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A high-throughput small-molecule screen to identify a novel chemical inhibitor of *Clostridium difficile*

David S. Katzianer^a, Takahiro Yano^b, Harvey Rubin^b, and Jun Zhu^{a,*}

^a Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

^b Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

Clostridium difficile, a highly drug-resistant Gram-positive, spore-forming bacterium, remains a leading cause of hospital-acquired diarrhoea and antibiotic-associated colitis. Clinically, only a handful of antibiotics are used for treating *C. difficile* infection (CDI), suggesting a necessity for the development of new treatment options. Here we performed a high-throughput screen of 2000 drug-like compounds for inhibition of *C. difficile*. From this screen, one compound, 5-nitro-1,10-phenanthroline (5-NP), showed potent bactericidal effects in vitro. In addition, this compound displayed high potency towards other *Clostridium* spp. as well as *Mycobacterium bovis* but not towards other tested Gram-positive and Gram-negative bacteria. Furthermore, we show that this inhibition may proceed through a metal chelation-dependent mechanism. More importantly, preliminary evidence suggests moderate efficacy for this compound in treating CDI in a murine infection model. These results present a possible basis for the further development of this compound as an antibiotic treatment for CDI.

Keywords

Clostridium difficile; High-throughput screen; Antibiotic discovery; Small-molecule library

1. Introduction

Clostridium difficile, a Gram-positive, spore-forming bacterium, is a prevalent cause of antibiotic-associated diarrhoea in hospitals and long-term care facilities worldwide [1]. In the USA alone, the rate of *C. difficile* infection (CDI) nearly tripled between 1996 and 2005

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* Corresponding author. Tel.: +1 215 573 4104; fax: +1 215 898 9557. junzhu@mail.med.upenn.edu (J. Zhu).

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[2]. Disease severity of CDI can range from self-limited diarrhoea to pseudomembranous colitis and, although death is uncommon, it does occur. Similarly, *C. difficile* remains an important economic burden to the healthcare industry, where an estimated \$4.8 billion in excess healthcare costs per year occur in acute care facilities alone [3].

CDI is commonly associated with broad-spectrum antibiotic usage [4,5]. Current theory suggests that disruption of the indigenous gut microbiota through antibiotics renders patients susceptible to *C. difficile* outgrowth in the lower intestine. Oral treatment with either metronidazole or vancomycin is indicated for CDI, although recurrence of infection is common [6]. Moreover, concern of evolved resistance in *C. difficile* towards these drugs is rising. Although resistance to vancomycin is generally regarded as uncommon, increased minimum inhibitory concentrations (MICs) and resistance to metronidazole have recently been reported [1]. Over the past few decades, the evolution of antibiotic resistance has been a major concern in clinical settings, with many antibiotics now ineffective against previously curable infectious diseases. In this study, we describe the use of high-throughput screening technology to screen a drug-like small-molecule library for antimicrobial activity against *C. difficile*. The results indicate that one particular compound, 5-nitro-1,10-phenanthroline (5-NP), can effectively inhibit *C. difficile* growth in vitro and in vivo. We further show that these inhibitory effects likely proceed through metal chelation. The results demonstrate the promise of this compound as a potential treatment option for CDI.

2. Materials and methods

2.1. Bacterial strains and growth

Clostridium difficile strain VPI10463 (ATCC 43255) was used for in vitro studies as well as in vivo animal experiments unless noted. Strains of other bacterial species used are indicated in Table 1. Unless otherwise noted, *C. difficile* strains were grown at 37 °C in an anaerobic workstation (Coy Laboratory Products Inc., Grass Lake, MI) in brain–heart infusion (BD Diagnostic Systems, Franklin Lakes, NJ) supplemented with 0.1% w/v L-cysteine (Sigma-Aldrich, St Louis, MO) and 5 mg/mL yeast extract (BD Diagnostic Systems) (referred to as BHIS), and cefoxitin was added to a final concentration of 8 µg/mL. An anaerobic atmosphere was maintained with 5% H₂, 5% CO₂ and balance nitrogen. For biofilm experiments, BHIS was supplemented with 0.1 M glucose in accordance with previous studies [7]. For all other bacterial species, strains were grown at 37 °C anaerobically or aerobically as indicated in BHIS (*Clostridium* spp.), TS (BD Diagnostic Systems) (*Staphylococcus aureus* and *Streptococcus pneumoniae*) or lysogeny broth [5 g/L yeast extract (BD Diagnostic Systems), 10 g/L sodium chloride (Fisher Scientific, Pittsburgh, PA) and 10 g/L tryptone (Fisher Scientific)] (all others). For anaerobic growth, media was pre-reduced in anaerobic conditions for 24 h prior to use.

