 µg/mL among strains tested herein, including four hypervirulent NAP-1 isolates. The *C. difficile*

476 MIC_{90} values for 5FDQD are equivalent to those reported for metronidazole, vancomycin, and
477 surotomycin, a phase III investigational agent for CDI therapy (56, 69). Although 5FDQD is less
478 potent against *C. difficile* than fidaxomicin, the available fecal concentration of 5FDQD after a
479 single oral dose is well above the MIC and at a concentration that rapidly kills *C. difficile in vitro*.
480 Accordingly, 5FDQD can completely prevent the onset of severe antibiotic-induced CDI in mice
481 at an oral dose of 10 mg/kg twice daily for five days. In three repeated side-by-side
482 comparisons, 10 mg/kg 5FDQD showed equivalent potency to 3 mg/kg fidaxomicin. Given the
483 high cecal bioavailability after dosing, it is likely that lower doses would also be effective. Future
484 studies could be designed to determine a minimal effective dose of 5FDQD. However, it might
485 also be beneficial to determine the maximal tolerated dose and whether higher concentrations
486 of drug might clear an infection sooner and with fewer doses. Overall, the antibacterial activity
487 and *in vivo* efficacy of 5FDQD against *C. difficile* are comparable to known CDI therapeutics or
488 development candidates.

489 At present, the mechanism of 5FDQD antibacterial activity has not been definitively
490 confirmed. Preliminary attempts to isolate 5FDQD-resistant *C. difficile* by single-step or serial
491 passage methods have not yielded isolates with a stably increased MIC. These effortsindicate
492 that the frequency of resistance development is $<1 \times 10^{-9}$ (data not shown). If confirmed, this
493 low propensity to develop resistance could suggest either that mutations in the primary target
494 are not well tolerated or that there are multiple targets. In support of a riboswitch-targeting
495 component of the mechanism, 5FDQD binds to and triggers the function of an FMN riboswitch
496 *in vitro* with potency comparable to that of the active phosphorylated form of RoF, whose
497 antibacterial activity is at least partly attributable to the repression of FMN riboswitch-
498 regulated genes (12, 13, 22). Moreover, among a collection of 5FDQD analogs, a correlation
499 exists between MIC and *in vitro* riboswitch binding potency (data not shown). However, it is
500 also conceivable that inhibition of flavoprotein-related processes could be a component of the
501 5FDQD antibacterial activity as has been suggested for RoF (70, 71). Efforts are currently
502 underway in the Breaker laboratory to isolate 5FDQD-resistant *C. difficile* and to definitively
503 characterize the mode of action for 5FDQD, including elucidating the relative importance of
504 FMN riboswitch binding.

It is well established that healthy, diverse intestinal flora protects against *C. difficile* colonization and reduces the likelihood of recurrence after an initial CDI episode (72-74). Although the specific taxa responsible for colonization resistance have not been unequivocally established, it is currently thought that bacteria of the *Bacteroides*, *Bifidobacterium*, and *Prevotella* genera as well as the *Ruminococcaceae*, *Lachnospiraceae*, and *Porphyromonadaceae* families are major contributors (75-80). Like fidaxomicin, 5FDQD is inactive against most of the aerobes and Gram negative anaerobes tested herein, including the *B. fragilis* group and *Prevotella* species. Favorably, 5FDQD is less active than fidaxomicin against the *Bifidobacterium*, *Lactobacillus*, and *Actinomyces* strains tested herein. Likewise, the MICs of 5FDQD against representatives of these genera are higher than the published MICs of fidaxomicin, vancomycin, metronidazole, and two clinical development candidates for CDI treatment—LFF-571 and surotomycin (39, 41, 58, 81). Thus, the spectrum of 5FDQD appears to be more narrow compared to established and developmental CDI therapeutics and should theoretically permit the regrowth of intestinal flora that protect against recurrence. At this time, the basis for the remarkable selectivity of 5FDQD is not entirely clear. Earlier analogs of 5FDQD were found to be modestly inhibitory toward an efflux-deficient *ΔnorA* strain of *S. aureus* (82) or chemically-permeabilized *E. coli* (83), suggesting that 5FDQD either does not readily enter or is effluxed from most of the bacterial species tested (data not shown). Future studies will seek to confirm the narrow spectrum of 5FDQD against a larger collection of representative species.

The narrower spectrum of 5FDQD compared to fidaxomicin and vancomycin was evident from the effect of each agent on the spectrum of bacteria cultured from mouse cecal flora. At comparable doses in healthy mice, 5FDQD was significantly less disruptive of cultured cecal flora than both fidaxomicin and vancomycin. No changes in excess of one order of magnitude were observed after a single oral dose of up to 50 mg/kg 5FDQD, whereas a single fidaxomicin dose caused reduced cecal *B. fragilis* density and *Clostridium* diversity and increased enterobacteriaceae density. Interestingly, overgrowth of enterobacteriaceae is positively correlated with a number of intestinal disorders including *C. difficile* colonization (74, 84-86). Although the taxa cultured herein represent a subset of the total flora, the measured changes are consistent with the known effects of fidaxomicin and vancomycin on bowel flora (32, 33).

