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Amixicile, a Novel Inhibitor of Pyruvate:Ferredoxin Oxidoreductase, Shows Efficacy against *Clostridium difficile* in a Mouse Infection Model

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***Clostridium difficile* infection (CDI) is a serious diarrheal disease that often develops following prior antibiotic usage. One of the major problems with current therapies (oral vancomycin and metronidazole) is the high rate of recurrence. Nitazoxanide (NTZ), an inhibitor of pyruvate:ferredoxin oxidoreductase (PFOR) in anaerobic bacteria, parasites, *Helicobacter pylori*, and *Campylobacter jejuni*, also shows clinical efficacy against CDI. From a library of ~250 analogues of NTZ, we identified leads with increased potency for PFOR. MIC screens indicated *in vitro* activity in the 0.05- to 2-μg/ml range against *C. difficile*. To improve solubility, we replaced the 2-acetoxy group with propylamine, producing amixicile, a soluble (10 mg/ml), nontoxic (cell-based assay) lead that produced no adverse effects in mice by oral or intraperitoneal (i.p.) routes at 200 mg/kg of body weight/day. In initial efficacy testing in mice treated (20 mg/kg/day, 5 days each) 1 day after receiving a lethal inoculum of *C. difficile*, amixicile showed slightly less protection than did vancomycin by day 5. However, in an optimized CDI model, amixicile showed equivalence to vancomycin and fidaxomicin at day 5 and there was significantly greater survival produced by amixicile than by the other drugs on day 12. All three drugs were comparable by measures of weight loss/gain and severity of disease. Recurrence of CDI was common for mice treated with vancomycin or fidaxomicin but not for mice receiving amixicile or NTZ. These results suggest that gut repopulation with beneficial (non-PFOR) bacteria, considered essential for protection against CDI, rebounds much sooner with amixicile therapy than with vancomycin or fidaxomicin. If the mouse model is indeed predictive of human CDI disease, then amixicile, a novel PFOR inhibitor, appears to be a very promising new candidate for treatment of CDI.**

Clostridium difficile is a Gram-positive spore-forming obligate anaerobe present in the intestinal microflora of much of the human population. Toxigenic *C. difficile* is associated with pseudomembranous colitis in patients receiving long-term broad-spectrum antimicrobials (3, 18) and is also recognized as the most common cause of hospital-associated severe diarrhea (28, 29). More worrisome is the emergence of hypervirulent binary toxin-producing strains such as the North American pulsed-field type 1 (NAP1, also referred to as NAP1/BI/027) prevalent in hospitals, nursing homes, and assisted-living facilities that can be acquired by otherwise healthy individuals (3, 42). These emerging hypervirulent strains are also associated with higher mortality rates (29, 42). Antibiotic interventions with oral vancomycin and metronidazole (MTZ) are effective treatments for severe and mild forms of the disease, respectively (8, 38); however, recurrence rates are as high as 30% and recurrences require multiple treatment cycles to resolve infection (8). Fidaxomicin, a recently FDA-approved drug that inhibits RNA polymerase (37), has been shown in clinical trials to substantially reduce recurrence compared with vancomycin in non-027-infected patients (20, 23). In contrast to vancomycin and metronidazole, fidaxomicin has been shown to have little effect on the human gut microbiota, which ordinarily provides competitive exclusion protection against *C. difficile* colonization (23, 35).

Nitazoxanide (NTZ), an FDA-approved therapeutic developed for treatment of intestinal infections caused by *Cryptosporidium parvum* and *Giardia intestinalis* (15), shows efficacy in the treatment of complex cases of *C. difficile* infection (CDI) (25, 39). Nitazoxanide shows selectivity for those anaerobic bacteria expressing pyruvate:ferredoxin oxidoreductase (PFOR), an essential enzyme of central metabolism (1, 11, 14, 16, 17, 26, 31). Previous studies have reported MICs as low as 50 ng/ml against *C. difficile*, and in other MIC-based comparative studies, NTZ outperformed most mainline antimicrobials (14, 25, 26). NTZ is poorly soluble in water and, like fidaxomicin, concentrates in the gut (33, 41).

NTZ targets the thiamine pyrophosphate (TPP) vitamin cofactor of PFOR by outcompeting the substrate pyruvate by nearly 2 orders of magnitude (K_i value of 5×10^{-6} M versus the K_m for pyruvate of 3×10^{-4} M) (2, 16). Nuclear magnetic resonance (NMR) studies revealed that the anion of NTZ is biologically active, and modeling studies suggest that the anionic 5-nitro group of NTZ is most favorable for abstracting a proton from the N4′ of the TPP pyrimidine, thus deactivating the enzyme (1, 2, 16). Importantly, by targeting the vitamin cofactor (small molecule) and not the enzyme *per se*, this class of drugs may escape the traditional path of mutation-based drug resistance (1, 2, 16, 31). McVay and Rolfe reported that NTZ was active against *C. difficile* in a hamster

