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Diarylacylhydrazones: Clostridium-selective antibacterials with activity against stationary-phase cells

Chao Chen

Northeastern University

Naveen K. Dolla

University of Wollongong, nkd547@uowmail.edu.au

Gabriele Casadei

Northeastern University

John B. Bremner

University of Wollongong, jbremner@uow.edu.au

Kim Lewis

Northeastern University

See next page for additional authors

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Abstract

Current antibiotics for treating Clostridium difficile infections (CDI), that is, metronidazole, vancomycin and more recently fidaxomicin, are mostly effective but treatment failure and disease relapse remain as significant clinical problems. The shortcomings of these agents are attributed to their low selectivity for C. difficile over normal gut microflora and their ineffectiveness against C. difficile spores. This Letter reports that certain diarylacylhydrazones identified during a high-throughput screening/counter-screening campaign show selective activity against two Clostridium species (C. difficile and Clostridium perfringens) over common gut commensals. Representative examples are shown to possess activity similar to vancomycin against clinical C. difficile strains and to kill stationary-phase C. difficile cells, which are responsible for spore production. Structure-activity relationships with additional synthesised analogues suggested a protonophoric mechanism may play a role in the observed activity/selectivity and this was supported by the well-known protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) showing selective anti-Clostridium effects and activity similar to diarylacylhydrazones against stationary-phase C. difficile cells. Two diarylacylhydrazones were shown to be non-toxic towards human FaDu and Hep G2 cells indicating that further studies with the class are warranted towards new drugs for CDI.

Keywords

stationary, against, activity, antibacterials, clostridium, selective, diarylacylhydrazones, cells, phase, CMMB

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Diarylacylhydrazones: *Clostridium*-Selective Antibacterials with Activity Against Stationary-Phase Cells

Chao Chen^{a†}, Naveen K. Dolla,^{b†} Gabriele Casadei^{a‡}, John B. Bremner^b, Kim Lewis^a, Michael J. Kelso^{b*}

^a*Antimicrobial Discovery Center, Department of Biology, Northeastern University, Boston, MA 02115, USA*, ^b*School of Chemistry, University of Wollongong, NSW 2522, Australia.*

[‡]*Present address: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna Sede Diagnostica Provinciale di Parma, 43126 Parma, Italy.*

[†]Authors contributed equally to this work

*To whom correspondence should be addressed. Phone: +61 2 4221 5085. Fax: +61 2 4221 4287. E-mail: mkelso@uow.edu.au

Keywords: *Clostridium difficile*, antibacterial, protonophore, diarylacylhydrazone, CCCP, stationary phase cells

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CDAD, *Clostridium difficile*-associated diarrhoea; CDI, *Clostridium difficile* infection; DACC, Data Analysis and Coordination Centre; 2,4-DNP, 2,4-dinitrophenol; DMSO, dimethyl sulfoxide; FDA, US Food and Drug Administration; HTS, high-throughput screening; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NCI, National Cancer Institute; NIH, National Institutes of Health; PBS, phosphate buffered saline; PCP, pentachlorophenol; PMC, pseudomembranous colitis.

Abstract

Current antibiotics for treating *Clostridium difficile* infections (CDI), i.e. metronidazole, vancomycin and more recently fidaxomicin, are mostly effective but treatment failure and disease relapse remain as significant clinical problems. The shortcomings of these agents are attributed to their low selectivity for *C. difficile* over normal gut microflora and their ineffectiveness against *C. difficile* spores. This paper reports that certain diarylacylhydrazones identified during a high-throughput screening/counter-screening campaign show selective activity against two *Clostridium* species (*C. difficile* and *C. perfringens*) over common gut commensals. Representative examples are shown to possess activity similar to vancomycin against clinical *C. difficile* strains and to kill stationary-phase *C. difficile* cells, which are responsible for spore production. Structure-activity relationships with additional synthesised analogues suggested a protonophoric mechanism may play a role in the observed activity/selectivity and this was supported by the well-known protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) showing selective anti-*Clostridium* effects and activity similar to diarylacylhydrazones against stationary-phase *C. difficile* cells. Two diarylacylhydrazones were shown to be non-toxic towards human FaDu and Hep G2 cells indicating that further studies with the class are warranted towards new drugs for CDI.

