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Screening for potent and selective anticlostridial leads among FDA-approved drugs

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Abstract

Clostridium difficile is a leading cause of morbidity and mortality particularly in hospital settings. In addition, treatment is very challenging due to the scarcity of effective therapeutic options. Thus, there remains an unmet need to identify new therapeutic agents capable of treating *C. difficile* infections. In the current study, we screened two FDA-approved drug libraries against *C. difficile*. Out of almost 3,200 drugs screened, 50 drugs were capable of inhibiting the growth of *C. difficile*. Remarkably, some of the potent inhibitors have never been reported before and showed activity in a clinically achievable range. Structure-activity relationship (SAR) analysis of the active hits clustered the potent inhibitors into four chemical groups; nitroimidazoles (MIC_{50}= 0.06 − 2.7 μM), salicylanilides (MIC_{50}= 0.2 − 0.6 μM), imidazole antifungals (MIC_{50}= 4.8 − 11.6 μM) and miscellaneous group (MIC_{50}= 0.4 − 22.2 μM). The most potent drugs from the initial screening were further evaluated against additional clinically-relevant strains of *C. difficile*. Moreover, we tested the activity of potent inhibitors against representative strains of human normal gut microbiota to investigate the selectivity of the inhibitors towards *C. difficile*. Overall, this study provides a platform that could be used for further development of potent and selective anticlostridial antibiotics.

Keywords

Clostridium difficile; Imidazole anticlostridials; salicylanilides anticlostridials; Gut microbiota; Drug library screening

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1. Introduction

Clostridium difficile infection (CDI) has recently drawn a significant worldwide attention. In 2011, CDI afflicted nearly half a million people and was a direct cause of death of over 29,000 patients in the United States alone (1). In Europe, the European Centre for Disease Prevention and Control estimated there were nearly 124,000 cases of healthcare-associated CDIs in acute care hospitals alone between 2011 − 2012 (2). Further, CDIs are not restricted to the healthcare setting only; community-acquired infections represent about 41% of all CDIs while 9% transpired in the residents of long-term care services such as retirement homes (3).

The United States Centers for Disease Control and Prevention (CDC) has classified *C. difficile* and CDIs as an urgent public health threat that necessitates immediate and rigorous action (4). However, despite numerous calls for effective preventive measures and potent treatments, only three drugs are used for the treatment of CDI, vancomycin, metronidazole and fidaxomicin. A challenge with treating CDI with either vancomycin or metronidazole is that both agents harm the gut microflora. A second limitation with metronidazole is this drug is completely absorbed from the intestinal tract leaving a very minute concentration at the site of infection. These drawbacks contribute to a high percentage of treatment failure and relapse (5). Fidaxomicin, FDA-approved for the treatment of CDI in 2011, has a better profile than both vancomycin and metronidazole as pertaining to bacterial specificity and oral bioavailability (6). Nonetheless, the clinical outcome is still unsatisfactory regarding treatment failure and relapse especially against the more virulent NAP1 strains of *C. difficile*. Furthermore, treatment with fidaxomicin costs 150 times more than metronidazole thus compounding the cost to treat CDI. This highlights the need to identify and develop new, safe, and effective anticlostridial drugs.

In spite of the massive advances in drug discovery technologies, developing a *de novo* drug takes up to 15 years and can cost up to 2 billion US dollars. Drug repositioning, or finding a novel indication for a known drug, is a way to lessen the time and cost of drug discovery since these drugs have well-characterized toxicity and pharmacokinetic profiles. Many successful drugs being used now are repurposed from their original indication (7–14). We used this approach in the current study to conduct a screening of about 3,200 drugs and clinical molecules to identify drugs or lead molecules with potent anticlostridial activity and limited effect against important bacterial species that comprise the gut microflora.

2. Materials and Methods

2.1. Bacterial strains and reagents

C. difficile and human gut microbiota strains used in this study (Supplementary Table 1) were acquired from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and the American Type Culture Collection (ATCC, Manassas, VA). Strains were cultured in brain heart infusion supplemented broth (BHIS, Brain heart infusion medium from Becton, Dickinson and Company, Cockeysville, MD),

supplemented with yeast extract, L-cysteine, vitamin K1 and hemin (Sigma-Aldrich, St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Corning (Corning, NY).

2.2. Compounds and libraries

The Pharmakon 1600 repositioning compound library was purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT) and the Johns Hopkins library was provided by Johns Hopkins University (Baltimore, MD). Both libraries were supplied in 96-well plates of 10 mM stocks of the compounds in either dimethyl sulfoxide or water and stored in −80° C. Ornidazole, miconazole nitrate, econazole nitrate, tioconazole, butoconazole, clotrimazole, metoprolol tartrate, metoclopramide hydrochloride, chloroquine diphosphate, miconazole, dimetridazole, nithiamide and methylthiouracil (Alfa Aesar, Ward Hill, MA), dichlorophen, triclabendazole, closantel, nitazoxanide (Ark Pharm Inc, Libertyville, IL), ronidazole, oxyclozanide (Sigma-Aldrich, St. Louis, MO), tinidazole (TCI, Portland, OR) and niclosamide (Cayman Chemical, Ann Arbor, MI) were all purchased separately to confirm the results of both libraries. Vancomycin hydrochloride (Gold Biotechnology, Olivette, MO) and metronidazole (BTC, Hudson, NH) were used as positive controls.