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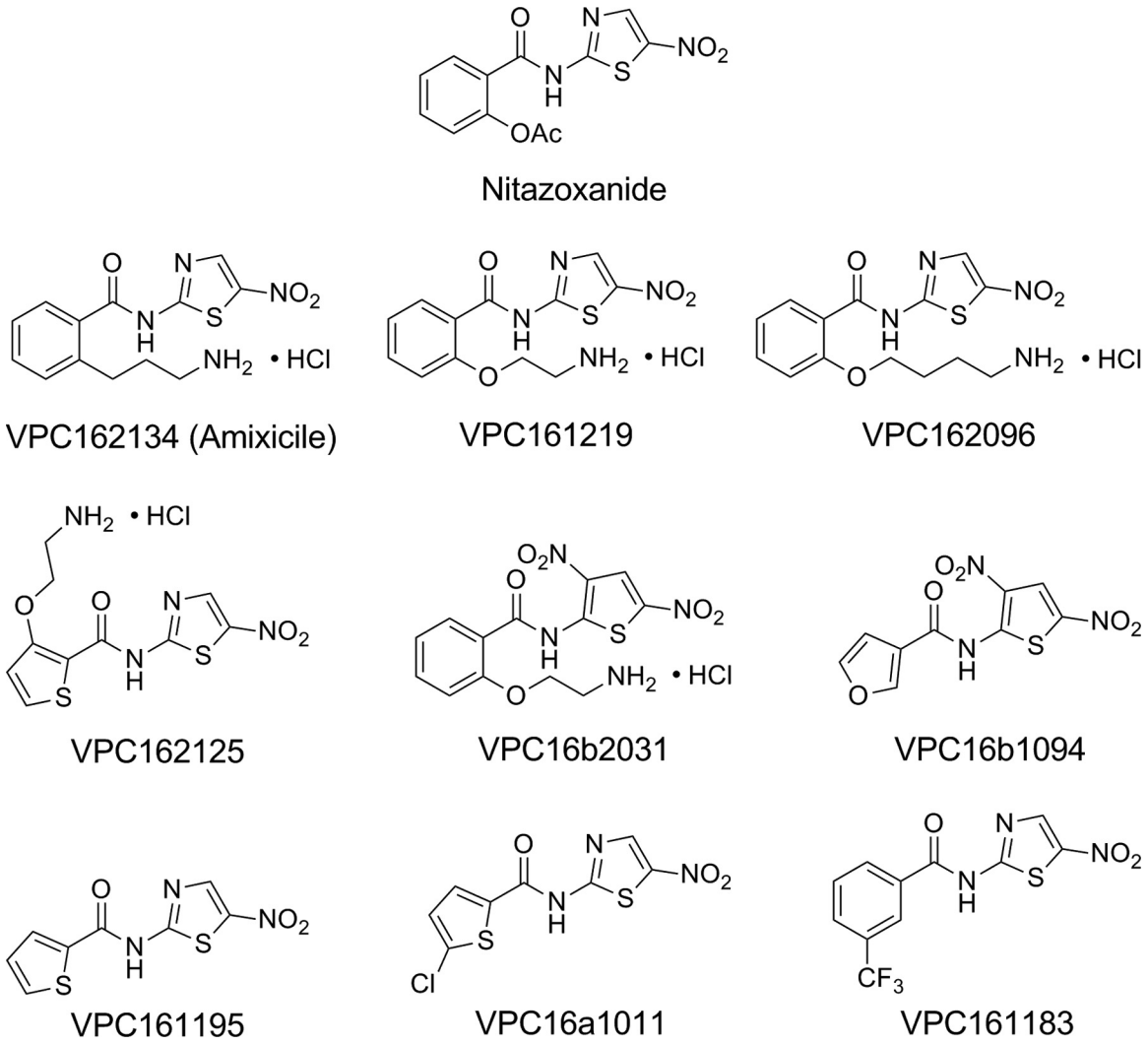


FIG 1 Chemical structures of compounds used in this study and parent compound nitazoxanide. Synthesis of amixicile and other analogues can be found in text S1 in the supplemental material and in references 1 and 2.

model at 15 mg/100 mg body weight (150 mg/kg) and 50% protective at 30 mg/kg (22). They further noted that, unlike vancomycin and metronidazole, pretreatment of hamsters with NTZ did not induce *C. difficile*, which can be explained by the selectivity of NTZ for PFOR and not for beneficial flora like *Lactobacillus*, *Bifidobacterium*, and enteric bacteria that express pyruvate dehydrogenase (PDH). These authors did note that cecal contents affected NTZ action and contributed to a higher MIC, which is consistent with the hydrophobicity and serum binding properties reported for NTZ (15, 33, 41). The effect of serum on NTZ action against *Mycobacterium tuberculosis* has also been noted (10).

In this study, we sought to develop derivatives of NTZ with more favorable pharmacodynamic properties, including improved solubility, spectrum, and potency for the PFOR drug target. We removed the 2-acetoxy group, which is rapidly lost upon hydration and the target of glucuronidation by the liver (15, 33). Replacement of the acetoxy group by an aliphatic amine produced leads that were water soluble, potent (PFOR inhibition), and nontoxic in mice. Here we show that one of these leads (amixicile) protected significantly more mice than did NTZ in a mouse CDI lethal challenge model but slightly fewer mice than did vancomycin or fidaxomicin on day 5, while outperforming both by day 12. Importantly, recurrence of infection was observed with both vancomycin and fidaxomicin but not with amixicile or NTZ. Our studies suggest that amixicile may be a promising new therapeutic for treatment of CDI.

MATERIALS AND METHODS

Chemistry. Analogues of NTZ used in these studies are depicted in Fig. 1 and in Table 1. Four of the compounds (VPC16a1011, VPC161195, VPC16b1094, and VPC161183) have been previously reported, including details on chemical synthesis (1, 2). Synthesis of amixicile and other analogues is presented as text S1 in the supplemental material. All analogues exhibited >98% purity by NMR and mass spectrometry (MS).

Solubility. Drug solubility in distilled water was determined by adding sufficient compound to excess of saturation in 1-ml volumes. Solutions were vortexed for 30 s to 1 min at room temperature. The saturated solutions were then centrifuged in a microcentrifuge to pellet insoluble compound. The supernatant was diluted in phosphate-buffered saline (PBS), pH 7.5, and read in a spectrophotometer at 413 nm. The molar extinction for amixicile was determined by preparing dilutions of a 10-mg/ml stock

TABLE 1 MIC and cytotoxicity testing of compounds

Compound	MIC for organism[a]:					CC_{50} for HFSK[b]	% inhibition of PFOR[c]
	C. difficile	*H. pylori*	*C. jejuni*	*E. coli*	*S. aureus*		
VPC162134	0.25–1.0	1.0	6.0	>32	>32	>32	75 ± 0.5
VPC16b2031	0.25–2.0	1.0	0.5	8	>32	16	84 ± 2
VPC16a1011	0.06–0.25	2.0	2.0	>32	2–4	16	34
VPC161183	0.125–0.25	1.1	1.0	12	1.0	16	36
VPC161219	0.5–1.0	0.5	4.0	>32	>32	16	61
VPC162096	0.5–1.0	1.0	2.0	>32	>32	16	ND
VPC162125	0.5–2.0	0.25	4.0	>32	>32	16	71 ± 8
VPC161195	0.125–0.5	0.75	0.75	>32	4	32	56 ± 6
VPC16b1094	0.5–2.0	0.75	0.125	>32	16	16	66 ± 16
Nitazoxanide	0.125–0.25	4.0	12	>32	16	16	52 ± 4

[a] MICs for *C. difficile* were determined by agar dilution, and those for the other bacteria were determined by microdilution as detailed in the text. The MIC is defined as the drug concentration (μg/ml) which inhibited growth. The MIC range depicted for *C. difficile* is from at least three independent determinations for each analogue.
[b] Human foreskin (HFSK) cells were used for cytotoxicity testing using resazurin as described in the text, and the drug concentration achieving 50% inhibition (CC_{50}) is reported in μg/ml. The 48-h time point is depicted. The > symbol indicates that there was no toxicity over controls at the highest concentration tested. The solubility of many of these compounds precludes testing at higher concentrations.
[c] The inhibitory activity of the analogues against PFOR is reported as percent inhibition at a fixed concentration of 40 μM and relative to 40 μM NTZ, which achieves 50% inhibition. IC_{50} data are presented in the text for amixicile and NTZ. ND, not determined.

solution. The extinction coefficient for amixicile was determined to be 20 mM^{-1} cm^{-1}, which is similar to that for NTZ (18 mM^{-1} cm^{-1}) (16).