Clostridium difficile-associated diarrhoea (CDAD), also known as *C. difficile* infection (CDI), is the leading cause of infectious nosocomial gastrointestinal illness.^{1,2} Steadily increasing CDI rates in US hospitals,¹ emergence of epidemic and hypervirulent strains (e.g. BI/NAP1/027),³ increased incidences of community acquired CDI⁴ and enormous costs to healthcare systems (estimated at \$3.2 billion/year in the US alone)⁵ have focused considerable attention on this disease over the past decade.^{6,7}

C. difficile is a Gram-positive, rod-shaped, spore-forming anaerobe transmitted via the oral-fecal route. In its vegetative form it is highly sensitive to oxygen but its spores are heat stable, insensitive to standard disinfectants, able to survive for long periods in the environment and they can passage intact through the acidic stomach. *C. difficile* typically resides asymptotically in the human gastrointestinal tract until normal microflora are disrupted, such as following broad-spectrum antibiotic treatments, after which it can overgrow producing three toxins; toxin A (TcdA), toxin B (TcdB) and the binary toxin CDT.. Ensuing CDI can range in severity from mild diarrhoea to life-threatening pseudomembranous colitis (PMC) and toxic megacolon.⁶

Treatments for CDI historically have involved antibiotic withdrawal followed by oral metronidazole **1** (500 mg t.i.d, 10-14 days) or vancomycin **2** (125-250 mg q.i.d, 10 days) but treatment failure remains as a significant and increasing problem (reportedly > 35% for metronidazole and 1-16% for vancomycin).² For patients who develop severe CDI (diarrhoea with leucocytosis, PMC or toxic shock) metronidazole is effective in 76% of cases and vancomycin 97%.² Upwards of 20-30% of patients can experience recurrent CDI with intermittent episodes arising over months and sometimes years.⁸ A major risk factor for recurrent CDI is failure to re-establish normal protective gut microflora due to the action of metronidazole and vancomycin on gut commensals and the ineffectiveness of these antibiotics against *C. difficile* spores.²

In 2011 the US Food and Drug Administration (FDA) approved fidaxomicin **3** (Dificid[®], Optimer Pharmaceuticals Inc.),⁹ an oral macrocyclic narrow-spectrum antibiotic developed specifically

for CDI. Fidaxomicin shows very high *in vitro* potency against *C. difficile* (minimum inhibitory concentrations (MICs) against clinical isolates 0.008–0.25 mg/L) and reduced activity against gut commensals, in particular *Bacteroides* species.¹⁰ This and lower post-treatment *C. difficile* spore counts¹⁰ are thought to contribute to reduced CDI recurrence with fidaxomicin.^{11,12} Relapse rates for infections caused by the BI/NAP1/027 hypervirulent strain, however, are the same for vancomycin and fidaxomicin^{13,14} and many hospitals have been slow to embrace the new drug due to its high cost (> US\$2700 per treatment, c.f. metronidazole US\$22 and vancomycin \$1270).¹⁵

While the proper place for fidaxomicin in clinical practice is still being established the search continues for alternative and, ideally, more cost-effective agents. Attractive new compound classes would include those that show high selectivity for *C. difficile* over gut commensals along with activity against the stationary-phase cells responsible for spore formation.¹⁶ In a recent high-throughput screening (HTS) and counter-screening campaign we identified that certain diarylacylhydrazones are *clostridium*-selective agents.¹⁷ This paper reports the activity and selectivity of diarylacylhydrazone screening hits, describes structure-activity studies around the class and reports that a representative member is active against stationary-phase *C. difficile* cells. Evidence is presented that selective anti-*Clostridium* activity in the class may arise, in part, through a protonophoric mechanism.