2.3. Screening assay

Libraries were screened at a fixed concentration of 16 μM against one strain of *C. difficile* (*C. difficile* NAP07) to identify active compounds (Table 1). Briefly, bacteria were streaked on BHIS agar plates and incubated anaerobically at 37 °C for 48 hours. Colonies were scraped off from the agar plates, suspended in PBS and diluted in BHIS broth at a concentration of about 5×10^5 CFU/mL. Compounds, at a concentration of 16 μM, were mixed with the bacterial suspension in 96-well plates and incubated anaerobically for 48 hours at 37 °C. Drugs that inhibited bacterial growth visually were considered as “hits”. Active drugs hits were purchased from commercial vendors and their activity was confirmed against *C. difficile* NAP07. Commercial drugs that did not show activity were excluded from the study.

2.4. Microdilution assay against *C. difficile* strains

The most potent hits were divided into four groups based on their chemical structure (Table 2) and tested for their minimum inhibitory concentrations (MICs) against a panel of ten *C. difficile* strains according to the Clinical and Laboratory Standards Institute guidelines (CLSI, M11-A8) (15). Drugs, at the required concentrations, were anaerobically incubated with bacterial suspensions (5×10^5 CFU/mL) at 37 °C for 48 hours in 96-well plates. After incubation, plates were examined for bacterial turbidity. MIC was defined as the lowest concentration where bacterial growth was halted and turbidity was unnoticeable.

2.5. Activity against human microbiota

Active compounds were evaluated for antibacterial activity against representative strains of human normal gut flora previously described (16). Different types of bacteria were used in this experiment; for anaerobic bacteria (*Bifidobacterium* and *Bacteroides*) and *Lactobacillus*, bacteria were first streaked on agar plates and incubated for 48 hours at 37 °C (anaerobically using BHIS agar for anaerobes and in 5% CO_2 using MRS agar plate for *Lactobacillus*).

Bacterial colonies were suspended in BHIS broth (for anaerobes) or in MRS broth (for *Lactobacillus*) to achieve a bacterial concentration of approximately 10^5 CFU/ml. Bacteria were then added to 96-well plates containing serial dilutions of the compounds and incubated as mentioned above for 48 hours. Regarding *Escherichia coli* and *Enterococcus faecalis*, bacteria were scraped off tryptic soy agar plates and suspended in tryptic soy broth to achieve a bacterial concentration of 5×10^5 CFU/mL. The bacterial suspensions were aerobically incubated with serial dilutions of the drugs at 37 °C for 16 − 20 hours. Reported MICs are the minimum concentration of the compounds that could inhibit visual growth of bacteria.

3. RESULTS AND DISCUSSION

3.1. Screening assay and Structure-activity relationship (SAR) analysis:

Two drug libraries consisting of approximately 3,200 FDA-approved drugs and clinical molecules were evaluated against one strain of *C. difficile* (NAP07, CDC#2007054, a reference strain in the human microbiota project). All molecules were initially tested at a single concentration, 16 μM, in order to pinpoint active compounds or “hits”. The initial screening revealed 116 compounds from Johns Hopkins library and 111 compounds from Pharmakon library that inhibited *C. difficile* at 16 μM (Supplementary Figure 1, Supplementary Tables 2 and 3). After excluding antiseptic and antibacterial agents and combining drugs from both libraries, 50 compounds were identified (Table 1). To confirm the screening results, the minimum inhibitory concentrations (MICs) of these 50 hits were determined against *C. difficile* NAP07. As depicted in Table 1, the MIC values for the active hits ranged from 0.06 μM to 16 μM.

At first glance, the active compounds seem to be highly scattered structurally. However, the vast majority of the active molecules are imidazole-containing structures. Among the imidazole-containing structures, the nitroimidazoles seem to be the most efficient as six nitroimidazoles (dimetridazole, secnidazole, ronidazole, ornidazole, tinidazole and benznidazole) possessed MIC values below 2 μM against *C. difficile* NAP07 (Table 1). Additionally, the anticlostridial activity was impacted by the position of the nitro group on the imidazole ring. In this vein, the 2-nitroimidazole derivative benznidazole was remarkably less active than all 5-nitromidazole analogs. This is also in accordance with the potent activity of the 5-nitrimidazole metronidazole which was used for a long time as a first-line therapy for CDIs (17). Moreover, the alkyl substitution seems to have less effect on the anticlostridial activity as dimetridazole (with only two methyl groups at positions 1 and 2) was nearly equipotent to the 5-nitroimidazole derivatives carrying more complex and bulkier substituents at positions 1 and 2, such as ornidazole and secnidazole (Figure 1). Apart from the nitroimidazole ring system, 5-nitrothiazole, a 5-nitroimidazole close bioisostere heterocyclic system, revealed very promising anticlostridial activity. In particular, nithiamide inhibited the growth of *C. difficile* NAP07 at a sub-micromolar concentration (the MIC value was 0.25 μM, Table 1). Increasing the bulkiness at thiazole position-2 (like in nitazoxanide) decreased the anticlostridial activity by a factor of 8 in where the MIC value of the antiprotozoal nitazoxanide was 2 μM (Figure 1).