2.2. High-throughput screening

Compounds from the TimTec ActiProbe-2K Screening Starter Kit Library (TimTec, Newark, DE) were purchased as 0.05 mg aliquots suspended in 50 µL of dimethyl sulphoxide (DMSO) for a concentration of ca. 3 mM based on average molecular weight. For screening, an overnight culture of *C. difficile* VPI10463 prepared in BHIS was diluted

1:100 into fresh medium and was aliquoted into 99 μL volumes in sterile, clear-bottomed 96-well plates (USA Scientific Inc., Ocala, FL). Then, 1 μL of each compound suspension was transferred to these plates for an approximate final compound concentration of 30 μM . For controls, DMSO was added to a final concentration of 1%. Plates were incubated anaerobically at 37 °C for ca. 24 h and the optical density at 600 nm (OD_{600}) was read (BioTek Synergy HT, Winooski, VT) as a proxy of growth. Bacterial growth inhibition was classified as none (OD_{600} same as control levels), partial (OD_{600} less than 50% of control levels) or total (no observed change in OD_{600}). To determine whether inhibition was due to bactericidal or bacteriostatic properties, overnight cultures grown in the presence of individual compounds were serially diluted and plated to determine viable bacterial numbers.

2.3. Calculation of minimum inhibitory concentrations

Approximate MICs were calculated for representative Gram-positive and Gram-negative species both anaerobically and aerobically where applicable. Overnight growth for each bacterial species (except *Mycobacterium bovis*) was tested in the presence of serial two-fold dilutions of 5-NP. To determine the MIC, the OD_{600} was measured and MICs were reported as the lowest concentration at which no bacterial growth was noted relative to media controls.

The MIC of 5-NP was then evaluated against *M. bovis* BCG using the microplate alamarBlue® assay (Invitrogen, Carlsbad, CA) as previously described [with 7H9 + glycerol + OADC (oleic acid–albumin–dextrose–and catalase) (all from Sigma-Aldrich)] [8]. Approximately 10^5 CFU/mL of bacteria were inoculated into the media. The MIC was defined as the lowest concentration affecting a reduction in fluorescence or absorbance of 90% relative to controls. Isoniazid was included as a positive quality control compound with an expected MIC range of 0.36–0.72 μM .

2.4. Chelation assays

To investigate the dependence of inhibition on chelation, *C. difficile* cultures were incubated with added concentrations of various metallic ions, and MICs under these conditions were measured. Briefly, overnight cultures of *C. difficile* were diluted 1:100 into fresh medium containing either FeSO_4 (40, 80, 120 and 200 $\mu\text{g/mL}$), ZnCl_2 (100 μM and 1 mM), MgCl_2 (10 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$) or CaCl_2 (13 $\mu\text{g/mL}$ and 130 $\mu\text{g/mL}$), aliquoted in 100 μL volumes in 96-well plates (USA Scientific Inc.) and grown in the presence of serial two-fold dilutions of 5-NP. After 24 h, plates were removed from the anaerobic chamber and the OD_{600} was measured.

2.5. Biofilm assays

To measure the effects of 5-NP on mature biofilms, the epidemic *C. difficile* UK1 strain was used due to enhanced biofilm formation relative to VPI10463 (data not shown). Briefly, an overnight *C. difficile* UK1 growth culture was diluted 1:100 in BHIS containing 0.1 M glucose, dispensed into 200 μL aliquots into a sterile, clear-bottomed 96-well plate (USA Scientific Inc.) and incubated at 37 °C with humidity to prevent evaporation. After 3 days of growth, the supernatant was discarded and 200 μL of sterile BHIS + 0.1 M glucose

containing either 15 μ M 5-NP (treatment) or 1% DMSO (vehicle) was added. Plates were incubated anaerobically at 37 °C for the time indicated. At each time point, plates were removed from the anaerobic chamber and biofilms were washed once with sterile phosphate-buffered saline and stained with 200 μ L of crystal violet for 5 min. Biofilms were then washed and re-suspended in DMSO. After 30 min, the OD₅₇₀ was measured. Data are the combination of eight repeats within the same 96-well plate.

2.6. Cytotoxicity assay

Adherence and growth of HeLa cells incubated with various concentrations of 5-NP for 24 h were examined to determine the cytotoxicity of this compound. For counting, cells were trypsinised and enumerated using a haemocytometer counting chamber slide (Hausser Scientific, Horsham, PA).