Bacterial strains and MIC testing. *Clostridium difficile* VPI strain 10463 was used in all experiments. An additional 17 clinical isolates were used, including 5 BI/NAP1 strains. An agar dilution method as described by the Clinical and Laboratory Standards Institute was used for MIC testing (6, 7). Analogues were serially diluted into 18 ml of Wilkins-Chalgren agar (Difco Laboratories, Detroit, MI) or 25 ml per plate of Anaerobe Broth MIC (Difco) at 50°C and poured into petri plates as previously described (2, 21). Dilution ranges included a screen (0 to 32 μg/ml) and a narrow range (0.02 to 8 μg/ml). Bacteria were grown anaerobically overnight in chopped meat medium and subcultured to chopped meat broth for 5 h at 37°C. The culture was standardized to an optical density at 600 nm (OD_{600}) of 0.1, and 10 μl was delivered to the surface of plates. The number of viable bacteria contained in each inoculum was 3.5×10^4 to 7×10^4 organisms. Plates were incubated in an anaerobic chamber and were read visually for growth or no growth at 12 h. Anaerobic plates containing no analogues were used as controls. All experiments were performed in triplicate and verified in 3 independent experiments. MIC testing for *Helicobacter pylori* strain 26695 and *Campylobacter jejuni* strain H840 was done by broth dilution in 96-well microtiter dishes as described previously (2). MIC testing for staphylococcal strains and for *Escherichia coli* strains was performed as previously described (2, 30, 36). Serial dilutions of analogues began at 32 μg/ml, and the MIC was determined both visually and by a plate reader (Molecular Dynamics) as the drug concentration that completely inhibited bacterial growth. All MIC testing was performed in triplicate and independently repeated at least once.

PFOR inhibition assays. PFOR enzymes show considerable variability at the subunit level, but the catalytic core is highly conserved through evolution (4, 16, 17, 31). The *Helicobacter pylori* PFOR enzyme complex was overexpressed and partially purified from *E. coli* as previously described (16, 31). Enzyme assays were carried out at 25°C in 1-ml cuvettes in a modified Cary-14 spectrophotometer equipped with an OLIS data acquisition and Olis Spectral Works software system (On Line Instrument Co., Bogart, GA). PFOR was assayed under anaerobic conditions in 100 mM potassium phosphate buffer (pH 7.4), 10 mM sodium pyruvate, 5 mM benzyl viologen (BV; $\varepsilon = 9.2$ mM^{-1} cm^{-1} at 546 nm), 0.18 mM coenzyme A (CoA), and 1 mM $MgCl_2$. Inhibitors and nitazoxanide were added at 40 μM: NTZ inhibition at this concentration is 50% of enzyme activity and served as an index for assessing the potency of the analogues. The enzyme reaction was started by addition of PFOR enzyme and recorded at 546 nm. The inhibitory activity was determined in triplicate and reported as a percentage of the uninhibited control. Fifty percent inhibitory concentrations (IC_{50}s) were determined for NTZ and amixicile over a range of drug concentrations from 0 to 48 μM.

Cytotoxicity. Compounds were screened for cytotoxicity against human foreskin (HFSK) cells by microdilution in a 96-well format (2). HFSK cells were grown in medium 106 containing low-serum supplement (Invitrogen), and 96-well plates were seeded with cells at 1.6×10^3 cells per ml and incubated overnight at 37°C in a CO_2 incubator to enable cells to adhere to the bottom of the wells. Test compounds were serially diluted in replicate plates (0 to 32 μg/ml), and following incubation for 24 h, 0.02% resazurin sodium salt was added to each well, incubated for 2 h, and then read on a plate reader at 570 nm. A second set of plates was similarly treated at the 48-h time point. All assays were performed in triplicate and in two independent assays. The cytotoxic concentration (CC_{50}) was reported as the drug concentration that inhibited 50% of the resazurin reduction by the untreated control. In these experiments, the dimethyl sulfoxide (DMSO) concentration did not exceed 0.6%.

Animal efficacy and toxicity studies. The protocols for toxicity and efficacy studies were approved by the Center for Comparative Medicine at the University of Virginia. C57BL/6 male mice were obtained from the Jackson Laboratory.

(i) Toxicity screening. Mice (20 to 22.5 g in weight) were fasted for 5 h prior to treatment with analogues at 0-, 20-, or 200-mg/kg/day doses. All analogues and NTZ were dissolved in dimethyl sulfoxide (DMSO), mixed into 1% methylcellulose, and administered by gavage or by intraperitoneal (i.p.) injection daily (0.1 ml) for 3 days. Clinical scores based on appearance (eyes and hair), weights, activity levels, and diarrhea as well as mortality were recorded, and stool samples were collected daily (see Table S2 in the supplemental material for the scoring system).