The last group of compounds with a similar scaffold was the imidazole antifungals as seven of them were active against *C. difficile* NAP07 with MIC values ranging between 1 and 8 μM (Table 1). This set of compounds shares more than the imidazole ring since most of them are 1-(2,4-dichlorophenylethyl)imidazole derivatives. From a structure-activity relationship position, the second chlorination at the side chain, benzyloxy moiety, drastically improves anticlostridial activity as observed with the dichlorinated miconazole (MIC value is 1 μM) and its monochlorinated analog econazole (MIC value is 8 μM). On the other hand, the type of linker seems to have less effect on anticlostridial activity. In this regard, econazole with an ether linker possessed the same MIC value against *C. difficile* NAP07 as its thioether analog sulconazole (MIC = 8 μM). This value is identical to the MIC for the first-generation imidazole-antifungal clotrimazole (Table 1). Additionally, the oxime linker seems to reduce the anticlostridial activity as observed with oxiconazole (MIC = 4 μM) whereas the ether analog miconazole had a MIC of 1 μM (Figure 2).

3.2. Activity against clinical *C. difficile* strains

The most potent drugs from the initial screening were further evaluated against additional clinically-relevant strains of *C. difficile*. These compounds were grouped based on their chemical structure and tested against additional ten *C. difficile* strains. For each compound, we calculated the minimum concentration that inhibited the growth of 50% of the tested strains, MIC_{50}, in order to use it to compare the activities of different drugs. Grouping of the hits yielded four distinct structural classes of molecules, namely nitroimidazole, imidazole antifungals, salicylanilide and a fourth group of potent inhibitors that did not belong to a specific chemical class (Table 2).

The first group we investigated was nitroimidazole-containing compounds. Members of this group included ronidazole, dimetridazole, ornidazole, secnidazole and tinidazole in addition to metronidazole, the positive control. Nitroimidazoles inhibited the growth of all tested *C. difficile* strains at low concentrations (MIC_{50}s ranged from 0.3 to 2.7 μM, Table 3).Nitroimidazole-containing compounds, and nitroheterocyclic drugs in general, are known to exert potent inhibitory activity against anaerobic bacteria (18). Although nitroimidazoles can diffuse into both aerobic and anaerobic bacterial cells, reductive activation occurs only in obligate anaerobes by pyruvate:ferredoxin oxidoreductase system. As a result, nitro group reduction produces imidazole radical and nitrite, both of which damage bacterial DNA leading to cell death. In addition, reduction reserves the concentration gradient around the bacterial cell envelop and allows more diffusion of the drug into the bacterial cells (19). Metronidazole was previously used as a first-line treatment for mild to moderated CDIs and is still recommended when vancomycin and fidaxomicin are not attainable (17, 20). However, the activity of metronidazole is limited by the high bioavailability of the drug, leaving a minute concentration of drug in the gut lumen where the infection is localized (21). As a result, the treatment outcome is not satisfactory. In addition, several cases of metronidazole-resistant CDIs have been reported (22). Still, nitroimidazole represents an attractive scaffold that could be modified in order to obtain a better anticlostridial drug (with decreased oral bioavailability). The activities of 5-nitroimidazole-containing compounds were thoroughly studied against parasites in comparison to metronidazole. In most cases, several nitroimidazoles (whether FDA-approved or not) were found to be more effective

than metronidazole and some of them possessed activity against metronidazole-resistant strains (23). On the contrary, fewer comparative studies have been conducted to evaluate the activity of 5-nitroimidazoles against anaerobic bacteria (only a handful of these studies involved *C. difficile*) (24). Tinidazole was previously shown to possess excellent *in vitro* activity against *C. difficile* and was found to be more effective than metronidazole against metronidazole-resistant strains (25). However a limitation with this study is that tinidazole was not assessed in an *in vivo* model of CDI (26). Ornidazole is an alternative therapy to metronidazole in the treatment of giardiasis and bacterial vaginosis. (27). Though ornidazole is not recommended for treatment of CDIs, it is reported to be used as a treatment for CDI in certain parts of the world (28). The most potent anticlostridial nitroimidazole was ronidazole, a veterinary antiprotozoal drug (29). Ronidazole inhibited the growth of all the tested *C. difficile* strains at a concentration of 0.6 μM or less. Indeed, ten out of the eleven tested strains were inhibited at 0.3 μM. Although ronidazole is anecdotally reported to have carcinogenic and embryotoxic effects, it was shown to be safe in albino rats and pigs at very high concentrations and for prolonged periods of time (30). Overall, nitroimidazoles warrant further investigation as more potent anticlostridial alternatives to metronidazole.

The second group to possess a potent anticlostridial activity was salicylanilide-related drugs/clinical molecules. Three drugs were included in this group, namely niclosamide, oxyclozanide and closantel. Salicylanilides inhibited the growth of *C. difficile* at concentrations that ranged from 0.2 to 1.2 μM, while their MIC_{50}s were 0.2 μM for closantel, 0.4 μM for niclosamide and 0.6 μM for oxyclozanide (Table 3). Salicylanilides were recently reported to exert potent activities against several Gram-positive bacterial pathogens including *C. difficile* and vancomycin-resistant *Enterococci* (VRE) (7, 31). Niclosamide and oxyclozanide exhibited activity against methicillin resistant *Staphylococcus aureus* through compromising the integrity of the bacterial cell envelope without causing cell lysis (32). Against *Helicobacter pylori*, niclosamide disrupted the bacterial proton motive force resulting in growth inhibition (33). Similar activity was observed against *C. difficile*, whereby salicylanilides were found to inhibit the growth of both logarithmic and stationary phase bacteria via dissipation of their membrane potential (31). However, the *in vivo* activity of this group of compounds in an animal model of CDI is yet to be tested. On the other hand, salicylanilides exhibited potent *in vitro* activity against VRE, while niclosamide was very effective in reducing the bacterial burden in a VRE colonization reduction mouse model (7). Knowing that VRE overgrowth is a major side effect of vancomycin and metronidazole when used to treat CDI (5, 34), the activity of salicylanilides against VRE can be exploited in the treatment of CDI.