The lower impact of fidaxomicin on human bowel flora compared to vancomycin is widely believed to be the basis for a lower rate of CDI recurrence. Therefore, it is conceivable that treatment with a selective agent like 5FDQD would lead to fewer recurrences in humans. Efforts are now underway to evaluate the likelihood of CDI recurrence after treatment utilizing a hamster model (87) and a recently developed mouse recurrence model (88).

In summary, our efforts to optimize new FMN riboswitch-targeting antibiotics resulted in the identification of a novel series of flavin analogs that are potent and highly selective *C. difficile* antibacterial compounds. The most potent of these analogs examined to date, 5FDQD, can prevent lethal CDI in mice without disrupting the regrowth of beneficial flora. Further exploration of this compound series appears to be a promising route for the development of new CDI therapeutics. The success of this program also underscores the potential for the development of riboswitch-targeting compounds for treating other bacterial infections.

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556 **References**

557 1. **Infectious Diseases Society of America.** 2008. The epidemic of antibiotic-resistant
558 infections: a call to action for the medical community from the Infectious Diseases
559 Society of America. Clin Infect Dis **46:**155-164.

560 2. **Silver LL.** 2011. Challenges of antibacterial discovery. Clin Microbiol Rev **24:**71-109.

561 3. **Mandal M, Breaker RR.** 2004. Gene regulation by riboswitches. Nat Rev Mol Cell Biol
562 **5:**451-463.

563 4. **Serganov A.** 2009. The long and the short of riboswitches. Curr Opin Struct Biol **19:**251-
564 259.

565 5. **Roth A, Breaker RR.** 2009. The structural and functional diversity of metabolite-binding
566 riboswitches. Annu Rev Biochem **78:**305-334.

567 6. **Smith AM, Fuchs RT, Grundy FJ, Henkin TM.** 2010. Riboswitch RNAs: regulation of gene
568 expression by direct monitoring of a physiological signal. RNA Biol **7:**104-110.

569 7. **Breaker RR.** 2011. Prospects for riboswitch discovery and analysis. Mol Cell **43:**867-879.

570 8. **Breaker RR.** 2012. Riboswitches and the RNA world. Cold Spring Harb Perspect Biol **4**.

571 9. **Koedam JC.** 1958. The mode of action of pyrithiamine as an inducer of thiamine
572 deficiency. Biochimica et Biophysica Acta **29:**333-344.

573 10. **Sudarsan N, Cohen-Chalamish S, Nakamura S, Emilsson Gail M, Breaker RR.** 2005.
574 Thiamine pyrophosphate riboswitches are targets for the antimicrobial compound
575 pyrithiamine. Chem Biol **12:**1253-1358.

576 11. **Sudarsan N, Wickiser JK, Nakamura S, Ebert Margaret S, Breaker Ronald R.** An mRNA
577 structure in bacteria that controls gene expression by binding lysine. Genes &

development FIELD Publication Date:2003 Nov 1 **17:**2688-2697. FIELD Reference Number: FIELD Journal Code:8711660 FIELD Call Number:.

12. **Lee ER, Blount KF, Breaker RR.** 2009. Roseoflavin is a natural antibacterial compound that binds to FMN riboswitches and regulates gene expression. RNA Biol **6:**187-194.

13. **Ott E, Stolz J, Lehmann M, Mack M.** 2009. The RFN riboswitch of *Bacillus subtilis* is a target for the antibiotic roseoflavin produced by *Streptomyces davawensis*. RNA Biol **6:**276-280.

14. **Blount KF, Wang XJ, Lim J, Sudarsan N, Breaker RR.** 2007. Antibacterial compounds that target lysine riboswitches. Nat Chem Biol **3:**44-49.

15. **Kim JN, Blount KF, Puskarz I, Lim J, Link KH, Breaker RR.** 2009. Design and antimicrobial action of purine analogues that bind guanine riboswitches. ACS Chem Biol **4:**915-927.

16. **Lünse CE, Schmidt MS, Wittmann V, Mayer G.** 2011. Carba-sugars activate the *glmS*-riboswitch of *Staphylococcus aureus*. ACS Chem Biol **6:**675-678.

17. **Blount KF, Breaker RR.** 2006. Riboswitches as antibacterial drug targets. Nat Biotechnol **24:**1558-1564.

18. **Blount KF, Puskarz I, Penchovsky R, Breaker RR.** 2006. Development and application of a high-throughput assay for *glmS* riboswitch activators. RNA Biol **3:**77-81.

19. **Deigan KE, Ferre-D'Amare AR.** 2011. Riboswitches: discovery of drugs that target bacterial gene-regulatory RNAs. Acc Chem Res **44:**1329-1338.

20. **Ster C, Allard M, Boulanger S, Lamontagne Boulet M, Mulhbacher J, Lafontaine DA, Marsault E, Lacasse P, Malouin F.** 2013. Experimental treatment of *Staphylococcus*

aureus bovine intramammary infection using a guanine riboswitch ligand analog. J Dairy Sci **96:**1000-1008.

