Host-targeted niclosamide inhibits *C. difficile* virulence and prevents disease in mice without disrupting the gut microbiota

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*Clostridium difficile* is the leading cause of nosocomial diarrhea and colitis in the industrialized world. Disruption of the protective gut microbiota by antibiotics enables colonization by multidrug-resistant *C. difficile*, which secrete up to three different protein toxins that are responsible for the gastrointestinal sequelae. Oral agents that inhibit the damage induced by toxins, without altering the gut microbiota, are urgently needed to prevent primary disease and break the cycle of antibiotic-induced disease recurrence. Here, we show that the anthelmintic drug, niclosamide, inhibits the pathogenesis of all three toxins by targeting a host process required for entry into colonocytes by each toxin. In mice infected with an epidemic strain of *C. difficile*, expressing all three toxins, niclosamide reduced both primary disease and recurrence, without disrupting the diversity or composition of the gut microbiota. Given its excellent safety profile, niclosamide may address an important unmet need in preventing *C. difficile* primary and recurrent diseases.

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Clostridium difficile is a spore-forming Gram-positive bacterium that causes a range of gastrointestinal diseases, typically in individuals that have taken a course of broad-spectrum antibiotics, which lowers the diversity of the protective resident GI microbiota. In the resulting aftermath, opportunistic C. difficile colonizes the lower GI tract of susceptible individuals, and secretes up to three gut-damaging toxins, including two large homologous toxins TcdA and TcdB, and in the case of epidemic strains of C. difficile—such as ribotype 027 (RT027) and ribotype 078 (RT078)—a third, unrelated binary toxin called CDT. In recent years, C. difficile has become a major public health concern, due to the proliferation and global spread of epidemic strains, which are associated with increased morbidity and mortality. The increased virulence of these strains has been attributed to several factors, including acquisition of mutations in gyrB that result in resistance to fluoroquinolones; increased expression of toxins; production of a more cytotoxic form of TcdB; and, expression of CDT.

Extensive experimental and epidemiological evidence support a role for toxins as the primary determinants of disease pathogenesis. Isogenic knockout studies, in which toxins were deleted individually, or in combination, showed that TcdA and TcdB alone are sufficient to cause fulminant disease in hamsters, whereas CDT appears to contribute to virulence in combination with TcdA or TcdB. Importantly, knockout of all three toxins renders C. difficile completely avirulent.

The inextricable link between antibiotic-induced dysbiosis of the GI microbiota and infection by C. difficile, together with the well-validated role of toxins in driving disease pathogenesis, provide strong rationale and validation for targeting the actions of the C. difficile toxins as a novel approach to treat or prevent C. difficile infection (CDI), despite early clinical setbacks with nonspecific polymers meant to sequester the toxins in the GI tract, the monoclonal antibody bezlotoxumab (Zinplava—Merck), which binds to and blocks TcdB following toxin-induced damage of the gut lining, was recently approved for use in CDI patients for reducing recurrence. This important clinical validation of toxin-targeting approaches for treating C. difficile recurrence has fueled efforts to develop next-generation anti-toxins that are orally-bioavailable (i.e., small molecules), have a greater spectrum of activity against all C. difficile toxins, and potentially be used prior to, or during a suspected primary infection. Ideally, such a therapeutic, in addition to having an impeccable safety profile in humans, would not itself affect the composition of the protective gut microbiota, which is ultimately required to prevent further re-infection. Moreover, with the emergence of new C. difficile ribotypes, such as RT033, that do not express TcdB, but are nevertheless pathogenic, it would be desirable to have a single agent with the above characteristics that is also able to prevent TcdA- and/or CDT-induced pathogenesis.

In this study, we screened libraries of approved drugs in a phenotypic screen of TcdB-induced cell rounding with the goal of identifying small molecules that may potentially be repurposed for treating CDI through direct toxin inhibition. Recent drug repurposing phenotypic screens have led to discoveries of potential new candidate therapies for a number of infectious diseases, including for giardiasis, Zika virus infection, Ebola virus disease, and Hepatitis C virus infection. Here, among the panel of hits identified that completely protected cells from TcdB intoxication, we identified niclosamide, a widely used anthelmintic drug approved by the US FDA for treating intestinal infections of tapeworms. Based on its excellent safety profile, and its preferential biodistribution in the colon resulting from its poor absorption in the GI tract, we investigated niclosamide as an oral toxin-neutralizing treatment for CDI.