The third group of *C. difficile* inhibitors included imidazole antifungal compounds, miconazole, econazole, tioconazole, butoconazole and clotrimazole. The MICs for these drugs varied from 2.4 to 23.2 μM and the MIC_{50} was 4.8 μM for miconazole, 4.9 μM for butoconazole, 10.3 μM for tioconazole, 10.5 μM for econazole and 11.6 μM for clotrimazole (Table 3). Azoles in general, and imidazoles in particular, represent an attractive scaffold for drug discovery. They can easily interact with enzymes through a wide array of noncovalent interactions. Imidazole compounds exert their antifungal activity through inhibition of ergosterol biosynthesis. Ergosterol depletion is primarily due to inhibition of cytochrome P-450-dependant 14α- demethylase activity and results in mitigating membrane integrity

and fungal inhibition (35). On the contrary, two mechanisms have been proposed for imidazoles activity as antibacterial agents. The first one is through the inhibition of enoyl acyl carrier protein reductase (FabI) with a resultant inhibition of bacterial fatty acid synthesis (36). Although this mechanism of action applies to several bacterial pathogens, e.g. *S. aureus* and *E. coli*, it cannot be expected in *C.difficile* due to the absence of FabI as a catalyst in fatty acid biosynthesis (37). The second proposed mechanism of bacterial inhibition by imidazoles is blocking of flavohaemoglobins-mediated metabolism of nitric oxide leading to bacterial cell death (38). Nevertheless, more investigation is required to confirm this activity in *C. difficile*. Although the antibacterial activity of imidazoles has been reported against other bacteria, this is the first report, to our knowledge, of the anticlostridial activity of imidazole antifungals.

The last group of *C. difficile* inhibitors contained compounds from scattered chemical classes. The most potent two compounds among this group were nitazoxanide and nithiamide. Both drugs contain a nitrothiazole ring structure. The MICs for nitazoxanide ranged from 0.2 to 1.6 μM and the MIC_{50} was 0.4 μM. On the other hand, nithiamide's MICs were between 0.3 and 0.7 μM against all the tested *C. difficile* strains and its MIC_{50} was 0.7 μM (Table 3). Nitazoxanide is an antiprotozoal drug that possesses, along with its active metabolite tizoxanide, a potent antibacterial activity against both aerobes and anaerobes. Although it is not included in CDI treatment guidelines, nitazoxanide has been reported to possess potent anticlostridial activity both *in vitro* and *in vivo* (39). In addition, nitazoxanide proved to be noninferior to both vancomycin and metronidazole in clinical studies against CDI (40). Despite the similarity between the spectrum of activity of metronidazole and nitazoxanide, studies have shown that nitazoxanide is mechanistically distinct from metronidazole. The anticlostridial activity of nitazoxanide is attributed to its noncompetitive inhibition of pyruvate:ferredoxin/flavodoxin oxidoreductases which in turn blocks the oxidative decarboxylation of pyruvate to acetyl coenzyme A and lead subsequently to bacterial killing (41). Nithiamide, on the other hand, has been used as an antiparasitic agent in both human and animal medicine (42). However nithiamide has never been tested against *C. difficile* and the data about its antibacterial activity is very erratic (43). In addition, nithiamide is structurally similar to nitazoxanide, hence it is expected that they might share the same mechanism of action, although this hypothesis will need to be confirmed experimentally. The last two drugs in the miscellaneous group (dichlorophene and triclabendazole) had less potent activity than nithiamide and nitazoxanide *in vitro*. Dichlorophene and triclabendazole are both antiparasitic drugs with no significant known antibacterial activity (44, 45). Dichlorophene inhibited the growth of *C. difficile* strains at concentrations ranging between 3.7 and 14.9 μM while triclabendazole inhibited the same strains at concentrations between 11.1 and 22.2 μM. The MIC_{50} values for dichlorophene and triclabendazole were 7.4 and 22.2 μM, respectively (Table 3).

3.3. Activity of the potent hits against hum an microbiota

Intestinal microbiota protects against *C. difficile* colonization through the production of short chain fatty acids (SCFAs). SCFAs stimulate the growth of gut epithelium, reduce inflammation through the induction of regulatory T cells (Tregs), induce antimicrobial peptides like thuricin CD and augment the mucus barrier through increasing production of

mucin (46). Furthermore, resident bacteria compete with invading *C. difficile* cells for intestinal niches and nutrients. In addition, the microbiota is involved in the transformation of primary bile acids, a germinant for *C. difficile* spores, into secondary bile acids resulting in a reduction of spore germination and inhibition of vegetative growth (46). Based on that, we sought to test the selectivity of the active anticlostridial hits by assessing their activity against representative strains of commensal bacteria present in the human gastrointestinal tract. Evaluated strains included anaerobic bacteria (*Bifidobacteria* and *Bacteroides*), microaerophilic bacteria (*Lactobacilli*), Gram-positive (*Enterococci*) and Gram-negative (*Escherichia coli*) bacteria.