21. **Gilbert SD, Reyes FE, Edwards AL, Batey RT.** 2009. Adaptive ligand binding by the purine riboswitch in the recognition of guanine and adenine analogs. Structure **17:**857-868.

22. **Mansjo M, Johansson J.** 2011. The riboflavin analog roseoflavin targets an FMN-riboswitch and blocks *Listeria monocytogenes* growth, but also stimulates virulence gene-expression and infection. RNA Biol **8:**674-680.

23. **Matsui K, Wang HC, Hirota T, Matsukawa H, Kasai S, Shinagawa K.** 1982. Riboflavin production by roseoflavin-resistant strains of some bacteria. Agr Biol Chem Tokyo **46:**2003-2008.

24. **Otani S, Takatsu M, Nakano M, Kasai S, Miura R.** 1974. Letter: Roseoflavin, a new antimicrobial pigment from *Streptomyces*. J Antibiot (Tokyo) **27:**86-87.

25. **Centers for Disease Control and Prevention (CDC).** 2013. Antibiotic Resistance Threats in the United States, 2013. Centers for Disease Control and Prevention (CDC). Atlanta.

26. **Johnson S.** 2009. Recurrent *Clostridium difficile* infection: causality and therapeutic approaches. Int J Antimicrob Agents **33 Suppl 1:**S33-36.

27. **Johnson S.** 2009. Recurrent *Clostridium difficile* infection: a review of risk factors, treatments, and outcomes. J Infect **58:**403-410.

28. **Kelly CP.** 2012. Can we identify patients at high risk of recurrent *Clostridium difficile* infection? Clin Microbiol Infect **18 Suppl 6:**21-27.

29. **Krook A.** 1981. Effect of metronidazole and sulfasalazine on the normal human faecal flora. Scand J Gastroenterol **16:**587-592.

30. **Edlund C, Barkholt L, Olsson-Liljequist B, Nord CE.** 1997. Effect of vancomycin on intestinal flora of patients who previously received antimicrobial therapy. Clin Infect Dis **25:**729-732.

31. **Van der Auwera P, Pensart N, Korten V, Murray BE, Leclercq R.** 1996. Influence of oral glycopeptides on the fecal flora of human volunteers: selection of highly glycopeptide-resistant enterococci. J Infect Dis **173:**1129-1136.

32. **Tannock GW, Munro K, Taylor C, Lawley B, Young W, Byrne B, Emery J, Louie T.** 2010. A new macrocyclic antibiotic, fidaxomicin (OPT-80), causes less alteration to the bowel microbiota of *Clostridium difficile*-infected patients than does vancomycin. Microbiology **156:**3354-3359.

33. **Louie TJ, Cannon K, Byrne B, Emery J, Ward L, Eyben M, Krulicki W.** 2012. Fidaxomicin Preserves the Intestinal Microbiome During and After Treatment of *Clostridium difficile* Infection (CDI) and Reduces Both Toxin Reexpression and Recurrence of CDI. Clinical Infectious Diseases **55:**S132-S142.

34. **Finegold SA, Molitoris D, Vaisanen ML, Song YL, Liu CX, Bolanos M.** 2004. *In vitro* activities of OPT-80 and comparator drugs against intestinal bacteria. Antimicrobial Agents and Chemotherapy **48:**4898-4902.

35. **Credito KL, Appelbaum PC.** 2004. Activity of OPT-80, a novel macrocycle, compared with those of eight other agents against selected anaerobic species. Antimicrob Agents Chemother **48:**4430-4434.

641 36. **Gerber M, Ackermann G.** 2008. OPT-80, a macrocyclic antimicrobial agent for the
642 treatment of *Clostridium difficile* infections: a review. Expert Opin Investig Drugs **17:**547-
643 553.
644 37. **Golan Y, Epstein L.** 2012. Safety and efficacy of fidaxomicin in the treatment of
645 *Clostridium difficile*-associated diarrhea. Therap Adv Gastroenterol **5:**395-402.
646 38. **Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, Gorbach S, Sears P,**
647 **Shue YK, Group OPTCS.** 2011. Fidaxomicin versus vancomycin for *Clostridium difficile*
648 infection. N Engl J Med **364:**422-431.
649 39. **Citron DM, Tyrrell KL, Merriam CV, Goldstein EJ.** 2012. *In vitro* activities of CB-183,315,
650 vancomycin, and metronidazole against 556 strains of *Clostridium difficile*, 445 other
651 intestinal anaerobes, and 56 *Enterobacteriaceae* species. Antimicrob Agents Chemother
652 **56:**1613-1615.
653 40. **Bakken JS.** 2009. Fecal bacteriotherapy for recurrent *Clostridium difficile* infection.
654 Anaerobe **15:**285-289.
655 41. **Debast SB, Bauer MP, Sanders IM, Wilcox MH, Kuijper EJ, Group ES.** 2013.
656 Antimicrobial activity of LFF571 and three treatment agents against *Clostridium difficile*
657 isolates collected for a pan-European survey in 2008: clinical and therapeutic
658 implications. J Antimicrob Chemother **68:**1305-1311.
659 42. **Regulski EE, Breaker RR.** 2008. In-line probing analysis of riboswitches. Methods Mol
660 Biol **419:**53-67.