(ii) Efficacy testing. The infection model used is a modification of the protocol published by Chen et al. (5) and depicted in Fig. 2. Mice are naturally resistant to infection by *C. difficile* but can be made susceptible by antibiotic treatment. Mice (8 weeks old) were pretreated with an antibiotic cocktail containing vancomycin (4.5 mg/kg), colistin (4.2 units/kg), gentamicin (3.5 mg/kg), and metronidazole (2.15 mg/kg) in drinking water from day 6 to day 4 before infection. One day prior to infection, clindamycin (32 mg/kg) was injected subcutaneously. Food and water were allowed *ad libitum* to the mice. *C. difficile* VPI 10463 was prepared from chopped meat overnight growth that was transferred to brain heart infusion (BHI) medium under anaerobic conditions, grown for 18 h, and standardized to an OD_{600} of 1.3 to 1.6 (~1×10^8 bacteria per ml). Dilutions were prepared to achieve either 1×10^4 or 1×10^5 CFU, which was administered to mice by oral gavage (0.1 or 0.2 ml). One day postinfection, treated mice were given vancomycin, fidaxomicin, NTZ, or one of

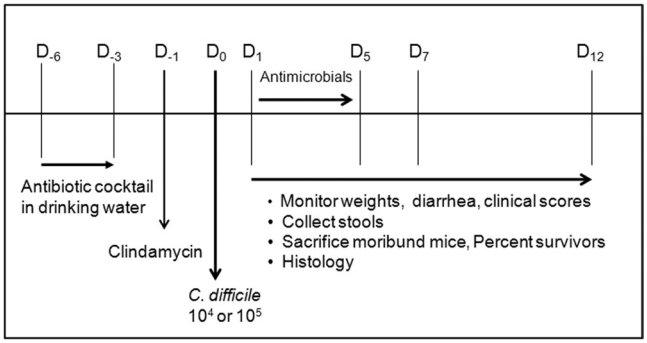


FIG 2 Experimental scheme for acute CDI mouse model. Details can be found in the text.

the analogues at 20 mg/kg in 10% DMSO and 1% methylcellulose by gavage daily for 5 days. Clinical scores were recorded daily (see Table S2 in the supplemental material). Moribund mice at any day of the experiment and all surviving mice at day 14 were sacrificed. Cecal and colonic tissues were harvested for hematoxylin-eosin staining (University of Virginia School of Medicine Research Histology Core) for histopathology. Histopathology was scored blindly based on inflammation, mucosal disruption, mucosal hypertrophy, exudate, and submucosa edema as we have previously reported (27).

Statistical analysis. The therapeutic efficacy (percent survival at day 5 or day 12) was evaluated using Fisher's exact test (two-tailed). A significant difference was defined as a P value of <0.05.

RESULTS

MIC testing. Previous studies determined that the 2-amino-5-nitrothiazole moiety of NTZ (2ANT) was responsible for the biological activity against PFOR (1, 2, 16) and enabled us to expand a library of benzene ring substitutions as well as couplings of heterocyclic moieties to 2ANT. Our drug development strategy began with screening new derivatives for potency against PFOR-containing bacteria, including *C. difficile*, *H. pylori*, and *C. jejuni*, and bacteria that do not express PFOR, such as *E. coli* and *Staphylococcus aureus*. Selected compounds exhibiting potency by MIC tests are depicted in Fig. 1, and their MIC values are presented in Table 1. The solubility of most analogues in water was similar to that of NTZ (<10 μg/ml). Analogues containing ether-linked aliphatic amines (VPC161219, VPC162096, VPC162125, and VPC16b2031) showed improved solubility (~0.4 mg/ml), with amixicile (VPC162134, direct carbon linkage of propylamine) showing the greatest solubility at 10 mg/ml.

While MIC tests for *C. difficile* were conducted by agar dilution and those for the other bacteria were conducted by broth dilution, MIC values were relatively consistent and supportive of a common PFOR target. As has been previously reported, *C. difficile* is more susceptible to NTZ and many of the analogues than are *H. pylori* and *C. jejuni*. In all cases, *E. coli*, which utilizes pyruvate dehydrogenase (PDH), is resistant to the inhibitory action of the majority of these analogues. However, *S. aureus* was susceptible to many of the analogues, some of which reached therapeutic levels (VPC16a1011 and VPC161183). Amixicile (VPC162134) exhibited no antimicrobial action against either *E. coli* or *S. aureus*. In the case of dinitrothiophene analogues, we explored the possibility that one or both of the nitro groups might be susceptible to nitroreduction. However, none of these compounds was nitroreduced by the NfsB nitroreductase from *E. coli* (data not presented). In the case of VPC161183, we have previously reported that this compound exhibits broad-spectrum antibacterial activity that in addition to *E. coli* includes *Staphylococcus aureus* and *Staphylococcus epidermidis* (2), neither of which contains PFOR. Off-target hits were not pursued in this study.

Amixicile and NTZ were tested against 17 clinical isolates of *C. difficile*, including NAP1/BI strains. As seen in Table 2, no clinical isolates were found to be resistant to amixicile or NTZ. Generally, NTZ was more potent than amixicile by MIC and its MICs ranged from ≤0.125 to 1 μg/ml versus ≤0.125 to 4 μg/ml for amixicile. These results indicate that amixicile, like NTZ, is inhibitory to the growth of a wide range of strains, including the NAP1/BI group of strains.

Cytotoxicity for HFSK cells. Previous studies have shown that NTZ is intrinsically cytotoxic for immortalized cell lines and of low toxicity for HFSK cells (2, 19). As seen in Table 1, most of the compounds were well tolerated by HFSK monolayers at 16 to 32 μg/ml (~50 to 100 μM). In this study, amixicile was less toxic for HFSK cells than was NTZ.

Target validation. To correlate MIC data with target specificity, we examined the inhibitory nature of these compounds in a direct PFOR enzyme assay (1, 16, 32). In this assay, inhibition of the reduction of BV is tracked at 546 nm. The assay is calibrated with 40 μM NTZ, which is sufficient to inhibit PFOR enzyme activity by 50% (1, 2). All compounds were tested at 40 μM, and data are presented as percent inhibition in Table 1. The most active PFOR inhibitors were VPC162134 (amixicile), VPC16b2031, VPC162125, and VPC16b1094. The IC_{50}s were determined for both NTZ and amixicile over a range of drug concentrations, and 50% enzyme inhibition was achieved with 8 μM amixicile and 24 μM for NTZ. *In silico* docking simulations (MOE; molecular operating environment 20010.10 release by Chemical Computing Group) with the crystal structure of PFOR from *Desulfovibrio africanus* (4) and analogues indicated that the 2ANT head group of NTZ most likely entered the active site of PFOR (lowest-energy state) with the 5-nitro group anionic and the most likely of five

TABLE 2 MIC testing of *C. difficile* strains[a]

Strain (type)	MIC (μg/ml)		
	Amixicile (AD)	Amixicile (BD)	NTZ (BD)
VPI10463	1	1	≤0.125
VPI26689	0.5	ND	ND
ATCC BAA-1382 (BI)	0.25	ND	ND
8864	0.5	0.5	≤0.125
F1470	0.5	≤0.125	1
67	0.5	2	0.5
39	0.5	2	0.5
5	1	2	0.5
4	0.5	4	1
9	0.5	2	1
19 (type K)	0.5	1	≤0.125
20 (BI-6, 8, 17)	0.25	0.5	≤0.125
22	0.25	1	≤0.125
24	0.25	2	≤0.125
29 (BI-6, 8, 17)	0.25	≤0.125	≤0.125
18 (BI-6, 8, 17)	0.25	1	≤0.125
54 (BI-6, 8, 17)	ND	≤0.125	0.5

[a] For agar dilution (AD), results were read at 12 h. For broth microdilution (BD), the MIC was determined at 14 h. ND, not determined.