In accordance with their reported activity against anaerobes, nitroimidazoles inhibited the growth of *Bifidobacteria* and *Bacteroides* (24, 47). Only one strain of *Bifidobacterium, B. breve* HM-856, was not inhibited by nitroimidazoles including metronidazole, the positive control (Table 4). Nitroimidazoles had minimal or no activity against the rest of the tested bacterial strains. Imidazole antifungals had a similar pattern of activity against *Bifidobacteria* and *Bacteroides*, in addition, they inhibited the growth of both enterococcal strains tested. The activity of the imidazole antifungal miconazole was reported previously against several Gram-positive bacteria e.g. *Staphylococcus, Streptococcus* and *Enterococcus* (48). Similarly, salicylanilides inhibited both anaerobes and had some activity against *Enterococcus*. Additionally, they inhibited growth of several *Lactobacillus* strains (Table 4). Closantel was the most potent inhibitor amongst salicylanilides potentially due to the difference in its physicochemical properties relative to other salicylanilides (7). All four drugs in the miscellaneous group inhibited the growth of anaerobes and some Gram-positive strains. The most selective drug in this group was nitazoxanide; it inhibited the growth of the microbiota at concentrations that were several folds higher than the drug's MIC_{50} against *C. difficile*. Nithiamide also exhibited a good selectivity profile against most of the tested strains of microbiota. The MIC of nithiamide against most of the inhibited microbiota strains was much higher than its MIC_{50} against *C. difficile* (Table 4).

To summarize, we screened two libraries consisting of FDA-approved drugs and clinical molecules against *C. difficile* in order to identify potent and selective inhibitors. We identified three distinct chemical classes of molecules that have potent inhibitory activity against *C. difficile*, nitroimidazoles, salicylanilides and imidazole antifungals. Additionally, we identified four drugs that do not belong to any of the previous chemical categories, nitazoxanide, nithiamide, dichlorophene and triclabendazole. All the active compounds were tested against a panel of *C. difficile* strains and were found to exhibit potent inhibitory activity. In addition, they were tested against normal intestinal microflora strains to investigate their selectivity for *C. difficile* over other beneficial bacteria. Overall, the current study can serve as a reference for anti-*C. difficile* drug developers and can provide leads for further development for the treatment of CDIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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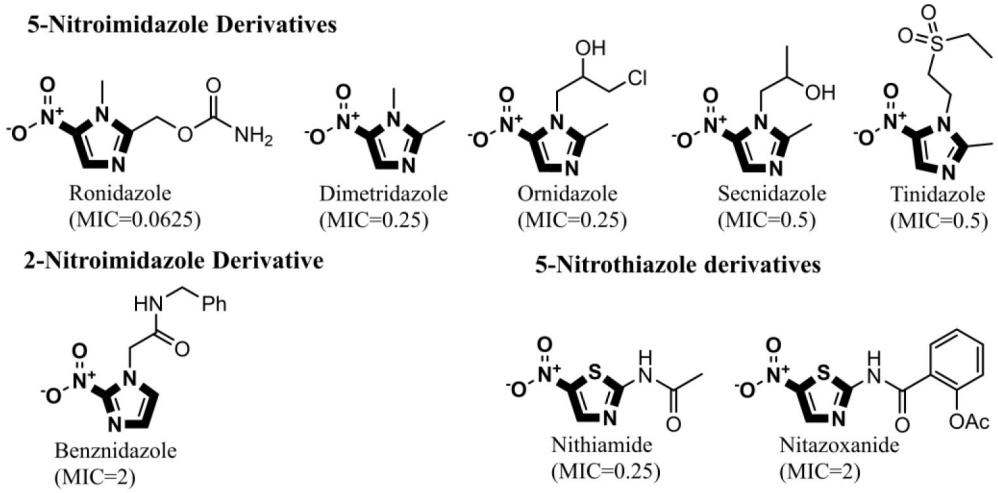


Figure 1: Effect of the nitro group position and alkyl substitution on the anticlostridial activity of nitroim idazoles and nitrothiazoles.

5-nitroimidazoles were found to be more potent than 2-nitroimdazle. Contrarily, variation in the alkyl substitution of 5-nitroimdazole did not significantly affect anticlostridial activity. Additionally, Increasing the size of the substitution at position 2 of the nitrothiazole ring increased the MIC from 0.25 μM in the case of nithiamide to 2 μM in case of nitazoxanide. MIC values are against *C. difficile* NAP07 and are expressed in μM.