43. **Landick R, Wang D, Chan CL.** 1996. Quantitative analysis of transcriptional pausing by *Escherichi coli* RNA polymerase: His leader pause site as a paradigm. Methods in Enzymology **274:**334-353.

44. **Clinical Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically--Ninth Edition: Approved Standard: M7-A7. CLSI, Wayne, PA.

45. **Clinical Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow anaerobically-ninth Edition: approved standard M7-A7. CLSI, Wayne, PA.

46. **Pearson RD, Steigbigel RT, Davis HT, Chapman SW.** 1980. Method of reliable determination of minimal lethal antibiotic concentrations. Antimicrob Agents Chemother **18:**699-708.

47. **Jousimies-Somer H, Summanen P, Citron DM, Baron EJ, Wexler HM, Finegold SM.** 2002. Wadsworth-KTL Anaerobic Bacteriology Manual, 6th ed. Star Publishing Company, Belmont, CA.

48. **Ferraris L, Aires J, Waligora-Dupriet AJ, Butel MJ.** 2010. New selective medium for selection of bifidobacteria from human feces. Anaerobe **16:**469-471.

49. **Hartemink R, Domenech VR, Rombouts FM.** 1997. LAMVAB - A new selective medium for the isolation of lactobacilli from faeces. Journal of Microbiological Methods **29:**77-84.

50. **Carman RJ, Wilkins TD.** 1991. *In vitro* susceptibility of rabbit strains of *Clostridium spiroforme* to antimicrobial agents. Vet Microbiol **28:**391-397.

683 51. **Borriello SP, Carman RJ.** 1983. Association of iota-like toxin and *Clostridium spiroforme*
684 with both spontaneous and antibiotic-associated diarrhea and colitis in rabbits. J Clin
685 Microbiol **17:**414-418.

686 52. **Soukup GA, Breaker RR.** 1999. Relationship between internucleotide linkage geometry
687 and the stability of RNA. RNA **5:**1308-1325.

688 53. **Serganov A, Huang L, Patel DJ.** 2009. Coenzyme recognition and gene regulation by a
689 flavin mononucleotide riboswitch. Nature **458:**233-237.

690 54. **Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, Bourgault AM,**
691 **Nguyen T, Frenette C, Kelly M, Vibien A, Brassard P, Fenn S, Dewar K, Hudson TJ, Horn**
692 **R, Rene P, Monczak Y, Dascal A.** 2005. A predominantly clonal multi-institutional
693 outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality.
694 N Engl J Med **353:**2442-2449.

695 55. **O'Connor JR, Johnson S, Gerding DN.** 2009. *Clostridium difficile* infection caused by the
696 epidemic BI/NAP1/027 strain. Gastroenterology **136:**1913-1924.

697 56. **Wong SS, Woo PC, Luk WK, Yuen KY.** 1999. Susceptibility testing of *Clostridium difficile*
698 against metronidazole and vancomycin by disk diffusion and Etest. Diagn Microbiol
699 Infect Dis **34:**1-6.

700 57. **Babakhani F, Gomez A, Robert N, Sears P.** 2011. Killing kinetics of fidaxomicin and its
701 major metabolite, OP-1118, against *Clostridium difficile*. J Med Microbiol **60:**1213-1217.

702 58. **Snydman DR, Jacobus NV, McDermott LA.** 2012. Activity of a novel cyclic lipopeptide,
703 CB-183,315, against resistant *Clostridium difficile* and other Gram-positive aerobic and
704 anaerobic intestinal pathogens. Antimicrob Agents Chemother **56:**3448-3452.

59. **Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP.** 2008. A mouse model of *Clostridium difficile*-associated disease. Gastroenterology **135:**1984-1992.

60. **Collignon A.** 2010. Methods for working with the mouse model. Methods Mol Biol **646:**229-237.

61. **Swanson RN, Hardy DJ, Shipkowitz NL, Hanson CW, Ramer NC, Fernandes PB, Clement JJ.** 1991. In vitro and in vivo evaluation of tiacumicins B and C against *Clostridium difficile*. Antimicrob Agents Chemother **35:**1108-1111.

62. **Rolfe RD, Helebian S, Finegold SM.** 1981. Bacterial interference between *Clostridium difficile* and normal fecal flora. J Infect Dis **143:**470-475.

63. **Chapin KC, Murray PR.** 1999. Media, p. 1687-1707. *In* Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (ed.), Manual of Clinical Microbiology, 9th ed. ASM Press, Washington, D.C.

64. **Savage DC, Dubos R.** 1968. Alterations in the mouse cecum and its flora produced by antibacterial drugs. J Exp Med **128:**97-110.

65. **Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB.** 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect Immun **77:**2367-2375.

66. **Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG.** 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. Infect Immun **80:**62-73.