**Results**

**High-throughput screen for inhibitors of TcdB intoxication.**

Intoxication by TcdB toxin is a multistep process, involving four functionally-distinct toxin domains, and several host-factors and processes (Fig. 1a). The intoxication of cells leads first to cytotoxic effects (i.e., rounding of cells) within 1–3 h, and later, cytotoxic effects (i.e., apoptosis) after 24 h. To identify small molecules that protected cells from TcdB, we employed a high-throughput assay of TcdB-induced cell rounding that we previously developed, which quantifies the extent of rounding of human lung fibroblasts treated with cytotoxic doses of TcdB (i.e., 1 pM for 3 h) using high-content imaging analysis.

From the 60 compounds that protected cells from TcdB by greater than the statistical cut-off of three standard deviations of the mean of the data (Supplementary Figure 1), we triaged drugs with undesirable mechanisms-of-action (i.e., antibiotics and antiseptics), and those that are known to be toxic or poorly tolerated in humans. Emerging from this prioritization were the three related salicylanilide anthelmintic drugs: niclosamide (71% inhibition), closantel (60% inhibition), and oxyclozanide (43% inhibition) (Fig. 1b, c); drugs that act on parasites within the GI lumen, and that have well-documented safety margins in humans. Among these salicylanilides, niclosamide was the most potent inhibitor of TcdB-induced cell-rounding, protecting cells with an EC₅₀ = 0.51 ± 0.03 μM (values represent mean ± s.e.m., n = 5) (Fig. 1d). Protection from TcdB-induced cell-rounding by niclosamide was complete; human IMR-90 fibroblasts that were co-incubated with niclosamide and TcdB were indistinguishable from cells that had not received toxin (Fig. 1e). To evaluate the extent of protection by niclosamide against different amounts of TcdB (reflecting the range of toxin levels that might be experienced during an infection), cells were treated with a range of TcdB concentrations at different fixed doses of niclosamide. In the absence of drug, TcdB dose-dependently induces cell rounding with an EC₅₀ = 0.8 PM (Fig. 1f). In the presence of increasing concentrations of niclosamide, the amount of TcdB required to reach equivalent levels of rounding increased dramatically. Remarkably, above the EC₅₀ of niclosamide, cells were completely protected from TcdB by over three orders-of-magnitude, corresponding to a protection factor (PF) >5000 (Fig. 1f).

Next, we tested the ability of niclosamide and the more water-soluble ethanolamine salt form of niclosamide, niclosamide ethanolamine (NEN) to maintain the integrity of human epithelial colorectal cells (CaCo-2 cells) that were treated with TcdB. Treatment of a confluent monolayer of CaCo-2 cells with TcdB results in disruption of monolayer integrity and loss of trans-epithelial resistance within hours of application as a result of GTD-induced actin depolymerization. Disruption of the monolayer integrity by TcdB was prevented by co-treatment with niclosamide (Supplementary Figure 2). Furthermore, NEN prevented the TcdB-induced disruption of Caco-2 monolayers maintaining barrier function to untreated levels (Fig. 1g).

**Mechanism of TcdB neutralization by niclosamide.**

To elucidate the mechanism by which niclosamide inhibits TcdB-induced cell rounding, we carried out a series of assays that evaluate each step of the intoxication pathway in isolation (Fig. 1a). Though
Niclosamide protects cells from all three C. difficile toxins. The determination that niclosamide inhibited TcdB at the level of the host endosome prompted us to consider the intriguing possibility...
that niclosamide might additionally block the actions of TcdA and CDT, both of which require endosomal acidification for pore-formation and intracellular entry. Indeed, niclosamide and NEN completely protected cells from TcdA-induced cell rounding, and from CDT-induced damage (i.e., depolymerization of the actin cytoskeleton), at the same doses that protect cells from TcdB pore-formation and intracellular entry. Indeed, niclosamide and NEN completely protected cells from TcdA-induced cell rounding, and from CDT-induced damage (i.e., depolymerization of the actin cytoskeleton), at the same doses that protect cells from TcdB pore-formation and intracellular entry.