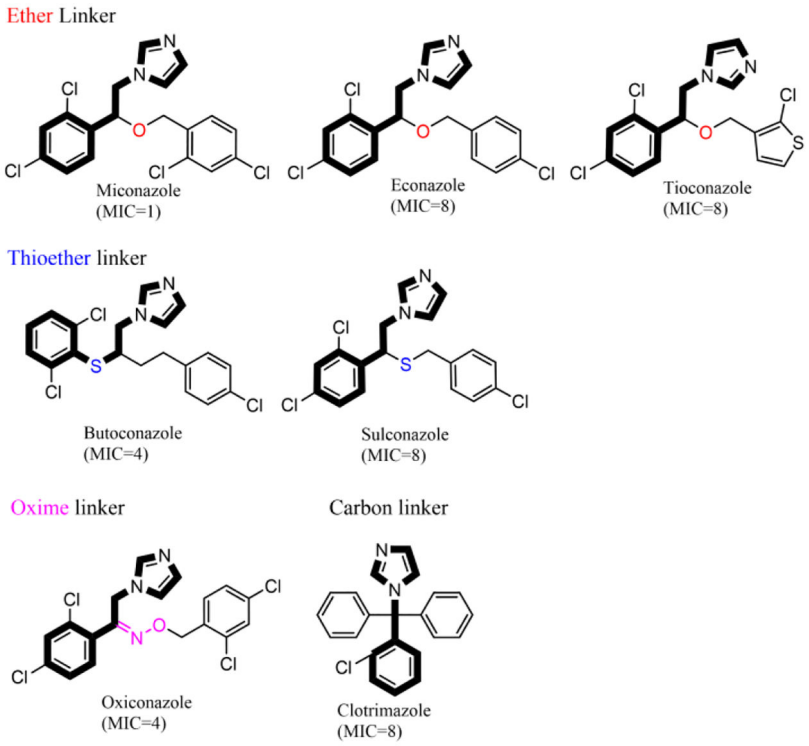
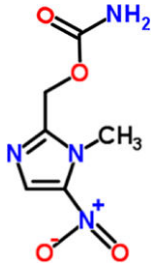
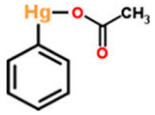
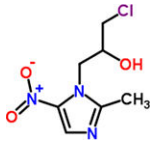
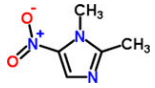
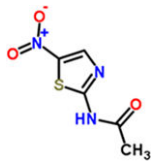
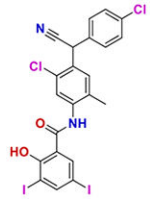


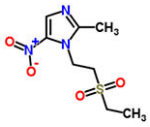
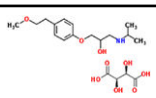
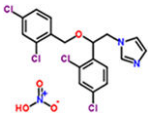
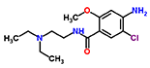
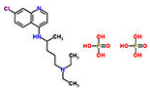
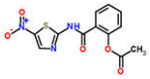
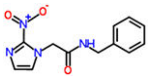
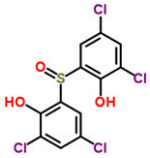
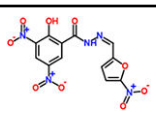
Figure 2: Effect of chlorination and linker type on the anticlostridial activity of imidazole antifungals.

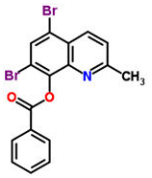
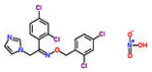
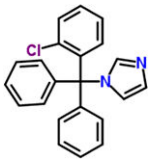
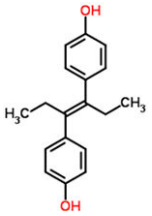
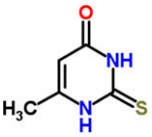
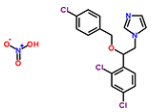
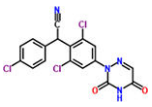
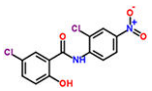
Dichlorinated miconazole is more potent than monochlorinated econazole. Additionally, the type of linker between the imidazole and phenyl rings does not affect anticlostridial activity. MIC values are against *C. difficile* NAP07 and are expressed in μM.

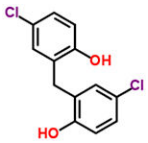
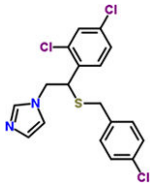
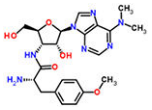
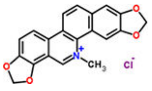
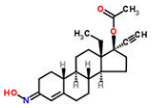
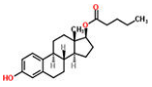
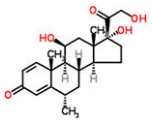
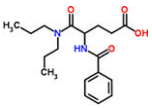
Table 1:

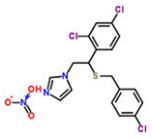
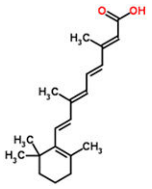
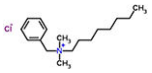
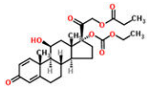
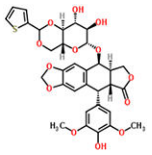
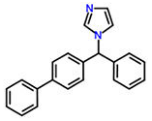
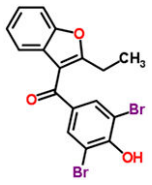
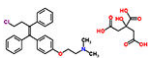
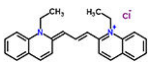
Active drugs “hits” identified from initial screening against *C. difficile* NAP07

	Compound name	Chemical structure	MIC (μM)	Main use
1	Ronidazole		0.06	Anthelmintic
2	Phenylmercuric acetate		0.25	Antifungal, antimicrobial
3	Ornidazole		0.25	Anthelmintic
4	Dimetridazole		0.25	Anthelmintic
5	Nithiamide		0.25	Antiprotozoal (trichomonas).
6	Closantel		0.25	Anthelmintic.
7	Bithionate sodium		0.50	Anthelmintic, antiseptic

	Compound name	Chemical structure	MIC (μM)	Main use
8	Secnidazole		0.50	Anthelmintic, antitrichomonas
9	Oxyclozanide		0.50	Anthelmintic
10	Tinidazole		0.50	Antiprotozoal
11	Metoprolol tartrate		1	Antihypertensive, antianginal
12	Miconazole nitrate		1	Antifungal (topical)
13	Metoclopramide hydrochloride		2	Antiemetic
14	Chloroquine diphosphate		2	Anthelmintic, antirheumatic, intercalating agent
15	Nitazoxanide		2	Anthelmintic
16	Benznidazole (n-benzyl-2-nitro-1h-imidazole-1-acetamide)		2	Antiprotozoal (trypanosoma)
17	Bithionol oxide (2,2′-sulfinyl-bis(4,6-dichlorophenol))		2	Anthelmintic
18	Nifursol		4	Anthelmintic