67. **Vicens Q, Mondragon E, Batey RT.** 2011. Molecular sensing by the aptamer domain of the FMN riboswitch: a general model for ligand binding by conformational selection. Nucleic Acids Res **39:**8586-8598.

68. **O'Shea R, Moser HE.** 2008. Physicochemical properties of antibacterial compounds: implications for drug discovery. J Med Chem **51:**2871-2878.

69. **Aspevall O, Lundberg A, Burman LG, Akerlund T, Svenungsson B.** 2006. Antimicrobial susceptibility pattern of *Clostridium difficile* and its relation to PCR ribotypes in a Swedish university hospital. Antimicrob Agents Chemother **50:**1890-1892.

70. **Langer S, Hashimoto M, Hobl B, Mathes T, Mack M.** 2013. Flavoproteins are potential targets for the antibiotic roseoflavin in Escherichia coli. J Bacteriol **195:**4037-4045.

71. **Langer S, Nakanishi S, Mathes T, Knaus T, Binter A, Macheroux P, Mase T, Miyakawa T, Tanokura M, Mack M.** 2013. The flavoenzyme azobenzene reductase AzoR from Escherichia coli binds roseoflavin mononucleotide (RoFMN) with high affinity and is less active in its RoFMN form. Biochemistry **52:**4288-4295.

72. **Wilson KH, Silva J, Fekety FR.** 1981. Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. Infect Immun **34:**626-628.

73. **Britton RA, Young VB.** 2012. Interaction between the intestinal microbiota and host in *Clostridium difficile* colonization resistance. Trends in Microbiology **20:**313-319.

74. **Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes **2:**145-158.

75. **Seekatz AM, Young VB.** 2014. *Clostridium difficile* and the microbiota. J Clin Invest **124:**4182-4189.

76. **Manges AR, Labbe A, Loo VG, Atherton JK, Behr MA, Masson L, Tellis PA, Brousseau R.** 2010. Comparative metagenomic study of alterations to the intestinal microbiota and risk of nosocomial *Clostridum difficile*-associated disease. J Infect Dis **202:**1877-1884.

77. **Hopkins MJ, Macfarlane GT.** 2002. Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. J Med Microbiol **51:**448-454.

78. **Rea MC, O'Sullivan O, Shanahan F, O'Toole PW, Stanton C, Ross RP, Hill C.** 2012. *Clostridium difficile* carriage in elderly subjects and associated changes in the intestinal microbiota. J Clin Microbiol **50:**867-875.

79. **Schubert AM, Rogers MA, Ring C, Mogle J, Petrosino JP, Young VB, Aronoff DM, Schloss PD.** 2014. Microbiome data distinguish patients with *Clostridium difficile* infection and non-*C. difficile*-associated diarrhea from healthy controls. MBio **5:**e01021-01014.

80. **Skraban J, Dzeroski S, Zenko B, Mongus D, Gangl S, Rupnik M.** 2013. Gut microbiota patterns associated with colonization of different *Clostridium difficile* ribotypes. PLoS ONE **8:**e58005.

81. **Citron DM, Tyrrell KL, Merriam CV, Goldstein EJ.** 2012. Comparative *in vitro* activities of LFF571 against *Clostridium difficile* and 630 other intestinal strains of aerobic and anaerobic bacteria. Antimicrob Agents Chemother **56:**2493-2503.

770 82. **Kaatz GW, Seo SM.** 1995. Inducible NorA-mediated multidrug resistance in
771 Staphylococcus aureus. Antimicrob Agents Chemother **39:**2650-2655.
772 83. **Leive L.** 1968. Studies on the permeability change produced in coliform bacteria by
773 ethylenediaminetetraacetate. J Biol Chem **243:**2373-2380.
774 84. **Hendrickson BA, Guo J, Laughlin R, Chen Y, Alverdy JC.** 1999. Increased type 1 fimbrial
775 expression among commensal Escherichia coli isolates in the murine cecum following
776 catabolic stress. Infect Immun **67:**745-753.
777 85. **Berg RD.** 1995. Bacterial translocation from the gastrointestinal tract. Trends in
778 Microbiology **3:**149-154.
779 86. **Gautreaux MD, Deitch EA, Berg RD.** 1994. Bacterial translocation from the
780 gastrointestinal tract to various segments of the mesenteric lymph node complex. Infect
781 Immun **62:**2132-2134.
782 87. **Douce G, Goulding D.** 2010. Refinement of the hamster model of *Clostridium difficile*
783 disease. Methods Mol Biol **646:**215-227.
784 88. **Sun X, Wang H, Zhang Y, Chen K, Davis B, Feng H.** 2011. Mouse relapse model of
785 *Clostridium difficile* infection. Infect Immun **79:**2856-2864.