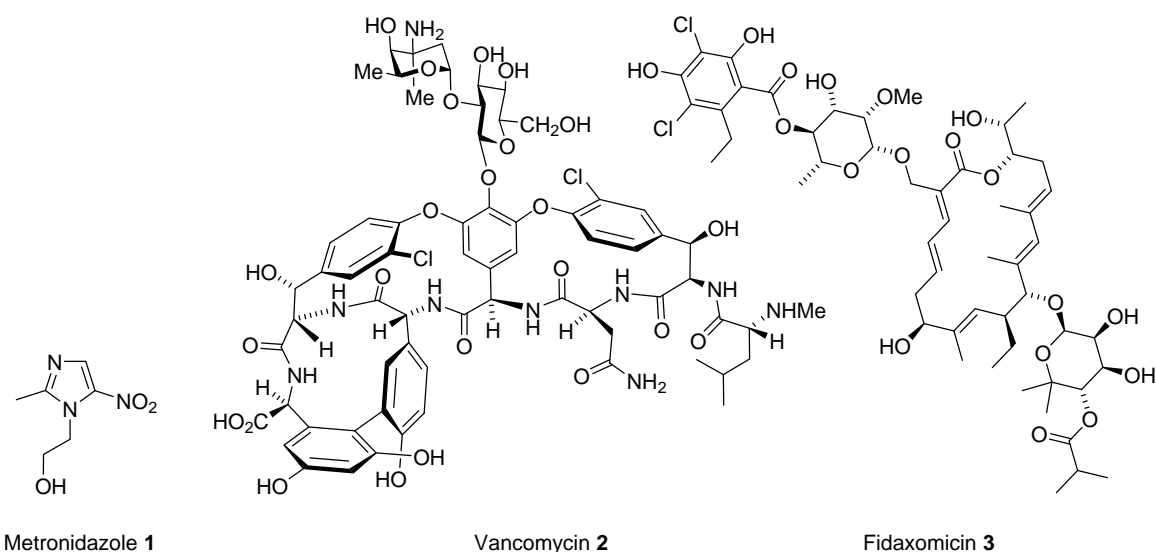


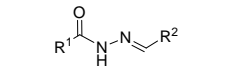
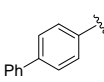

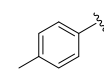

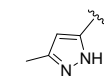

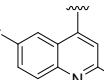

Figure 1. Current antibiotic treatments for *C. difficile* infections.

High-throughput screening was carried out to identify hits against *C. difficile* CD196.¹⁷ Hits were subjected to a counter-screening panel of ten bacterial species representing the major taxonomic groups from the human gut environment in order to identify *Clostridium*-selective compounds. Counter-screening species were chosen from the Data Analysis and Coordination Centre (DACC) for the Human Microbiome Project (<http://www.hmpdacc.org/>), part of the National Institutes of Health (NIH) Roadmap for Medical Research, and included abundant members of the gut flora, e.g. *Bacteroides thetaiotaomicron*, organisms of clinical significance, e.g. *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*, along with representatives from each of the predominant phyla. *Clostridium perfringens* was included to identify species-specific anti-*C. difficile* compounds.

Four acylhydrazones carrying aryl substituents at R¹ and R² (i.e. diarylacylhydrazones **4-7**, Figure 2) were initially identified as hits. Follow-up measurements showed that the minimum inhibitory concentrations (MIC) of **4-7** against *C. difficile* CD196 ranged from 1.56-6.25 µg/mL and that the compounds exhibited similar MICs against *Clostridium perfringens*. Minimum bactericidal concentrations (MBC) of two examples chosen for further study (i.e. compounds **5** and **6**, see below) against CD196 were equivalent to their MICs, confirming that the compounds are bactericidal.

Quinolinium acetate, chloride and mesylate salts of **7** showed identical activity to the free base **7**. Compound **4** was shown to have activity similar to vancomycin (< 2-fold difference in MIC) against five *C. difficile* clinical isolates (Supporting Information, Figure S1).¹⁷ Importantly, compounds **4-7** were essentially inactive (MIC > 50 µg/mL) across the gut commensal panel with only **5** and **6** showing weak activity (MIC = 12.5-25 µg/mL) against *B. fragilis* and *B. thetaiotaomicron*. In contrast, metronidazole **1**, vancomycin **2** and fidaxomicin **3** all showed high potency against *C. difficile* accompanied by significant activity against commensals (Figure 2).

Compound		MIC (µg/mL)										
		<i>C. difficile</i> CD196	<i>C. perfringens</i>	<i>B. fragilis</i>	<i>B. thetaiotaomicron</i>	<i>B. longum</i>	<i>L. casei</i>	<i>L. reuteri</i>	<i>S. aureus</i>	<i>S. mutans</i>	<i>E. coli</i>	<i>E. faecalis</i>
1	Metronidazole	1.56	3.13	≤ 0.78	≤ 0.78	≤ 0.78	> 50	> 50	> 50	> 50	> 50	> 50
2	Vancomycin	1.00	≤ 0.78	12.5 - 25	12.5 - 25	≤ 0.78	> 50	> 50	3.13	1.56	> 50	3.13
3	Fidaxomicin	0.18	≤ 0.0075	> 50	> 50	≤ 0.78	> 50	> 50	3.13 - 6.25	3.13	> 50	12.5