2.7. Animal experiments

Clostridium difficile mouse infection experiments were performed following a previously published protocol with modifications [5]. A detailed timeline of the experiment is included in Supplementary Fig. S1. Briefly, 6-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were administered an antibiotic cocktail (kanamycin 0.4 mg/mL, gentamicin 0.035 mg/mL, colistin 850 U/mL, metronidazole 0.215 mg/mL, vancomycin 0.045 mg/mL and cefoperazone 0.5 mg/mL in sterile drinking water containing 5% sucrose) for 5 days and were then switched to regular drinking water for the remainder of the experiment. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally 1 day prior to *C. difficile* challenge.

Immediately prior to infection, overnight cultures of *C. difficile* VPI10463 were mixed 1:1 with DMSO (vehicle control) or with 2 mg/mL or 4 mg/mL 5-NP and then 100 μ L of these mixtures were inoculated into mice via oral gavage (ca. 10^7 CFU). For treatment groups, either 0.1 mg or 0.2 mg of the compound was administered in 50% DMSO at 4, 24 and 48 h post infection. Treatment doses were chosen based on the limited solubility of 5-NP in 50% DMSO. Animals were monitored and recorded every 12 h for signs of disease including weight loss, diarrhoea, hunched posture and lethargy.

3. Results and discussion

3.1. High-throughput screen of *Clostridium difficile* inhibitors

To screen for possible inhibitors of *C. difficile* growth, the TimTec ActiProbe-2K Screening Starter Kit Library containing 2000 organic compounds randomly synthesised to contain functional groups common in known drugs was used. Each compound was screened at ca. 30 μ M concentration for antimicrobial properties. Supplementary Fig. S2 shows the flow chart for this screening process. After confirmation of individual compounds displaying growth inhibition, 19 molecules were obtained that limited *C. difficile* overnight growth by >50% of control levels. Of these 19 compounds, 11 compounds had some bactericidal properties at the tested concentration based on CFU counting, whereas the remaining 8 delayed bacterial growth in liquid culture. The potency of the 11 bactericidal compounds was further investigated and 2 compounds that had inhibitory activity at significantly low

concentrations were found. One of these compounds, 5-NP (Fig. 1A), displayed efficacy in vivo (see below). Importantly, 5-NP is predicted to be orally active based on Lipinski's 'Rule of 5' owing to its few hydrogen bond donors and acceptors and low molecular weight [9]. Therefore, 5-NP was chosen for further characterisation.

3.2. 5-Nitro-1,10-phenanthroline efficiently inhibits select Gram-positive pathogens

To examine the potency of 5-NP towards *C. difficile* as well as its spectrum of inhibitory effects, MICs against different bacterial species grown under anaerobic and/or aerobic conditions were measured. 5-NP could inhibit the growth of all three tested *C. difficile* strains efficiently (MIC $\approx$ 2 μ M) (Table 1). Observed MICs for *Clostridium perfringens* and *Clostridium sporogenes* were similarly low. Much higher concentrations were necessary for growth inhibition of other tested species, including both Gram-positive and Gram-negative bacteria such as *Bacillus*, *Staphylococcus*, *Streptococcus*, *Vibrio*, *Escherichia coli*, *Pseudomonas* and the prevalent intestinal commensal *Bacteroides*, implying narrow-spectrum activity towards Clostridia. Interestingly, high levels of inhibitory activity towards *M. bovis* were also observed. With the emergence of multidrug resistance in strains of *Mycobacterium* and *Clostridium* pathogens, development of this molecule or similarly structured compounds could provide an important basis for antibiotic discovery towards these highly resistant pathogens.

3.3. 5-Nitro-1,10-phenanthroline inhibition is partially overcome by addition of iron

The non-substituted parent molecule, 1,10-phenanthroline, has previously been shown to have antimicrobial properties through complexing with metal ions and has additionally been shown to interfere with the acetyl-coenzyme A synthesis pathway of *C. difficile* when cloned and expressed in *E. coli* [10–12]. Furthermore, recent findings have shown that complexes of copper(II) and 5-NP act synergistically with ofloxacin, decreasing bacterial MICs to this fluoroquinolone in Gram-negative but not Gram-positive species [13]. To investigate the activity dependence of the nitro-substituted daughter molecule on chelation, various concentrations of separate metal solutions were added to growth media as described and the OD₆₀₀ was measured to determine increased MICs of 5-NP in the presence of these compounds. Interestingly, whilst the divalent cations ZnCl₂, MgCl₂ and CaCl₂ displayed no significant effect on MICs, a dose-dependent response was noticed for FeSO₄ (Fig. 1B). These data suggest that a potential mechanism of inhibitory action for 5-NP is partially through the chelation of free iron atoms. Further investigation will be performed to determine additional bactericidal mechanisms of this compound.