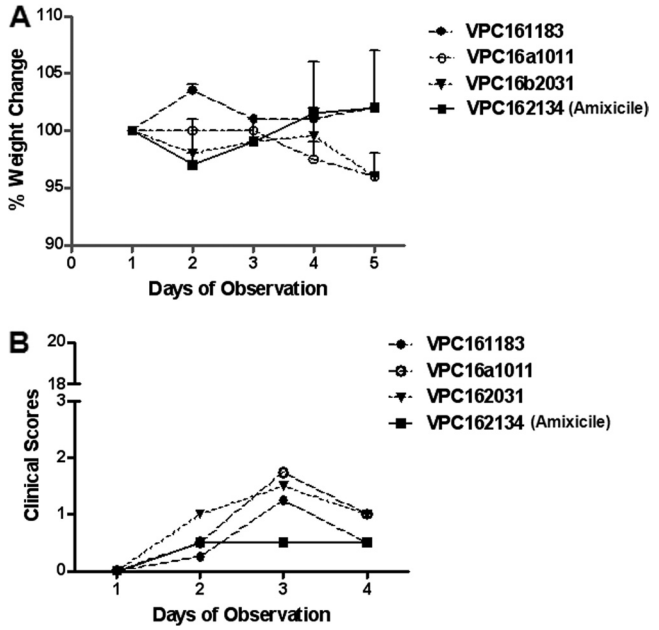
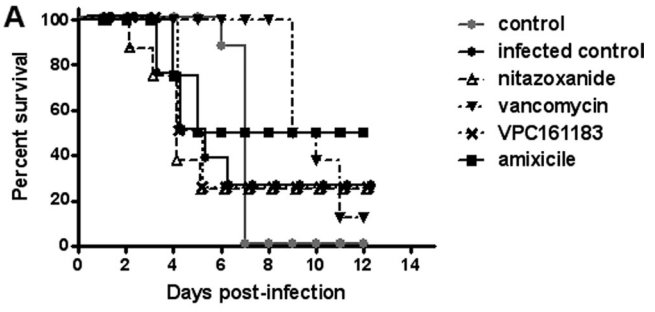


FIG 3 Mouse cytotoxicity and clinical score. (A) Weight loss. Analogues were screened at 200 mg/kg, and body weights were recorded daily. (B) Clinical score. Clinical scores were computed on a scale of 0 (normal) to 20 (death) and evaluated on weight loss, eyes/nose, coat, activity, posture, and diarrhea against a severity scale for each of 0 to 4 with 4 being worst. The clinical score matrix is presented in Table S2 in the supplemental material.



B

Compound	Percent Survival	
	Day 5 (N)	Day 12 (N)
Uninfected Control	100 (8/8)	0 (0/8)
Infected Control	25 (2/8)	25 (2/8)
Vancomycin	100 (8/8)	12.5 (1/8)
Amixicile	50 (4/8)	50 (4/8)
VPC161183	25 (2/8)	25 (2/8)
Nitazoxanide	25 (2/8)	25 (2/8)

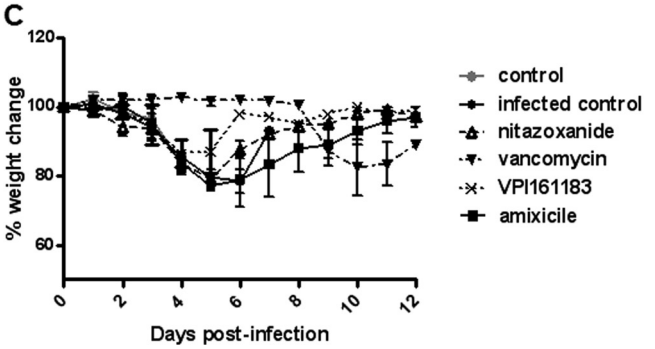


FIG 4 Mouse survival study. (A) Survival curve. Groups of 8 mice were infected on day 0 with 10^4 CFU/100 μl and on day 1 received the indicated compounds at 20 mg/kg body weight/day for 5 days. Animals were followed for 12 days postinfection. The results are depicted as percent survivors. (B) The percent survivors in each group are tabulated at days 5 and 12 postinfection. N, number of survivors/group. (C) Body weights. The mean weights of animals (groups of 8 or survivors) were measured each day for 12 days.

resonance forms to interact with TPP (references 1 and 2 and data not presented). Consistent with MIC tests, these simulations supported the notion that the tail group was not critical for the biological activity. While MIC and IC_{50} do not necessarily have to correlate, we found that the most active analogues retain potency for the biological target. As predicted by *in silico* docking studies with the crystal structure of PFOR (4), the inhibitory action of amixicile was improved by 3-fold over that of the parent drug, NTZ. However, the MIC for this analogue was slightly higher than predicted from the percent PFOR inhibition and might reflect differences in efficiency of drug uptake.

Mouse toxicity screening. The various analogues were tested for toxicity in mice by gavage at concentrations of 20 and 200 mg/kg/day. These doses were administered for 3 days, and survivors were followed out to 10 days. All mice survived the 3-day course with these analogues at 200 mg/kg/day. Some compounds (not depicted) were toxic at this high concentration, and one of the listed compounds (VPC161219) was toxic at 500 mg/kg/day (data not presented). In contrast, NTZ was not toxic by gavage at 500 mg/kg/day. As seen in Fig. 3, most analogues were well tolerated. While not depicted, VPC16b1094 produced a clinical score of 8 at 200 mg/kg/day and was not further tested. At the maximum dose of 200 mg/kg/day, amixicile (VPC162134) caused transient weight loss of less than 5% (Fig. 3A) and had the lowest clinical score (0.5), though there was no statistical difference among any of the compounds tested (Fig. 3B). Activity levels of mice remained normal, and there were no deaths. Amixicile was well tolerated by intraperitoneal injection at 200 mg/kg/day, producing a clinical score of <1. Several other drugs, including VPC16a1011 and VPC161282 (bromine substitution of VPC16a1011), were toxic at 200 mg/kg by the i.p. route (death of all animals by day 3) but not when administered by gavage. The absence of toxicity with amixicile might be attributable to improved solubility and tissue dispersion.