	Compound name	Chemical structure	MIC (μM)	Main use
19	Butoconazole		4	Antifungal
20	Broxaldine		4	Anthelmintic, antifungal
21	Oxiconazole nitrate		4	Antifungal
22	Clotrimazole		8	Antifungal
23	Diethylstilbestrol		8	Estrogen
24	Methylthiouracil		8	Antithyroid agent
25	Econazole nitrate		8	Antifungal
26	Diclazuril		8	Coccidiostat
27	Niclosamide		8	Anthelmintic, teniacide

	Compound name	Chemical structure	MIC (μM)	Main use
28	Triclabendazole		8	Anthelmintic
29	Dichlorophene		8	Anthelmintic (cestodes)
30	Sulconazole		8	Antifungal
31	Tioconazole		8	Antifungal (topical)
32	Puromycin		8	Antineoplastic
33	Sanguinarium chloride		16	Antineoplastic, antiplaque agent
34	Norgestimate		16	Progestin
35	Estradiol valerate		16	Estrogen
36	Methylprednisolone		16	Anti-inflammatory, glucocorticoid
37	Proglumide		16	Anticholinergic and cholecystokinin antagonist

	Compound name	Chemical structure	MIC (μM)	Main use
38	Sulconazole nitrate		16	Antifungal
39	Tretinoin		16	Keratolytic, antiacne, antineoplastic
40	Chloroxine		16	Chelating agent, antiseborrheic
41	Benzalkonium chloride		16	Preservative
42	Prednicarbate		16	Anti-inflammatory, glucocorticoid
43	Teniposide		16	Antineoplastic
44	Bifonazole		16	Antifungal, calmodulin antagonist
45	Benzbromarone		16	Uricosuric
46	Toremifene citrate		16	Antineoplastic, antiestrogen
47	Quinaldine blue (pinacyanol chloride)		16	Antineoplastic

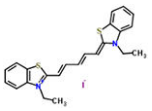
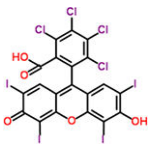
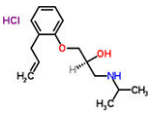
	Compound name	Chemical structure	MIC (μM)	Main use
48	Dithiazanine iodide		16	Anthelmintic (nematodes)
49	Rose bengal		16	Diagnostic aid (corneal trauma indicator).
50	Alprenolol hydrochloride		16	Antihypertensive

Table 2:

Classification of the most potent anticlostridial hits

Class			Drug	Use(s)
A	**Nitroimidazole**	1	Ronidazole	Antiprotozoal in veterinary medicine
		2	Dimetridazole	Antiprotozoal in veterinary medicine
		3	Tinidazole	Antiparasitic
		4	Ornidazole	Antiparasitic
		5	Secnidazole	Antiparasitic
B	**Salicylanilides**	5	Closantel	Anthelmintic and pesticide
		6	Oxyclozanide	Anthelmintic in veterinary medicine
		7	Niclosamide	Anthelmintic in humans and animals
C	**Imidazole antifungal**	8	Miconazole nitrate	Antifungal
		9	Econazole	Antifungal
		10	Tioconazole	Antifungal
		11	Butoconazole	Antifungal
		12	Clotrimazole	Antifungal
D	**Miscellaneous**	13	Dichlorophene	Antiparasitic in veterinary medicine
		14	Triclabendazole	Antiparasitic in human and animal medicine
		15	Nitazoxanide	Antiparasitic and antiviral
		16	Nithiamide	Antiprotozoal

Table 3:

MICs (μM) of the active compounds against clinical strains of *C. difficile*

C. difficile Strain	NR number	Nitroimidazoles					Imidazole antifungals				
		Ronidazole	Dimetridazole	Ornidazole	Tinidazole	Secnidazole	Miconazole	Econazole	Tioconazole	Butoconazole	Clotrimazole
P 3	32884	0.3	1.8	0.6	2	2.7	4.8	10.5	10.3	4.9	23.2
P 5	32885	0.3	1.8	0.6	2	2.7	4.8	5.2	5.2	4.9	5.8
P 6	32886	0.3	1.8	0.3	2	1.4	4.8	10.5	10.3	4.9	11.6
P 7	32887	0.3	1.8	0.6	16.2	5.4	4.8	10.5	10.3	4.9	11.6
P 19	32895	0.3	1.8	0.6	4	5.4	4.8	10.5	10.3	4.9	11.6
P 30	32904	0.3	1.8	0.6	8.1	5.4	2.4	10.5	10.3	4.9	23.2
Isolate 7	13433	0.3	1.8	0.6	4	2.7	2.4	10.5	5.2	4.9	11.6
Isolate 11	13437	0.3	1.8	0.3	2	2.7	4.8	10.5	10.3	9.7	11.6
Isolate 13	13553	0.6	1.8	0.6	16.2	2.7	9.6	5.2	20.6	4.9	11.6
ATCC BAA 1801		0.3	1.8	0.6	2	1.35	9.6	21	20.6	19.4	23.2
MIC_{50}		**0.3**	**1.7**	**0.6**	**2**	**2.7**	**4.8**	**10.5**	**10.3**	**4.9**	**11.6**