3.4. 5-Nitro-1,10-phenanthroline promotes *Clostridium difficile* biofilm dispersal

Bacterial biofilms impose serious health issues because of their antibiotic-resistant properties [14,15]. To test whether 5-NP has any effect on *C. difficile* biofilms, biofilm-associated cells were incubated with either DMSO or the compound for different times and the remaining biofilm was quantified by crystal violet staining. The biofilm-associated biomass was significantly reduced in those treated for 6 h and 24 h compared with controls ($P = 0.090$ and 0.044 , respectively) (Supplementary Fig. S3), suggesting that 5-NP may promote *C. difficile* biofilm dissolution.

3.5. 5-Nitro-1,10-phenanthroline displays modest in vivo effects against *Clostridium difficile* infection

To examine the therapeutic effects of 5-NP on CDI, the cytotoxicity of this compound was first tested towards eukaryotic epithelial cells. It was found that HeLa cells incubated with concentrations two- to four-fold higher than *C. difficile* MICs for 24 h were still capable of adherence and attachment (Supplementary Fig. S4). These findings suggest that this compound has low cytotoxicity to epithelial cells.

The in vivo efficacy of 5-NP in a murine model was then investigated. Mice were infected with *C. difficile* and were treated with either 5 mg/kg or 10 mg/kg doses of the compound in 50% DMSO. As a proxy for illness, weight loss was monitored for the duration of the experiment and was reported as percentage change per cage relative to baseline weight (Fig. 2A). Whilst untreated mice lost ca. 20% of their weight 2 days post infection, significantly less weight loss was observed in both treatment groups. Furthermore, although 100% of mice were moribund following 2 days of infection, treatment groups likewise survived significantly better (Fig. 2B). All mice in treatment groups were euthanised 72 h post infection owing to illness, although it is possible that enhanced dosage regimens and drug solubility could improve in vivo efficacy. Taken together, these data suggest that 5-NP has a modest effect on CDI in vivo.

4. Discussion

Widespread antibiotic resistance among bacterial pathogens remains a major public health threat. To combat this, there has been recent impetus to discover and develop new antibiotic therapies. Ideally these compounds would show desired effects at low concentrations and relatively low cytotoxicity to host cells. Here we report the potential utility of an iron chelator, 5-NP, in treating CDI. We show that this molecule has a relatively low spectrum of bacterial inhibition, displaying high specificity towards Clostridia and Mycobacteria groups, and is also capable of inhibiting *C. difficile* growth at concentrations comparable with the first-line drug metronidazole. In addition, we show that 5-NP slows CDI disease progression compared with control groups in a murine animal model. We speculate that enhanced dosing methods through further development and chemical optimisation may provide a potential new treatment option for combating Clostridia pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Huang H, Weintraub A, Fang H, Nord CE. Antimicrobial resistance in *Clostridium difficile*. *Int J Antimicrob Agents*. 2009; 34:516–22. [PubMed: 19828299]
2. Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. *N Engl J Med*. 2008; 359:1932–40. [PubMed: 18971494]
3. Dubberke ER, Olsen MA. Burden of *Clostridium difficile* on the healthcare system. *Clin Infect Dis*. 2012; 55(Suppl 2):S88–92. [PubMed: 22752870]
4. Kelly CP, LaMont JT. *Clostridium difficile* infection. *Annu Rev Med*. 1998; 49:375–90. [PubMed: 9509270]
5. Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection. *Gut Microbes*. 2011; 2:145–58. [PubMed: 21804357]
6. Zar FA, Bakkanagari SR, Moorthi KM, Davis MB. A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. *Clin Infect Dis*. 2007; 45:302–7. [PubMed: 17599306]
7. Ethapa T, Leuzzi R, Ng YK, Baban ST, Adamo R, Kuehne SA, et al. Multiple factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*. *J Bacteriol*. 2013; 195:545–55. [PubMed: 23175653]
8. Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. *Antimicrob Agents Chemother*. 2006; 50:2853–6. [PubMed: 16870785]
9. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001; 46:3–26. [PubMed: 11259830]
10. Khorasani-Motlagh M, Noroozifar M, Moodi A, Niroomand S. Biochemical investigation of yttrium(III) complex containing 1,10-phenanthroline: DNA binding and antibacterial activity. *J Photochem Photobiol B*. 2013; 120:148–55. [PubMed: 23384614]
11. Zhu X, Li T, Gu X, Zhang S, Liu Y, Wang Y, et al. Structural and functional investigation into acetyl-coenzyme A synthase and methyltransferase from human pathogen *Clostridium difficile*. *Metallomics*. 2013; 5:551–8. [PubMed: 23599026]
12. Liu X, Li X, Zhang Z, Dong Y, Liu P, Zhang C. Studies on antibacterial mechanisms of copper complexes with 1,10-phenanthroline and amino acid on *Escherichia coli*. *Biol Trace Elem Res*. 2013; 154:150–5. [PubMed: 23716177]
13. Patel MN, Parmar PA, Gandhi DS. Antibacterial, SOD mimic and nuclease activities of copper(II) complexes containing ofloxacin and neutral bidentate ligands. *Appl Organomet Chem*. 2010; 25:27–33.
14. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001; 358:135–8. [PubMed: 11463434]
15. Martinez JL, Rojo F. Metabolic regulation of antibiotic resistance. *FEMS Microbiol Rev*. 2011; 35:768–89. [PubMed: 21645016]