Mouse efficacy testing. In the murine model of CDI (Fig. 2), infected mice develop diarrhea, lose weight, and succumb on days 2 to 6 after oral inoculation with 10^4 to 10^5 CFU of *C. difficile*. Untreated infected mice that survived acute infection typically do not develop recurrence of the disease. With the exception of analogue VPC16b1094, all analogues depicted in Fig. 1, including NTZ, were tested for efficacy in groups of 8 mice per analogue. Figure 4 depicts a representative result from one challenge experiment in which mice were infected with *C. difficile* at 10^4 CFU. In these challenge experiments, uninfected control animals housed in the same enclosure and room with infected animals often, but not always, succumbed to *C. difficile* infection by day 7. Under these conditions, animals treated with nitazoxanide and compound VPC161183 fared poorly, with a survival rate of 25% at day 12. In contrast, mice treated with vancomycin fared well, with no deaths up to day 8, and thereafter succumbed to recurrence of

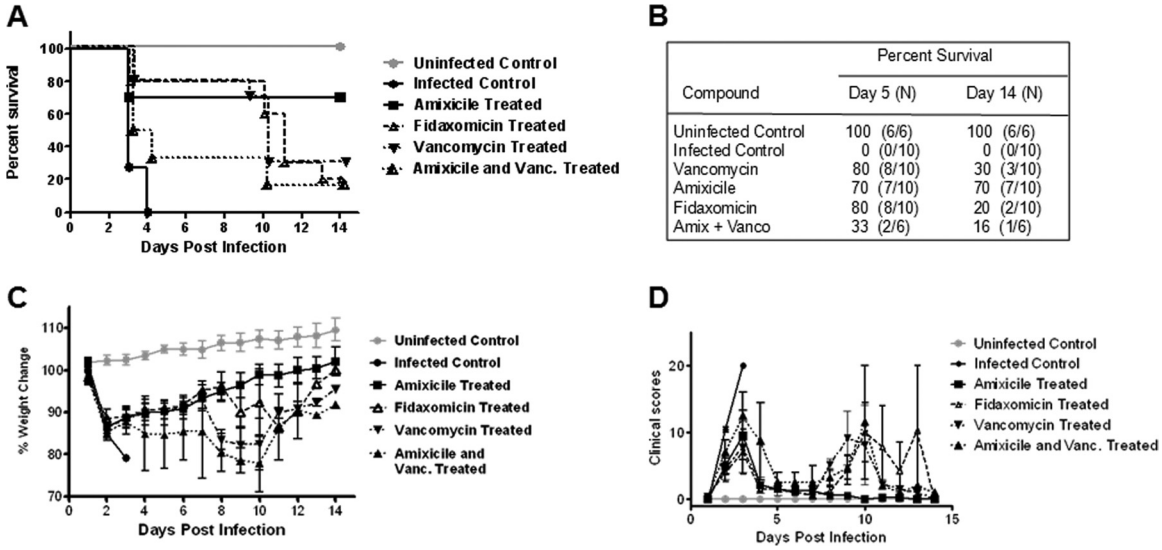


FIG 5 Mouse head-to-head challenge with fidaxomicin and vancomycin. (A) Survival curves. The data are combined from two experiments, one of 4 animals per group and the other of 7 per group. All animals received 1×10^5 CFU in 100 μl. Three animals experienced gavage-related deaths (one in each group) unrelated to CDI, so the denominator for the combined study is 10 (except for the uninfected-control and vancomycin-plus-amixicile groups, each with $n = 6$). All drugs were administered by gavage at 20 mg/kg body weight/day for 5 days. (B) The percent survivors are tabulated at days 5 and 14. N, number of survivors/group. (C) Body weights. The mean weights (groups of 6 to 10) were measured each day for 14 days. (D) Clinical scores are assessed as indicated in the text and detailed in Table S2 in the supplemental material.

infection with a survival rate of 12.5%. In these experiments, amixicile-treated mice fared worse (relative to those treated with vancomycin) early, with 50% death by day 4, but did not succumb to recurrence of infection. While not depicted, all of the other tested analogues, including NTZ controls, showed efficacy of 25% or less at 20 mg/kg. By combining these results with four replicates (including results depicted in Fig. 5), we established that untreated infected mice had a median mortality rate of 87% (75 to 100%) (Table 3) but that those that survived the first few days postinfection completely recovered by the end of observation (day 12). Infected mice treated with vancomycin had a 94% survival rate and less weight loss and less diarrhea at day 5 than did amixicile-treated mice. By day 12 postinfection (Table 3), the median survival rate of vancomycin-treated mice was 15% (0 to 30%), compared with 22% (0 to 25%) for NTZ-treated mice and 56% (50% to 70%) for amixicile-treated mice. Amixicile was superior to NTZ ($P = 0.008$) at both days 5 and 12, while vancomycin was superior to amixicile at day 5 ($P = 0.0001$); however, by day 12 amixicile was superior to vancomycin ($P = 0.0003$) (Table 4). While the vancomycin-treated mice showed little weight loss out to day 8 (Fig. 4C), weight loss was rapid thereafter and correlated with poor outcome. In contrast, all animals treated with analogues and NTZ showed substantial weight loss (~20%) up to day 4 or 5 postinfection, followed by recovery and no recurrence (Fig. 4C). We noted that vancomycin-treated animals showed less abnormal histopathology (lower scores) at day 5 than did animals treated with amixicile, but at day 12, histopathology scores for survivors were indistinguishable (data not presented). It is noteworthy that in all of our efficacy studies, none of the infected mice treated with NTZ, amixicile, or other tested analogues relapsed ($P < 0.001$), even in studies that were carried out to 18 days postinfection. The absence of recurrence might be related to a postantibiotic effect or more likely to an absence of toxicity for CDI-protective resident flora that do not contain the PFOR target (e.g., lactic acid bacteria, *Bifidobacterium*, and enteric bacteria) (1, 22, 26).