Demonstrating protection against CDT, a toxin that bears no structural or functional similarities to TcdA and TcdB, other than requiring low pH to escape endosomes, further supports the mechanism-of-action for niclosamide. More importantly, this finding suggests that niclosamide, as a single entity, could potentially protect from infection and disease by all pathogenic C. difficile strains, expressing any combination of toxins, in vivo.

NEN reduces disease by an epidemic strain of C. difficile in mice. Mice preconditioned with antibiotics and challenged with C. difficile develop typical CDI (weight loss, diarrhea, death) in the absence of any therapeutic countermeasures. To test the hypothesis that niclosamide is capable of preventing disease induced by strains expressing multiple toxins, we evaluated the efficacy of NEN in protecting against CDT in a murine model challenged with the hypervirulent strain UK1 (RT027), which expresses TcdA, TcdB, and CDT. Infected mice were treated with either water (control) or NEN (at different doses; 2, 10, and 50 mg per kg) via oral gavage 4 h post spore challenge and for 3 consecutive days after spore challenge (Fig. 3a). Typical symptoms of CDI in murine models include severe weight loss on days 2 and 3 post-challenge accompanied with diarrhea and high mortality rate in sham groups. All doses of NEN tested significantly protected mice from weight loss compared to control group (Fig. 3b). NEN protected mice from death in a dose-dependent manner.
Niclosamide ethanolamine (NEN) is protective in primary and recurrent CDI. a Protocol schematic for primary CDI model. b Weights of mice after challenge with C. difficile spores (10^5 CFU/mL) on day 0. Mice were treated with vehicle (5% DMSO) or NEN (in 5% DMSO) at different doses (2, 10, and 50 mg per kg). Each point is the mean SE from day 0. c Survival of infected mice treated and un-treated with NEN (50 mg per kg). d Diarrhea score of infected mice. *p < 0.05; **p < 0.01 using unpaired Student t-test. Data represent 10 mice/group.

**Table 2** MIC values for niclosamide, NEN, and vancomycin on Clostridial species

| Organism (strain) | Vancomycin (μg/mL) | NEN^a (μg/mL) |
|-------------------|---------------------|---------------|
| C. difficile R20291 (027) | 1 | >19 |
| C. difficile M68 (017) | 2 | >19 |
| C. clostridioforme ATCC25537 | 4 | >19 |
| C. sporogenes ATCC3584 | 8 | >19 |

Data are n = 3
^a9 μg/mL = 50 μM

with all mice in the 50 mg per kg group remarkably surviving infection, compared to only 45% for control group (Fig. 3c). These results closely tracked the wet tail and diarrhea scores, which were significantly lower in NEN-treated groups (Fig. 3d).

Symptomatic recurrence of CDI, which occurs in approximately one-in-four individuals, is a characteristic feature of CDI that complicates eradication and management of C. difficile. We assessed whether NEN (50 mg per kg) could prevent recurrence in a mouse model of recurrent CDI in which infected mice are treated with vancomycin (0.5 mg per mL) in their drinking water starting on day 1 ongoing for 6 days after spore challenge (Fig. 3e). NEN treatment was given via oral gavage 4 h post spore challenge and for 3 consecutive days after spore challenge. Both groups (NEN-treated and un-treated) started losing weight on day 4 after receiving vancomycin water (i.e., day 11 post spore challenge) (Fig. 3f). As above, all mice in the NEN group survived from C. difficile challenge, whereas more than 60% of mice in the control group became moribund (Fig. 3g, h). After resolution of symptoms, both groups began to lose weight at day 11; however, NEN-treated mice displayed less severe diarrhea scores, and importantly all NEN-treated mice survived recurrence.

**NEN does not affect C. difficile growth in vitro.** As salicylanilide derivatives have been reported previously to have antimicrobial activity against certain Gram-positive bacteria, we next carried out a series of experiments to address, whether NEN, specifically, had any antibacterial activity against C. difficile that may have contributed to the protective effects seen in vivo. To this end, we measured the minimum inhibitory concentrations (MICs) of NEN on individual strains of Clostridium species using the gold-standard anaerobic agar dilution assay. No antimicrobial activity was seen for NEN up to 19 μg/mL (i.e., 50 μM NEN) against either of the two strains of C. difficile tested (017 and 027), or against the two non-pathogenic Clostridium species tested (Table 2). These results were confirmed against a larger panel of C. difficile strains, where we saw no activity for NEN up to 32 μg/mL (i.e., 84 μM) (Table 3). While these data indicate that
NEN does not affect *C. difficile* growth directly, an important feature to demonstrate for NEN, or any would-be *C. difficile* therapeutic, is the lack of effect on the gut microbiota.