													
	R ¹	R ²											
4			1.56	3.13	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50
5			6.25 (6.25)*	1.56	12.5	25	> 50	> 50	> 50	> 50	> 50	> 50	> 50
6			3.13 (3.13)*	≤ 3.13	12.5	25	> 50	> 50	> 50	> 50	> 50	> 50	> 50
7[#]			6.25	6.25	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50

*Values in parentheses represent minimal bactericidal concentrations (MBC). [#]Quinolinium acetate, chloride and mesylate salts showed identical activity to **7**.

Figure 2. Activity of metronidazole **1**, vancomycin **2**, fidaxomicin **3** and diarylacylhydrazones **4-7** against *C. difficile* CD196 and a panel of gut commensals.

Three structural motifs common in hits **4-7** were: (1) an acyl hydrazone, (2) an aryl ring at R¹ and (3) an aryl ring at R² carrying an *ortho*-hydroxy substituent. Follow-up similarity searches of our libraries identified six additional diarylacylhydrazones containing the 2-hydroxynaphthalene group at R² available for examination (Supporting Information, Figure S2, compounds **S1-S6**). Compounds **S1-**

S3, which incorporate phenyl, *m*-bromophenyl and *o*-hydroxynaphthyl groups at R¹, respectively, displayed MICs against CD196 in the range 6.25-12.5 µg/mL. Compounds **S4-S6**, which carry 4-nitrophenyl, 3-nitrophenyl and 3,5-dinitrophenyl substituents at R¹, respectively, all showed no activity against CD196. These findings indicate that substituents on the aryl group at R¹ can dramatically impact potency. A further nine compounds with structures related to **4-7** but lacking one or more of the above criteria (Supporting Information, Figure S3, **S7-S15**) were selected from the libraries for testing and found to be inactive. Diarylacylhydrazones with aryl rings at R¹ and *ortho*-hydroxy substituted aryl rings at R² thus appear to present the minimal structural requirements for selective anti-*Clostridium* activity in the class.

Encouraged by these results, analogues **8-19** (Figure 3) were synthesised to answer specific structure-activity questions about the class. The targeted compounds were all prepared by heating the requisite R¹-acylhydrazines (prepared by reacting precursor methyl esters with hydrazine) and R²-aryl aldehydes overnight in ethanol (Figure 3). Yields ranging from 70-90% of the pure compounds were obtained after silica-gel column chromatography and/or recrystallization. Compound **8** (INP0400) carrying a *p*-chlorophenyl group at R¹ and a 2-hydroxynaphthyl group at R² was targeted because it had previously been reported as an inhibitor of type III secretion in the common bacterial pathogen *Chlamydia trachomatis*.¹⁸ The MIC of **8** against CD196 was found to be 6.25 µg/mL and it showed higher potency against *C. perfringens* (MIC ≤ 0.78 µg/mL). The compound's selectivity for *Clostridium* species over gut commensals was similar to **5** showing only weak activity against *B. fragilis* and *B. thetaiotaomicron* (MIC = 12.5 µg/mL) and no other activity across the panel.

Compound **9**, which substituted the *ortho*-hydroxynaphthyl group present at R² in **5** with the *ortho*-vanillyl moiety of **7**, was synthesized to probe the effect of interchanging the two different R² groups present in diarylacylhydrazone screening hits. *Clostridium*-selective activity was observed with **9** which showed MICs of 12.5 and 6.25 µg/mL against *C. difficile* and *C. perfringens*, respectively, and

no other activity across the panel. Replacing the *ortho*-hydroxynaphthyl group at R² of **6** with the *ortho*-vanillyl moiety (i.e. compound **10**), however, abolished all activity, including against both *Clostridium* species. Total loss of activity across the panel was similarly observed with **11**, where the *ortho*-vanillyl group at R² in **7** was replaced with the *ortho*-hydroxynaphthyl group. These results demonstrate that the *Clostridium*-selective activity of diarylacylhydrazones is structure-dependent and is affected by substituents on both aryl groups at R¹ and R².