Fig. 1. 5-Nitro-1,10-phenanthroline (5-NP) chemical structure and chelation-dependent mechanism of action. (A) 5-NP structure. (B) Minimum inhibitory concentration (MIC) fold changes of *Clostridium difficile* grown in the presence of various concentrations of the divalent cations Zn²⁺, Mg²⁺, Ca²⁺ and Fe²⁺.

Fig. 2.

In vivo effects of 5-nitro-1,10-phenanthroline (5-NP) towards *Clostridium difficile* infection. (A) Weight of untreated and treated mice infected with *C. difficile* VPI10463 2 days post infection. Data were analysed using Student's *t*-test: * $P < 0.05$ and *** $P < 0.0005$. (B) Kaplan–Meier survival plot. Survival curves are significantly different [$P = 0.0006$ by the log-rank (Mantel–Cox) test].

Table 1

Estimated minimum inhibitory concentrations (MICs) of 5-nitro-1,10-phenanthroline (5-NP) against different bacteria grown anaerobically and/or aerobically

Species	Strain	Gram designation ^a	Growth ^b	MIC (μM) ^c
<i>Clostridium difficile</i>	VPI10463	+	O ₂ ⁻	2
	CD630		O ₂ ⁻	2
	UK1		O ₂ ⁻	2
<i>Clostridium perfringens</i>	ATCC3624	+	O ₂ ⁻	4
<i>Clostridium sporogenes</i>	ATCC3584	+	O ₂ ⁻	1
<i>Bacillus cereus</i>		+	O ₂ ⁻	60
			O ₂ ⁺	150
<i>Bacillus subtilis</i>	3610	+	O ₂ ⁺	150
<i>Staphylococcus aureus</i>	Newman	+	O ₂ ⁺	150
<i>Streptococcus pneumoniae</i>	TIGR4	+	O ₂ ⁺	150
<i>Mycobacterium bovis</i>	BCG	(+)	O ₂ ⁺	2
<i>Pseudomonas aeruginosa</i>	PAO1	-	O ₂ ⁺	>150
	PA14		O ₂ ⁺	>150
<i>Escherichia coli</i>	O:157	-	O ₂ ⁺	>150
			O ₂ ⁺	>150
<i>Vibrio cholerae</i>	C6706	-	O ₂ ⁻	60
<i>Bacteroides</i>	ATCC	-	O ₂ ⁺	150
<i>thetaitaomicron</i>	29741		O ₂ ⁻	60

MICs were calculated as the lowest concentration of 5-NP at which no overnight bacterial growth was observed through absorbance readings relative to control values. The compound was evaluated for MIC against *Mycobacterium bovis* BCG using the microplate alamarBlue® assay as previously described [8]. Data presented are the mean of three independent experiments.

^a +, Gram-positive; -, Gram-negative; (+), Gram-positive like.

^b Bacteria were grown anaerobically (O₂⁻) in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) or aerobically (O₂⁺) at 37 °C for 24 h.

^c Overnight growth for each bacterial species was tested in the presence of serial two-fold dilutions of 5-NP. The optical density at 600 nm (OD₆₀₀) was then measured.