**NEN effects on the gut microbiota in vivo.** To directly address whether NEN had any effects on the gut microbiota that may contribute to disease pathogenesis, we next evaluated the effect of NEN treatment on the composition and structure of the gut microbiota in mice under various situations. First, in mice that were not infected with *C. difficile*, we investigated the effects of NEN at the highest dose tested in the efficacy study (i.e., 50 mg per kg), and compared this with both vancomycin and vehicle control. As shown in Fig. 4a, gut microbiota diversity in NEN-treated and control mice are only slightly but statistically significant different on day 3, however, the difference is minimal compared to the large reduction observed after vancomycin treatment. This small difference disappears on day 6, while that observed in the vancomycin-treated group remains high. Further, ordination analysis shows the minimal effect of NEN and the large effect of vancomycin on the gut microbiota structure at all time points post-treatment compared to controls (Supplementary Figure 5a). Vancomycin treatment dramatically lowered the diversity of the microbiota, shifting the composition to high relative abundance of *Lactobacillaceae* and *Enterobacteriaceae* (Fig. 4a) as seen previously. Thus, we conclude that NEN treatment has a minimal effect on the gut microbiota.

Next, we evaluated the effects of NEN on the gut microbiota in *C. difficile*-infected mice that had been pre-treated with an antibiotic cocktail, and clindamycin the day prior to infection (Fig. 4b–d). The diversity of the microbiota following NEN treatment (50 mg per kg) on days 1, 2, and 3 post-infection was indistinguishable from the water control group (Fig. 4b). Further, the composition and structure of the gut microbiota on day 6 post-infection did not differ from water treatment (Fig. 4b and Supplementary Figure 5), comprising of high relative abundance of *Lactobacillaceae, Bifidobacteriaceae, Clostridiaceae*, and *Bacteroidales*, and decreased relative abundance of *Enterobacteriaceae* (mainly *Escherichia coli*) (Supplementary Figure 5 and Supplementary Figure 6). Linear discriminant analysis (LDA) effect size (LEfSe) analysis only identified members of the genus *Bacteroides* out of 154 phyotypes as significantly more abundant on day 6 in water control than in NEN treated mice (Supplementary Figure 7).

Having shown that NEN does not affect the structure or composition of the microbiota, we next asked whether NEN (alone or combination with vancomycin) was potentially able to help restore the gut microbiota during the resolution phase of infection. To this end, we compared the effects of NEN (50 mg per kg) + vancomycin (0.5 mg per kg), and NEN (50 mg per kg) alone, to vancomycin alone, in the recurrent *C. difficile* model. As expected, the diversity of the gut microbiota after vancomycin treatment alone remained low throughout the treatment cycle and was dominated by *Lactobacillaceae* (Fig. 4c, d). Whereas the addition of NEN (50 mg per kg) to vancomycin (0.5 mg per kg) showed no benefits to the microbiota compared to vancomycin alone (Fig. 4c), treatment with NEN (50 mg per kg) alone resulted in a significant increase in diversity post-treatment and during resolution of infection (Fig. 4d), indicating that NEN may have additional benefits on the gut microbiota as a stand-alone therapy.

**Discussion**

The global spread of epidemic strains of *C. difficile* capable of causing outbreaks and life-threatening infections is a recent phenomenon that has been brought on, in part, by modern human practices. The widespread introduction of the food additive trehalose, shortly before the emergence of epidemic RT027 and RT078, has been proposed to have contributed to selecting for these strains and increasing their virulence. Similarly, the use and misuse of antibiotics have further accelerated the enrichment of multidrug-resistant variants of *C. difficile*, whilst disrupting the protective microbiota that normally prevents such infections. As a result, *C. difficile* continues to increasingly become more widespread, more virulent, and more difficult to treat with traditional eradication approaches (i.e., antibiotics). The notion of targeting the virulence determinants of *C. difficile* has emerged as an attractive alternative strategy to treat CDI, especially given the role that toxins play in all aspects of disease pathogenesis. The recent clinical demonstration of disease recurrence attenuation by the injectable TcdB-targeted antibody bezlotoxumab supports these approaches and has fueled efforts to identify next generation antivirulence therapeutics. In particular, more convenient oral agents (i.e., small molecules) that can be dosed at all stages of disease are highly sought after. Moreover, although targeting TcdB appears to be capable of decreasing recurrence, blocking the actions of TcdA and binary toxin, both of which contribute to disease pathogenesis in hypervirulent strains and appear to be sufficient for causing disease in strains lacking TcdB in certain cases, would be a highly desirable feature of any comprehensive would-be antivirulence strategy.