The importance of the *ortho*-hydroxy group at R² was established next with compounds **12-15**. Transferring the hydroxyl group of **5** to the naphthyl 4-position (compound **12**) led to total loss of activity, as did removing the *ortho*-hydroxy group altogether from compounds **5**, **6** and **7** (compounds **13**, **14**, and **15**, respectively). Methylation of the acylhydrazone 'amide' nitrogen of **5** (compound **16**), its *ortho*-phenolic group at R² (i.e. compound **17**) or both of these groups (compound **18**) similarly removed all activity.

It was noted that diarylacylhydrazones structurally resemble nitrofurans, an older class of broad spectrum antibiotics that have seen widespread historical use in humans and in veterinary medicine.¹⁹ Nitrofurans typically contain an acylhydrazone substituted with a 5-nitrofuranyl group at the position corresponding to R² in diarylacylhydrazones. A variety of substituents, both aryl and non-aryl, can be present at the position corresponding to R¹ (e.g. R¹ = *p*-hydroxyphenyl, nifuroxazide; R¹ = NH₂, nitrofurazone; R¹ = hydantoin, nitrofurantoin; R¹ = oxazolidinone, furazolidone). The novel nitrofuran **19**, carrying a 3-methylpyrazole group at R¹ and thus high structural similarity to **6**, was synthesised to explore the possibility that nitrofurans and diarylacylhydrazones might share overlapping antibacterial mechanism(s) of action. However, **19** showed broad spectrum activity across the panel, including high potency against *C. difficile* CD196 (MIC = 0.78 µg/mL, Figure 3), confirming that diarylacylhydrazones and nitrofurans exert their antibacterial effects via different mechanisms.

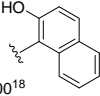
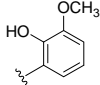
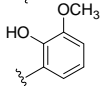
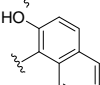
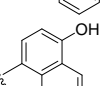
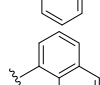
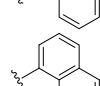
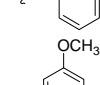
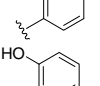
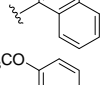
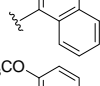
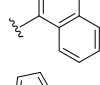
$R^1-C(=O)-N(R^3)-NH_2 + H-C(=O)-R^2 \xrightarrow[\Delta]{EtOH} R^1-C(=O)-N(R^3)-N=C(R^2)$			MIC ($\mu\text{g/mL}$)												
R ¹	R ²	R ³	<i>C. difficile</i> CD196	<i>C. perfringens</i>	<i>B. fragilis</i>	<i>B. thetaiotaomicron</i>	<i>B. longum</i>	<i>L. casei</i>	<i>L. reuteri</i>	<i>S. aureus</i>	<i>S. mutans</i>	<i>E. coli</i>	<i>E. faecalis</i>		
8	 INP0400 ¹⁸	H	6.25	≤ 0.78	12.5	12.5	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
9		H	12.5	6.25	> 50	> 50	> 50	> 50	> 50	> 50	1.56	> 50	> 50		
10		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
11		H	> 50	25	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
12		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
13		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
14		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
15		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
16		CH ₃	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
17		H	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
18		CH ₃	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50	> 50		
19		H	0.78	≤ 12.5	≤ 3.13	≤ 3.13	≤ 0.78	6.25	12.5	1.56	1.56	12.5	12.5		

Figure 3. Activity of synthesized diarylacylhydrazones **8-19** against *C. difficile* CD196 and a panel of gut commensals.

Demonstrating that nitrofurans invoke different antibacterial mechanisms and that anti-*Clostridium* activity is lost in diarylacylhydrazones upon methylation of the ‘amide’ nitrogen and/or the *ortho*-phenolic group present at R² led to speculation that the compounds might be exerting their selective effects through a protonophoric mechanism. To test this hypothesis, MIC measurements were obtained with three well-known membrane-active protonophores; carbonyl cyanide *m*-

chlorophenylhydrazone (CCCP),²⁰ pentachlorophenol (PCP),²¹ and 2,4-dinitrophenol (2,4-DNP)²² (Figure 4) against CD196 and the gut panel. It was reasoned that if *Clostridium*-selective activity were observed with one or more of these it would support a protonophoric mechanism for diarylacylhydrazones. CCCP was found to be highly active against CD196 (MIC = 1.56 µg/mL) and also showed high selectivity, affecting only one other panel member (*B. longum*, MIC = 3.25 µg/mL). In contrast to diarylacylhydrazones, CCCP showed no activity against *C. perfringens*. PCP and 2,4-DNP showed only weak activity across the panel and no selectivity towards *Clostridium*, indicating that selective action against *Clostridium* is not a general effect of protonophores. The similarity between the activity/selectivity observed with CCCP and diarylacylhydrazones implies that a protonophoric mechanism probably plays some role in the mechanism. That CCCP and diarylacylhydrazones both carry hydrazone moieties may be important but this remains to be determined.