Efficacy comparisons with vancomycin and fidaxomicin. To assess the competitive efficacy of amixicile, we used an optimized mouse lethal challenge model in which infected mice die of *C. difficile* infection by day 4 postinfection. Moreover, we altered animal housing protocols to minimize the risk of contaminating

TABLE 3 Composite survival data

Compound	% survival (no. surviving/total no.)	
	Day 5	Day 12
Uninfected control	81 (18/22)	45 (10/22)
Infected control	14 (6/42)	12 (5/42)
Vancomycin	94 (32/34)	15 (5/34)
Amixicile	56 (23/42)	56 (23/42)
Nitazoxanide	22 (7/32)	22 (7/32)
Fidaxomicin	80 (8/10)	20 (2/10)

TABLE 4 Statistical analysis[a]

Comparison	*P* value	
	Day 5	Day 12
UC vs IC	0.0001	0.0033
Van vs IC	0.0001	0.745
Amix vs IC	0.0002	0.0001
NTZ vs IC	0.5393	0.3424
Fidax vs IC	0.0001	0.6079
Amix vs NTZ	0.008	0.008
Amix vs Van	0.0001	0.0003
Amix vs Fidax	0.1739	0.0777

[a] Fisher's exact test (two-tailed). The *P* value for amixicile versus fidaxomicin is derived from the data presented in Fig. 5. Abbreviations: UC, uninfected control; IC, infected control; Van, vancomycin; Amix, amixicile; Fidax, fidaxomicin.

uninfected animals. NTZ was not evaluated in this study due to its poor efficacy. As seen in Fig. 5A, all uninfected control mice survived the entire study period. In contrast, 100% of infected mice succumbed to CDI by day 4. During acute infection, animals treated at 20 mg/kg/day for each drug fared well, with 80% of animals treated with vancomycin or fidaxomicin and 70% treated with amixicile surviving at day 5, which was not statistically significant (Tables 3 and 4) ($P = 0.1739$). As seen in Fig. 5A and tabulated in Fig. 5B, recurrence of infection was observed by day 10 for animals treated with fidaxomicin and vancomycin, which resulted in poor outcomes by day 14 of 20% and 30%, respectively. In contrast, recurrence of infection was not observed with amixicile (70% survival at 14 days postinfection from Fig. 5), a result that approached statistical significance relative to fidaxomicin (Tables 3 and 4) ($P = 0.0777$). Animals treated with each of these drugs showed comparable early weight loss (Fig. 5C) and, in the case of amixicile, a steady weight gain. Similarly, clinical scores were comparable until recurrence of infection in the groups receiving vancomycin and receiving fidaxomicin (Fig. 5D). Interestingly, when amixicile was combined with vancomycin in a group of 6 infected mice, animals fared worse than with either drug individually, with a 33% survival rate at day 5 and recurrence in one additional mouse by day 14 (16% survival rate). In the mouse model, which is considered predictive of human outcomes, amixicile compares favorably with vancomycin and fidaxomicin and shows a benefit in inhibiting recurrence of CDI.

DISCUSSION

We established an acute infection model in mice to evaluate the efficacy of analogues of nitazoxanide (NTZ) as potential therapeutics for CDI. In this model, we found that amixicile (VPC162134), a water-soluble derivative of NTZ, compared favorably in a head-to-head challenge with vancomycin and fidaxomicin. A comparison of weight losses/gains and clinical scores also showed similar data for each of the therapeutics until the point of recurrence. Importantly, no recurrence of infection was observed in animals treated with amixicile, whereas recurrence and death were common for mice treated with vancomycin or fidaxomicin. The absence of recurrence of CDI was also noted for animals receiving NTZ or other analogues, suggesting that this class of drugs might exhibit an extended postantibiotic effect or perhaps is less harmful to reestablishment of normal gut microflora. Amixicile, the propylamine derivative of NTZ, exhibited low cytotoxicity for HFSK cells (>100 μM), was well tolerated in mice by i.p. and oral routes (200 mg/kg), and in direct PFOR enzyme assays showed potency improved over that of NTZ (IC_{50}, 8 μM versus 24 μM for NTZ). A screen of clinical isolates of *C. difficile* revealed no intrinsic drug resistance, consistent with PFOR as an essential drug target that mechanistic studies had determined might resist mutation-based development of drug resistance (16). Moreover, our MIC results with clinical isolates compare favorably to those of similar studies reported by Finegold et al. and Pankuch and Appelbaum (14, 26).

While the adverse effects of vancomycin on gut microflora are well documented in both mice and humans (5, 22, 28, 29, 34, 40), one might have expected NTZ, based on its broad-spectrum action against PFOR-expressing anaerobic gut flora (11, 26), to have produced similar adverse effects. Certainly, in our mouse studies, as depicted in Fig. 4, the opportunity for reinfection was quite high, often leading to the death of all the uninfected control mice and the recurrence of disease in and the death of most vancomycin- and fidaxomicin-treated animals. In reviewing the fate of uninfected mice and untreated infected survivors from all studies, it was apparent that infected survivors treated with NTZ or analogues also showed no recurrence of CDI in mice surviving past day 6. The resistance to recurrence of CDI in these animals is most likely due to recovery of protective gut flora. Thus, the recurrence of CDI in fidaxomicin-treated animals was unexpected, since in clinical trials fidaxomicin fared better than vancomycin (20, 24) and is believed to cause less harm to normal gut flora (35). Therefore, the protective effect of amixicile must be considered impressive and perhaps underscores the importance of reestablishment of non-PFOR-expressing gut microbiota in protection from CDI recurrence in mice (12, 13). While the postantibiotic recovery of gut flora in mice was not specifically investigated in our study, the gut flora is known to rebound relatively quickly (32, 38).

Our studies also found that amixicile was very well tolerated by mice whether administered by gavage or by i.p. injection at 200 mg/kg, with clinical scores of <1. In contrast, animals receiving 200 mg/kg of VPC16a1011 by the i.p. route were all dead by day 3. While NTZ is well tolerated by gavage at 500 mg/kg, it is lethal for mice by i.p. injection at 150 mg/kg (9). The low toxicity of amixicile relative to NTZ and VPC16a1011 may be due to its solubility and ability to disperse in tissue. Both NTZ and VPC16a1011 are sparingly soluble in water (<10 μg/ml). Studies are in progress to assess drug absorption properties for amixicile.