786

FIG 1. Riboswitch binding and functional activity of 5FDQD [5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido[3,4-b]quinoxaline-1,3(2H,5H)-dione]. (A) The chemical structures of 5FDQD, FMN, roseoflavin, and roseoflavin phosphate. (B) Sequence and secondary structure model for the 165 nucleotide aptamer region of the *B. subtilis ribD* FMN riboswitch. Nucleotides where spontaneous cleavage activity decreases upon addition of FMN or 5FDQD are identified with gray circles (regions 1-6, corresponding to the bands identified in C). The nucleotide position where spontaneous cleavage increases upon addition of 5FDQD is designated with an open circle (arrowhead in C). (C) PAGE analysis of an in-line probing assay with 5′ ^{32}P-labeled 156 *ribD* RNA exposed to various concentrations of FMN or 5FDQD (18 pM to 300 nM in 4-fold increments). NR, −OH, and T1 and designate no reaction and partial digestion with either hydroxide ions (cleaves after any nucleotide) or RNase T1 (cleaves after guanosine nucleotides), respectively. Precursor RNA (Pre) and certain RNase T1 cleavage product bands are identified. Locations of spontaneous RNA cleavage changes upon addition of FMN or 5FDQD (regions 1 through 6) are identified by numbered bars. The location of an increase in spontaneous RNA cleavage upon addition of 5FDQD is identified by a gray arrowhead. (D) Plot depicting the average of the normalized fraction of RNA cleaved at regions 1-6 versus the concentration of either FMN or 5FDQD. The curves indicate the best fit of the data to an equation for a two-state binding model (see Materials and Methods). (E) PAGE analysis of an *in vitro* transcription termination assay using the *ribD* FMN riboswitch from *B. subtilis* with the addition of various concentrations of 5FDQD (6 nM to 100 µM in 4-fold intervals). T, riboswitch-terminated RNA transcript; FL, full-length transcript. (F) Plot depicting the fraction of transcripts in the

terminated form versus the concentration of 5FDQD or FMN. The curves indicate the best fit of the data to a two-state model for ligand-dependent termination activity.

FIG 2. Time-kill analysis of 5FDQD, fidaxomicin, and vancomycin. Representative time-kill plot with DMSO, fidaxomicin, vancomycin, or 5FDQD treatment of *C. difficile* VPI 10463. At 6 h, 4X and 8X MIC 5FDQD reduced the density of *C. difficile* VPI 10463 below the 200 CFU/mL lower limit of detection (dashed black line).

FIG 3. Efficacy of 5FDQD, fidaxomicin, and vancomycin in a mouse model of antibiotic-induced *C. difficile* infection. (A) Survival of *C. difficile* VPI 10463-infected C57BL/6 mice that were treated twice daily with 10 mg/kg 5FDQD, 3 mg/kg fidaxomicin (Fid), 0.5% methylcellulose vehicle control (Vehicle), or 50 mg/kg vancomycin once daily (Van). All treatments were administered orally for five days beginning 1 h after inoculation, and each treatment group included 10 mice. 100% survival was observed for 8 d with 5FDQD, fidaxomicin, and vancomycin as highlighted with an asterisk (*). (B) Average daily weight of surviving *C. difficile* VPI 10463-infected mice that received the treatments described in part A, normalized to the average weight of mice in each treatment group immediately prior to inoculation (set to a value of 1). Treatment groups are labeled as in A. The weights of vehicle-treated mice that died during the study were omitted from the calculation of average weights after they died.