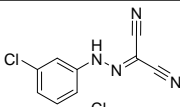
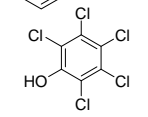
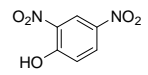
Protonophore	MIC (µg/mL)											
	<i>C. difficile</i> CD196	<i>C. perfringens</i>	<i>B. fragilis</i>	<i>B. thetaiotaomicron</i>	<i>B. longum</i>	<i>L. casei</i>	<i>L. reuteri</i>	<i>S. aureus</i>	<i>S. mutans</i>	<i>E. coli</i>	<i>E. faecalis</i>	
Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone (CCCP) 	1.56	> 50	> 50	> 50	3.25	> 50	> 50	> 50	> 50	> 50	> 50	
Pentachlorophenol 	50	6.25	6.25/12.5	6.25	12.5	50	50	< 0.78	25	> 50	> 50	
2,4-dinitrophenol 	50	> 50	12.5/25	25	25	> 50	> 50	25	> 50	> 50	> 50	

Figure 4. Activity of three protonophores against *C. difficile* CD196 and a panel of gut commensals.

Recent reports indicate that perturbing membrane function represents a promising strategy towards novel anti-*C. difficile* therapeutics,²³ especially since some membrane-active agents affect the quiescent/stationary-phase cells responsible for spore formation.²⁴ Two membrane-active agents oritavancin (vancomycin analogue)²⁵ and CB-183,315 (daptomycin analogue)²⁶ are in clinical

development for CDI. Several membrane-active compounds (including CCCP) were recently shown to kill *C. difficile* cells in both logarithmic- and stationary-phase cultures.²⁷ The activity of diarylacylhydrazones **5** and **6** was thus examined against stationary-phase *C. difficile* CD196 cells alongside CCCP, with metronidazole **1**, vancomycin **2** and fidaxomicin **3** included for comparison.

CD196 cells were grown to stationary phase according to an in-house procedure (Supporting Information) and treated with 1x, 5x, 10x and 20x the previously determined MIC ($\mu\text{g/mL}$) concentrations of each compound. Metronidazole **1**, vancomycin **2** and fidaxomicin **3** showed little or no activity at all concentrations tested (Figure 5a-c). Compound **5** showed some ability to kill stationary-phase cells but the data was not reproducible (not shown) and it was suspected that the poor solubility of **5** in the assay medium may have been partially responsible. Switching to the more soluble **6** produced reproducible dose-dependent cell-killing effects that were remarkably similar to those observed with CCCP (Figure 5d-e), supporting the postulate that diarylacylhydrazones and CCCP may indeed exert their selective anti-*Clostridium* effects through overlapping (protonophoric) mechanisms.