Our strategy for developing NTZ analogues was based on previous studies where we identified the 2-aminonitrothiazole (2ANT) head group as responsible for the biological activity (1, 2). From an expanded library of some 250 compounds, we determined that benzene ring substitutions and carboxylic acid couplings of aliphatic and heterocyclic moieties to 2ANT that preserved the anionic nature of the nitrothiazole ring retained their inhibitory activity against PFOR and PFOR-containing bacteria (1, 2, 16). Moreover, docking simulations with the crystal structure of PFOR from *Desulfovibrio africanus* (4) predicted the anionic nitro group as the most likely of 5 possible resonance states to interact with the 4-aminopyrimidine group of the thiamine pyrophosphate vitamin cofactor of PFOR (1, 2, 16). These models show that 2ANT is positioned proximal to TPP with the benzene tail group pointing outwards and with considerable freedom of motion. Modeling with amixicile suggested slightly improved binding relative to that of NTZ, which correlated with an ~1.5-fold-increased inhibitory action against PFOR (Table 1). The improved binding might result from interactions of the propylamine tail with amino acids within the PFOR pocket. It is noteworthy, though, that increased inhibition of PFOR does not necessarily correlate with the MIC against *C. difficile*, as exemplified by analogues VPC16a1011 and VPC161183, which were poor direct inhibitors of PFOR while potent inhibitors of *C. difficile* growth. For compounds like VPC161183 and VPC16b2031, which exhibit broad-spectrum antibacterial activity (inhibitory to *E. coli* and *S. aureus*), the low MIC values for *C. difficile* might reflect inhibitory action against non-PFOR targets. We suggest that the propylamine substitution on the benzene ring of amixicile (VPC162134) improves selectivity for microorganisms containing the PFOR target and not most of the beneficial flora that express PDH.

A second objective of our discovery strategy was to improve drug characteristics of NTZ. NTZ is poorly soluble in water, be-

comes 99% bound to plasma proteins, is inactivated by glucuronidation by the liver, and is inactivated by interaction with cecal material (22, 33, 41). The addition of propylamine to the ortho group of the benzene ring of NTZ resulted in a water-soluble molecule (>10 mg/ml). We note that the solubility of amixicile decreases somewhat with alkalinity to ~5 mg/ml at pH 7.4 in PBS. Our studies with substitutions of aliphatic amines also determined that linkages via ether oxygen are toxic for mice at doses above 200 mg/kg (VPC161219). We believe that the amixicile scaffold can be further optimized through additions of halide electron withdrawing groups and amine structures that strengthen the anionic state of the nitro group.

With the exception of metronidazole (MTZ), mainline therapeutics (vancomycin, fidaxomicin, and NTZ) have poor gut absorption and their effectiveness in eradicating primary infections with *C. difficile* is attributed to concentration of these drugs in the intestine (24, 41). Metronidazole, a water-soluble prodrug, is absorbed in the small intestine, achieves high levels in blood, and must diffuse through the inflamed intestinal epithelium into the gut. MTZ is recommended for mild primary cases of CDI but is less effective in severe cases (8, 39). The relative solubility of amixicile might be considered a disadvantage if indeed the drug is first absorbed. Nevertheless, the head-to-head studies with fidaxomicin and vancomycin are encouraging regardless of whether the drug is absorbed or not in the mouse CDI model.

In addition to water solubility and lack of measurable side effects in mice, amixicile also appears to be highly specific for PFOR. Unlike NTZ, which we have shown to inhibit assembly of pili by the chaperone/usher pathway in enteroaggregative strains of *E. coli* (EAEC) (30) and to inhibit biofilm formation by *Staphylococcus epidermidis* (36), amixicile does not inhibit piliation by EAEC or the formation of biofilm by *S. epidermidis*. Most of the other analogues depicted in Fig. 1 are either potent pilicides or exhibit potent antibacterial action against *S. aureus* and *S. epidermidis*. Since the nitrothiazolide group of analogues is not inhibitory to bacteria that lack PFOR (16, 26), beneficial gut flora, including the *Lactobacillus* group, *Bifidobacterium*, and members of the *Enterobacteriaceae*, would not be depressed. Unlike vancomycin and metronidazole, the nitrothiazolides (amixicile and NTZ) would not be expected to render individuals more susceptible to recurrence of CDI by damaging normal flora (35). This was noted by McVay and Rolfe, who reported that pretreatment of hamsters with NTZ did not predispose these animals to CDI, as was observed with animals similarly treated with vancomycin or metronidazole (22). While clinical trials indicate that the recurrence rate with fidaxomicin is slightly lower than that for vancomycin, our studies in mice showed near equivalence, suggesting that the microflora of mice may be more susceptible to fidaxomicin than is human gut flora.

A key therapeutic advantage to the nitrothiazolide (NTZ and amixicile) class of antimicrobials is that by targeting the activated form of TPP (vitamin cofactor of PFOR and related enzymes) and not the enzymes directly, the inevitable mutation-based development of drug resistance, common to all of these other anti-CDI therapeutics, including fidaxomicin (13), is minimized, if not avoided entirely (2, 16, 31). Moreover, a review of two large *in vitro* MIC-based screens that included NTZ among other antimicrobials against anaerobic gut flora established, without exception, that NTZ was active against all species that contained PFOR and with no evidence of intrinsic drug resistance (11, 14, 26). Unlike NTZ, the solubility of amixicile might lead to development of more bioavailable PFOR inhibitors for treatment of systemic anaerobic infections and infections caused by *Campylobacter jejuni*, parasites, and *H. pylori*.

In summary, we have evaluated a water-soluble analogue of NTZ for efficacy in a CDI infection model in which untreated mice succumb to infection by day 4. In this model, amixicile showed equivalence to treatments with vancomycin and fidaxomicin early in infection and superiority posttreatment by protecting against recurrence of CDI. To our knowledge, this is the first time that fidaxomicin has been evaluated in an acute CDI mouse model, and while the drug was equivalent to vancomycin in efficacy, fidaxomicin did not prove beneficial to recurrence of CDI. We suggest that amixicile may be a promising lead for treatment of CDI based on its solubility, low toxicity, low impact on resident flora, and specific molecular mechanism of PFOR inhibition of this compound class for which no drug resistance has been observed clinically.

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