In this study, we performed a high-throughput screen of libraries containing FDA- and EMEA-approved drugs to identify small-molecules that protected cells from TcdB intoxication that could potentially be repositioned as orally-bioavailable therapeutics for treating CDI. Among the dozens of hits identified in the primary screen (Supplementary Figure 1), we noted that several were approved anti-parasitic drugs. From the most potent inhibitors of cell rounding in this class, niclosamide was selected for further characterization based on its impeccable safety profile in humans, and known preferential distribution in the lower GI after oral dosing, which we anticipated would be beneficial for targeting the gut-damaging toxins of *C. difficile*. Niclosamide is a remarkably well-studied molecule that has been shown to have a number of other biological activities in vitro that have prompted other investigations into translation into diseases including cancer, diabetes as well as other infectious diseases. In most cases, however, the low systemic exposure of niclosamide,

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**Table 3.** *C. difficile* MIC values for NEN, vancomycin, metronidazole, and fidaxomicin

| Organism ATCC (MMX No.)a | MIC in μg/mL | Fidaxomicin | Vancomycin | Metronidazole | NEN |
|--------------------------|-------------|-------------|------------|---------------|-----|
| *C. difficile 70005* (4381) | 0.06b | 2c | 0.5d | >32 |
| *C. difficile (8261)* | 0.06 | 2 | 0.5 | >32 |
| *C. difficile (8262)* | 0.12 | 4 | 0.25 | >32 |
| *C. difficile (8263)* | 0.06 | 2 | 0.25 | >32 |
| *C. difficile (8341)* | 0.06 | 1 | 0.25 | >32 |
| *C. difficile (8336)* | 0.12 | 4 | 0.5 | >32 |
| *C. difficile (8337)* | 0.06 | 1 | 0.25 | >32 |
| *C. difficile (8338)* | 0.12 | 4 | 2 | >32 |
| *C. difficile (8339)* | 0.12 | 4 | 4 | >32 |
| *C. difficile (8340)* | 0.12 | 2 | 0.5 | >32 |

a Micromyx Isolate Number
b CLSI QC range (0.06–0.25)
c CLSI QC range (0.5–4)
d CLSI QC range (0.125–0.5)
which is likely a major contributor to its overall safety, has hampered its use for indications outside of the GI tract. Nevertheless, efforts have been undertaken to improve the bioavailability of niclosamide, using different salt forms, chemical modifications, and the use of nanoparticles51–53.

Here, we found that niclosamide provided protection to a variety of human cells from both TcdB-induced cell-rounding, and TcdB-induced necrosis. Moreover, due to its mechanism of inhibition of TcdB (i.e., targeting host endosomal pH through a proton-shuttle mechanism), niclosamide had the added major inhibition of TcdB (i.e., targeting host endosomal pH through a mechanism of action that also requires low pH for entry into the host). These features provided the unique opportunity to test niclosamide as a standalone agent against epidemic strains of C. difficile that are triple-positive for TcdA, TcdB, and CDT. In mouse models of CDI, we showed that NEN dose-dependently improved disease symptoms associated with both primary infection and recurrence, with full protection seen at 50 mg per kg NEN. Of note, the reported median oral lethal dose (LD50) for NEN in rats is 10,000 mg per kg body weight27, further emphasizing the large therapeutic index for NEN in treating CDI. Finally, an important and significant finding in this study was that NEN had no major deleterious effects on the structure and composition of the microbiota (Fig. 4). Increased diversity of the gut microbiota was observed after NEN treatment, a feature that was thought to be unique to fecal microbiota transplantation and key to its success. Recovery of a diverse microbiota might in part contribute to its curative properties. Beyond this study and this indication, the findings
presented here pave the way to future studies aimed at investigating the effects of Nen on other toxin-driven enteric diseases. Also, given the increasingly appreciated role that helminths play in interacting with and modulating the microbiome\textsuperscript{54,55}, the results here highlight the importance of future studies aimed at understanding the effects of Nen on the microbiota during a helminth infection.