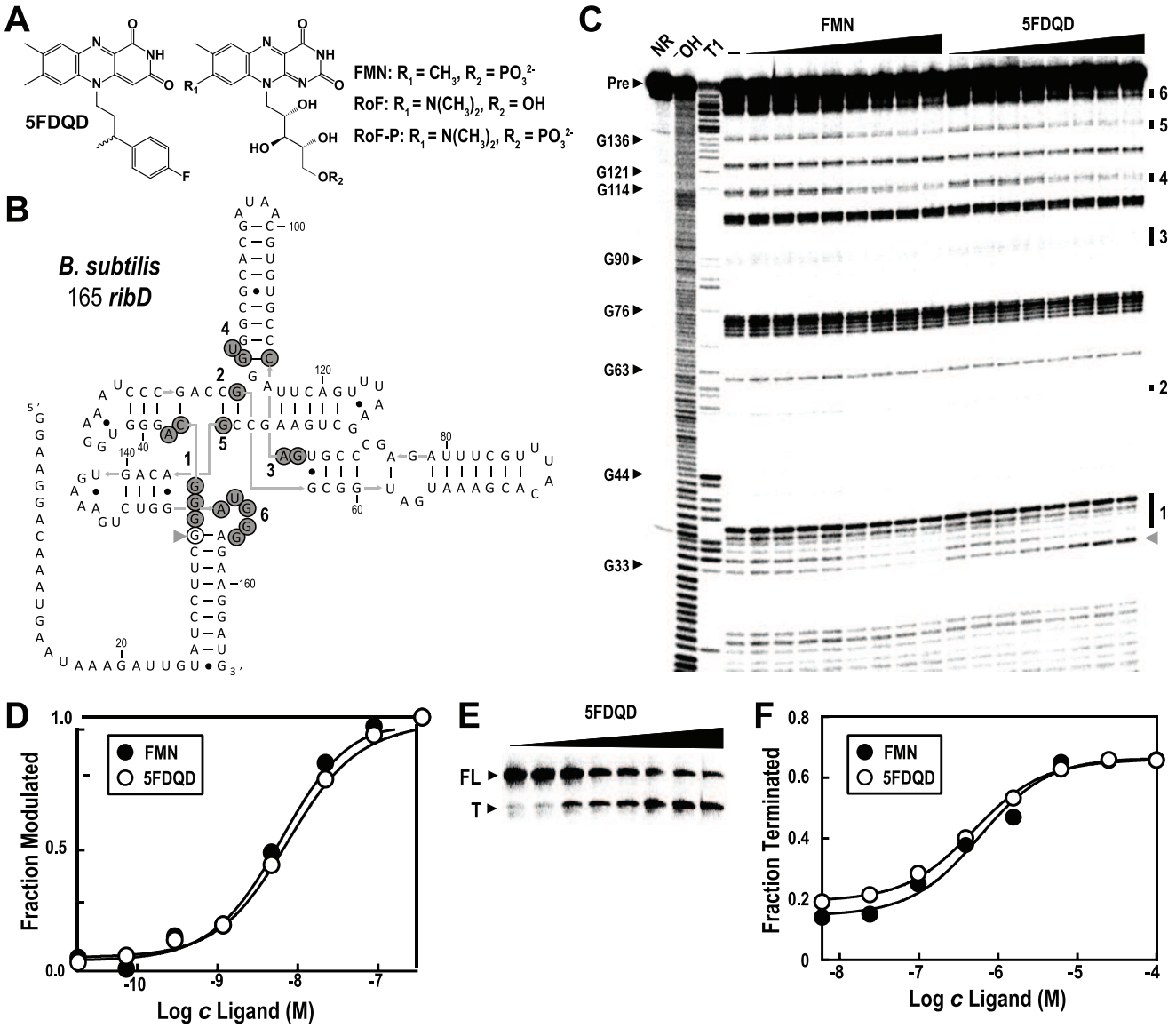
828 **FIG 4. The effect of 5FDQD, fidaxomicin, and vancomycin on culturable cecal flora in mice.**
829 Ceca from healthy C57BL/6 mice were isolated 24 h after the mice were administered a single
830 dose of each indicated treatment and dose, and cecal contents were dilution plated onto seven
831 types of selective agar media to determine the densities of the indicated bacterial taxa in units
832 of CFU/g cecal contents. All colony types counted were presumptively identified to genus or
833 species level as described in MATERIALS AND METHODS. Data shown for clostridia are a
834 measure of cecal spore density as determined by dilution plating an aliquot of ethanol-treated
835 cecal contents. Data from vehicle treated mice are shown as a range of the average +/- one
836 standard deviation (gray bars).

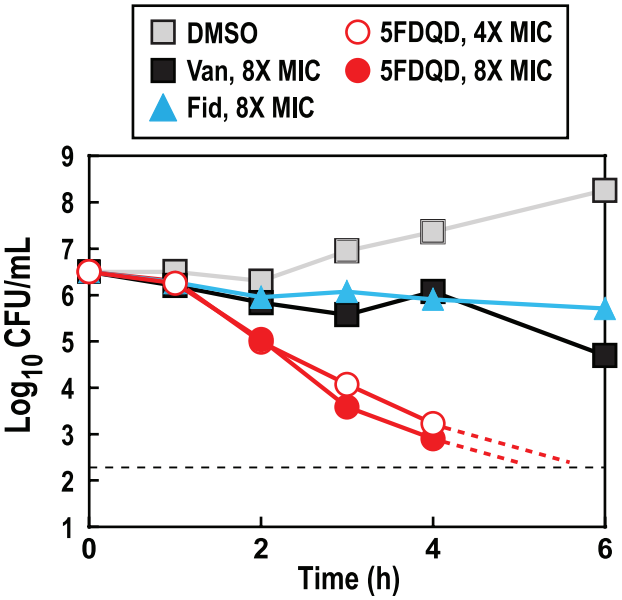
837

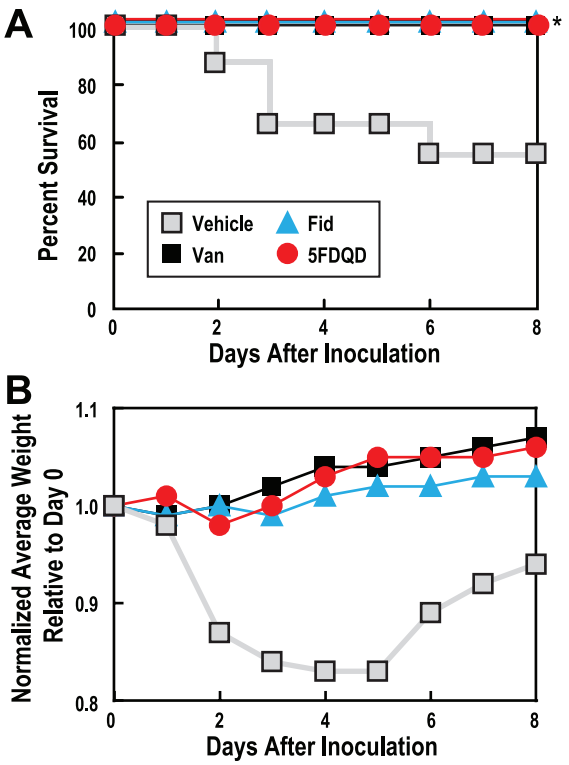
Table 1. Antibacterial activity of 5FDQD and fidaxomicin.