C. difficile Strain	NR number	Salicylanilides			Miscellaneous				Vancomycin	Metronidazole
		Closantel	Oxyclozanide	Niclosamide	Nitazoxanide	Dichlorophene	Triclabendazole	Nithiamide		
P 3	32884	0.4	1.2	0.8	0.8	7.4	22.2	0.7	0.7	1.5
P 5	32885	0.2	0.3	0.2	0.8	7.4	22.2	0.7	0.7	0.7
P 6	32886	0.2	1.2	0.8	0.8	3.7	11.1	0.3	0.7	0.7
P 7	32887	0.4	1.2	0.8	1.6	7.4	22.2	0.7	0.7	0.7
P 19	32895	0.2	0.6	0.4	0.2	14.9	11.1	0.7	1.4	1.5
P 30	32904	0.2	0.6	0.4	0.2	7.4	22.2	0.3	0.3	1.5
Isolate 7	13433	0.2	0.3	0.4	0.4	7.4	22.2	0.7	0.3	1.5
Isolate 11	13437	0.4	1.2	0.4	0.4	14.9	22.2	0.7	0.7	1.5
Isolate 13	13553	0.8	0.6	0.4	0.8	14.9	22.2	0.7	0.7	1.5
ATCC BAA 1801		0.2	0.3	0.4	0.2	7.4	11.1	0.3	0.3	0.7
MIC_{50}		**0.2**	**0.6**	**0.4**	**0.4**	**7.4**	**22.2**	**0.7**	**0.7**	**1.5**

Table 4:

MICs (μg/mL) of the active compounds against human normal gut flora

Bacterial Strain	Strain ID	Nitroimidazole				Imidazole antifungals				
		Ronidazole	Dimetridazole	Ornidazole	Tinidazole	Miconazole	Econazole	Tioconazole	Butoconazole	Clotrimazole
Lactobacillus casei	ATCC-334	>640	>907	>583	>518	308	335	330	311	371
Lactobacillus acidophilus	ATCC-314	640	>907	>583	>518	308	335	>330	>311	371
Bifidobacterium bifidum	ATCC-11863	≤5	≤7	≤5	32	10	21	21	19	23
Bifidobacterium breve	ATCC-15700	≤5	227	146	518	38	42	41	39	46
Bifidobacterium longum	HM-845	≤5	≤7	36	16	38	42	41	5	46
Bifidobacterium breve	HM-856	>640	>907	>583	>518	38	42	>330	10	46
Bacteroides fragilis	HM-711	≤5	28	≤5	≤4	38	42	5	10	46
Bacteroides fragilis	HM-709	≤5	28	≤5	≤4	19	21	≤3	10	46
Lactobacillus crispatus	HM-371	640	907	>583	>518	308	335	>330	>311	371
Lactobacillus gasseri	HM-407	320	907	>583	>518	308	335	>330	>311	371
Escherichia coli	ATCC-35150	640	113	>583	>518	>308	>335	>330	78	>371
Escherichia coli	1411	640	113	>583	>518	>308	>335	>330	78	>371
***Enterococcus faecalis* -- TX0104**	HM-201	640	454	>583	>518	38	42	>330	19	>371
***Enterococcus faecalis* -- TX1322**	HM-202	>640	907	>583	>518	19	42	>330	19	46

Strain	Strain ID	Salicylanilides			Miscellaneous				Vancomycin	Metronidazole
		Closantel	Oxyclozanide	Niclosamide	Nitazoxanide	Dichlorophene	Triclabendazole	Nithiamide		
Lactobacillus casei	ATCC-334	193	80	196	>417	>476	>356	>684	>88	>748
Lactobacillus acidophilus	ATCC-314	97	40	>391	>417	>476	>356	>684	0.7	>47
Bifidobacterium bifidum	ATCC-11863	≤1.5	40	≤3	≤3	30	11	≤5	0.7	5.8
Bifidobacterium breve	ATCC-15700	≤1.5	10	≤3	≤3	30	22	≤5	2.8	47
Bifidobacterium longum	HM-845	≤1.5	≤2.5	≤3	52	30	5.5	≤5	0.3	23
Bifidobacterium breve	HM-856	3	40	≤3	26	30	22	≤5	0.3	>748
Bacteroides fragilis	HM-711	3	5	6	26	30	44	43	88	≤5.8
Bacteroides fragilis	HM-709	3	5	≤3	26	30	22	11	44	≤5.8
Lactobacillus crispatus	HM-371	12	>319	49	417	476	>356	342	1.4	>748

Strain	Strain ID	Salicylanilides			Miscellaneous				Vancomycin	Metronidazole
		Closantel	Oxyclozanide	Niclosamide	Nitazoxanide	Dichlorophene	Triclabendazole	Nithiamide		
Lactobacillus gasseri	HM-407	12	>319	49	417	476	>356	342	1.4	>748
Escherichia coli	ATCC-35150	>193	>319	>391	>417	>476	>356	>684	ND	ND
Escherichia coli	1411	193	>319	>391	>417	>476	>356	>684	ND	ND
***Enterococcus faecalis* -- TX0104**	HM-201	24	>319	>391	26	59	22	11	>88	ND
***Enterococcus faecalis* -- TX1322**	HM-202	24	>319	>391	26	59	11	11	0.7	ND

ND= Not detected