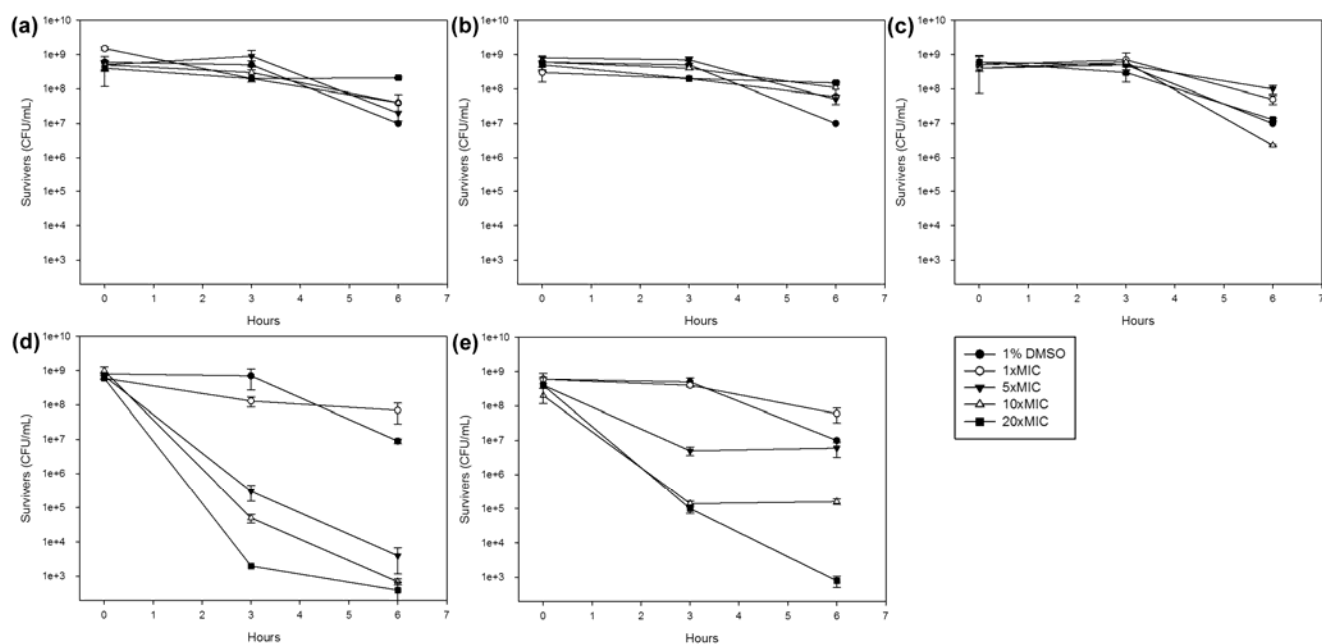


Figure 5. Activity against stationary-phase *C. difficile* CD196 cells of: (a) metronidazole **1** (b) vancomycin **2** (c) fidaxomicin **3** (d) **6** (e) CCCP. DMSO was present at a final concentration of 1% v/v in all assay solutions.

Given the known effects of CCCP as a protonophoric uncoupler of oxidative phosphorylation,²⁸ it was of interest to examine human cell cytotoxicity as a preliminary indicator of druggability (or otherwise) in the diarylacylhydrazone class. Compounds **5** and **6** were examined alongside miconazole (positive control), metronidazole **1**, vancomycin **2** and CCCP for cytotoxicity against human FaDu and Hep G2 cells using a standard 96-well resazurin-based cell viability assay (Supporting Information). The positive control miconazole showed the expected cytotoxic concentration (50 µg/mL) against both cell lines. Metronidazole **1** and vancomycin **2** showed no toxicity at or below 200 µg/mL against either cell line. Compounds **5** and **6** showed no cytotoxicity against either cell line at 100 µg/mL while CCCP was found to be toxic at 50 µg/mL (Figure 6).

Compound	Cytotoxic conc. (µg/mL)	
	FaDu	HepG2
Miconazole	50	50
Metronidazole		
1	>200	>200
Vancomycin		
2	>200	>200
5	>100	>100
6	>100	>100
CCCP	50	50

DMSO showed no cytotoxicity at 2% (v/v).

Figure 6. Cytotoxicity against human FaDu and HepG2 cells.

This study demonstrates that certain diarylacylhydrazones are narrow-spectrum antibacterials with selectivity for *C. difficile* and *C. perfringens* over other gut commensals. The demonstrated structure-dependence of the selectivity is significant because other diarylacylhydrazones have previously been shown to have a wider spectrum of antibacterial activity.²⁹ The activity of **6** against stationary-phase *C. difficile* cells and its low human cell cytotoxicity indicate that further investigations with the class are warranted towards creating cost-effective antibiotics for CDI that may reduce treatment failure and relapse rates. Identifying that CCCP shows similar activity to diarylacylhydrazones against the gut panel and against stationary phase *C. difficile* cells suggests that a protonophoric mechanism may play a role in the selectivity, although many questions remain. One avenue we are exploring is the possible involvement of dynamin-like proteins, which are important mediators of membrane remodelling in bacteria (called dynamins in eukaryotes).³⁰ Inhibition of dynamins by diarylacylhydrazones is well characterised³¹ and compounds structurally very similar to those reported here were recently shown to prevent uptake of *C. difficile* TcdA into eukaryotic cells.³²

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