**Methods**

**Cell lines, consumables, and reagents.** Plasticware used for cell culture and enzyme digestion were from Corning. Streptavidin-Hi-bind plates, Superblock buffer, SuperSignal West, and Quantablu peroxidase substrate were purchased from Thermo Pierce (Rockford, IL). Cell lines CHO-K1 (ATCC, Cat #CCL-61), Vero (ATCC, Cat #CCL-81), CaCo-2 (ATCC, Cat #HTB-37), HCT-116 (ATCC, Cat #CCL-247), and IMR90 (ATCC, Cat #CCL-186) were purchased from ATCC (Manassas, VA). Anti-TcdB antibody (used at 1:2000 dilution) was purchased from R&D Systems (Cat #AF6246). Anti-Rac1 antibody Mab102 (used at 1:1000 dilution) was purchased from BD Biosciences (Mississauga, ON). The LOPAC1280 was purchased from Sigma-Aldrich (Oakville, ON).

**Protein expression and purification.** Isolation of recombinant proteins were as described by Yang et al.\textsuperscript{56}. Briefly, transformed Bacillus megaterium was inoculated into LB containing tetracycline and grown to an A600 of 0.7, followed by overnight xylene induction at 37 °C. Bacterial pellets were collected, resuspended with 20 mM Tris pH 8/0.5 M NaCl, and passed twice through an Emulsiflex C3 microfluidizer (Avestin, Ottawa, ON) at 15,000 psi, then clarified by centrifugation at 18,000g for 20 min. TcdB was purified by nickel affinity chromatography followed by anion exchange chromatography using HisTrap FF and HiTrap Q (GE Healthcare, Baie D’Urfé, QC), respectively. Fractions containing TcdB were verified by SDS-PAGE, then pooled and dialyzed with a 100,000 MWCO ultrafiltration device (Conning) into 20 mM Tris pH 7.5/150 mM NaCl. Finally, glycerol was added to 15% v/v, the protein concentration was estimated by A280 (using coefficient 281160), divided into single-use aliquots, and stored at −80 °C.

For TcdA, cell lysates were prepared as described for full-length TcdB, and purification of the protein was by nickel affinity chromatography using HisTrap FF columns.

**Arrayscan high content phenotypic screen.** IMR90 cells were grown in 6-well plates at a density of 300,000 cells/well. The next day, TcdB (0.5 μM final) and NEN (2A pharmachen) were added to the wells in serum-free media. After 60 min the cells were harvested and lysed in Laemmli buffer. SDS PAGE and western blotting were performed to detect glycolylated Rac1 using mAb102 (BD Biosciences), total Rac1 using mAB23A8 (Abcam), and loading controls using anti-EEL1 and anti-ERK1/2 (EMD Millipore). Raw Western Blot images are shown in Supplementary Figure 8.

**UDP-Glo\textsuperscript{TM} UDP-glucose hydrolase assay (Promega).** Experiments were performed as per the manufacturer’s instructions. Briefly, 100 nM of GTF enzyme was incubated in a glycolylation buffer (see above) with various concentrations of inhibitor in a final volume of 16 μL. Reactions were started with the addition of 4 μL of UDP-glucose (50 μM final). Reactions were allowed to proceed at room temperature.
temperature for 15 min. To stop the reaction, 10 μL were removed and added to a white, polystyrene 96-well half-area plate (Costar) containing 10 μL of UDG detection reagent. Plates were incubated at room temperature for 1 h, then luminescence was recorded on a SpectraMax M5e plate reader (Molecular Devices) with an integration time of 750 ms. Results were analyzed with SoftMax Pro 6.2.2 and GraphPad Prism 5.0.

**MIC assays to determine effects of NEN on *C. difficile* strains.** For results shown in Table 2: Minimal Inhibitory Concentration (MIC) testing was performed by the National Research Council of Canada (Ottawa), using the following bacterial strains: *Clostridium difficile* R20291 (UK) O27 ribotype, *Clostridium difficile* M6089 017 ribotype, *Clostridium clostridiotrophi* ATCC25537, and *Clostridium sporogenes* ATCC3584. Compounds were dissolved in DMSO and added to Brucella supplemented blood plates. Niclosamide and NEN from a 10 mM stock solution in DMSO, was tested at 50 μM starting concentration (16 and 19 μg/mL, respectively), with 1 in 3 serial dilutions. Vancomycin was tested as a positive control starting at 8 μg/mL, with 1 in 2 serial dilutions.