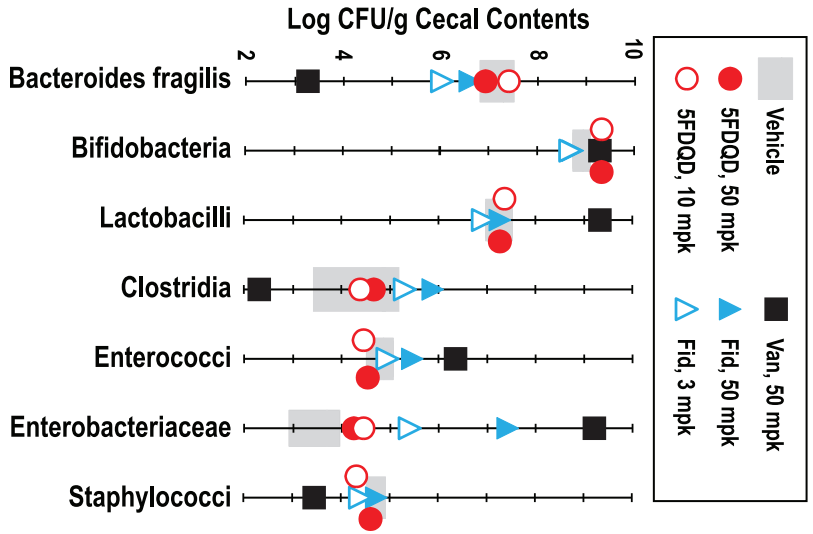
Organism	Strain designation	MIC µg/ml	
		5FDQD	Fidaxomycin
Clostridium difficile (21)*a*		0.5-1	0.03-0.5
Bacteroides fragilis group spp. (10)*b*		>32	>32
Prevotella melaninogenica	3437	>32	>32
Prevotella bivia	3447	>32	>32
Porphyromonas asaccharolytica	3553	4	0.12
Porphyromonas levii	ATCC 29147	4	4
Fusobacterium nucleatum	ATCC 25586	>32	>32
Fusobacterium necroforum	ATCC25286	>32	>32
Veionella parvula	ATCC 17745	>32	0.06
Bifidobacterium bifidum	ATCC 15696	>32	0.015
Bifidobacterium breve	ATCC 15698	>32	0.008
Bifidobacterium infantis	ATCC 15702	>32	0.008
Bifidobacterium longum	ATCC 15707	>32	0.03
Lactobacillus plantarum	ATCC 39268	>32	4
Lactobacilus crispatus	ATCC 33820	>32	4
Lactobacillus acidophilus	ATCC 4356	>32	>32
Lactobacillus lactis	ATCC 3711	>32	2
Lactobacvillus acidophilus	ATCC 4356	>32	1
Lactobacillus gasseri	ATCC 33323	>32	8
Lactobacillus jensenii	ATCC 25258	>32	32
Lactobacillus casei	ATCC 393	>32	2
Lactobacillus rhamnosus	ATCC 7469	>32	4
Actinomyces naeslundii	ATCC 14699	>32	1
Actinomyces naeslundii	ATCC 12104	>32	0.004
Actinomyces viscosus	ATCC 43146	>32	0.008
Actinomyces israelii	ATCC 12102	>32	>32
Escherichia coli (5)		>8*c*	NT*d*
Pseudomonas aeruginosa (5)		>8	NT
Klebsiella pneumoniae (5)		>8	NT
Enterococcus faecalis (5)		2->8	NT
Streptococcus pneumonia (5)		>8	NT
Staphylococcus aureus (6)		>8	NT

[a]ATCC 700057, ATCC 43255, BAA-1802 (NAP-1), BAA-1805 (NAP-1), BAA-1870 (NAP-1), and 16 recent clinical isolates. [b]*B. fragilis* (5), *B. thetaiotaomicron* (3), *B. ovatus*, *B. vulgatus*. [c]Precipitation was observed at 16, 32, and 64 µg/mL in Mueller-Hinton II broth and Mueller-Hinton II + 3% lysed horse blood. [d]Not tested.








Log CFU/g Cecal Contents
2
4
6
8
10
Bacteroides fragilis
Bifidobacteria
Lactobacilli
Clostridia
Enterococci
Enterobacteriaceae
Staphylococci
Vehicle
5FDQD, 50 mpk
Van, 50 mpk
5FDQD, 10 mpk
Fid, 50 mpk
Fid, 3 mpk