Inoculum preparation: strains were grown on Brucella supplemented blood agar plates in an anaerobic chamber for 16 h. For each strain, cells were harvested into saline to OD 0.1. The CFU/mL was confirmed by serial dilution and plating on Brucella supplemented blood or Baziars agar for each experimental group. For MIC Testing, 10 μL of each strain was spotted three times onto each agar plate and incubated at 37 °C in an anaerobic chamber. Each plate was examined for visible growth at 16, 24, 32, and 48 h. No further change in growth pattern was observed for any strain from 16 to 48 h growth. MIC determinations were performed in duplicate and reported as the concentration that completely inhibits growth. For results represented in Table 3: Minimal Inhibitory Concentration (MIC) testing was performed by Micromyx. NEN and comparator antibiotics were prepared on the day of testing using solvents recommended by CLSI. Stock solutions of all compounds were made at 100x the final testing concentration. Test organisms consisted of clinical isolates from the American Type Culture Collection (ATCC) and Micromyx repository. Drug dilutions and drug-supplemented agar plates were prepared manually. After pouring the Supplemented Brucella agar plates, they were allowed to dry, pre-reduced in the Bactron II anaerobic chamber, then spotted and incubated using the Steers Replicator to yield final cell concentration of 1 × 10^6 colony-forming units/spot. After the inocula had dried, the drug-supplemented plates were incubated at 35 °C for 16, 24, 32, and 48 h under anaerobic conditions. The MIC was read per CLSI guidelines as the concentration at which growth was significantly inhibited relative to the growth control.

**In vivo studies.** All procedures involving animals were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore. For the primary CDI Model41-44, C57BL/6 mice (10 per group) were orally administered 10^6 CFU of *C. difficile* spores from the UK1 (BUNAP1027) strain after receiving antibiotic treatment in the drinking water for 3 days, as shown in Fig. 3. For each antibiotic treatment, inocula were prepared containing: 100 μg/mL of DMSO (0.035 mg per mL), colistin (850 U per mL), metronidazole (0.215 mg per mL), and vancomycin (0.045 mg per mL). This corresponds to the approximate concentration of the 16S rRNA gene V4 hypervariable region was sequenced from the UK1 (BI/NAP1/027) strain characterized resulting in a total of 6,619,465 high-quality non-chimeric reads with an average length of 245 bp. Trimmed reads were used to infer ribosomal sequence variants and their relative abundance in each sample after removing chimera. A total of 205 fecal samples were sequenced by the National Research Council of Canada (Ottawa), using the following bacterial strains: *Clostridium dif".

**Microbiota analysis.** DNA was extracted using the MagAttract PowerMicrobiome DNA/RNA kit (Qiagen) from the fecal pellet of all samples. Briefly, the glass bead plate was used to mix fecal material and lysis solution, and inhibitor was subse- quently removed from the supernatant. ClearMag Beads suspension was then mixed with 450 μL of the supernatant to purify the extracted DNA. DNA was extracted from all samples (150 μg) using the MagAttract PowerMicrobiome DNA/RNA kit (Qiagen) implemented on a Hamilton STAR robotic platform and after a bead-beating step on a TissueLyser II (Qiagen) in 20 μL of a 25 μM Tris-EDTA buffer. The DNA was extracted from a 100 μL sample using the MagAttract PowerMicrobiome DNA/RNA kit (Qiagen). In order to remove the yeast cells, the supernatant was centrifuged at 20,000 × g for 10 min. The resulting supernatant was used for the subsequent steps. The DNA was eluted with 30 μL of Elute Buffer and stored at −80 °C for further analysis. The extracted DNA was quality-checked using the Agilent 2100 Bioanalyzer (Agilent Technologies) and stored at −80 °C for further analysis.

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**Author contributions**

J.T., T.H., H.F., and R.A.M. designed the research. J.T., T.H., B.M., K.C., and G.L.B. performed the research. J.T., T.H., B.M., K.C., G.L.B., J.R., H.F., and R.A.M analyzed the data and R.A.M. wrote the paper.

**Additional information**

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