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Identification of *N*-(5-Nitrothiazol-2-yl)-3-oxopyrazolidine-4-carboxamide Derivative as a Potent Inhibitor of *Clostridioides difficile*

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Abstract: The persistent need to introduce novel antibacterial agents remains a challenging predicament facing the drug development industry due to the major problem of bacterial resistance. Herein, we report the identification of a new *N*-(5-nitrothiazol-2-yl)-3-oxopyrazolidine-4-carboxamide derivative (**RW18**) as a potent inhibitor of *Clostridioides difficile*. *C. difficile* is a spore-forming, gram-positive bacterium that is announced as an urgent threat by the CDC. It is the principal cause of nosocomial diarrhea ranging from mild diarrhea to lethal colitis, spreading worldwide with the lack of many treatment alternatives. Altogether, *C. difficile* is ranked as an alarming concern to the health system. **RW18** was discovered during our synthetic efforts to discover MurA inhibitors targeting cell wall synthesis in bacteria. **RW18** exhibited significant inhibition of MurA, with IC_{50} value of 9.8 μM as well as demonstrated nearly the same inhibitory activity against MurA C115D, the mutant version established by resistant *E. coli* against fosfomycin. Notably, **RW18** displayed potent activity against several *Clostridioides difficile* clinical isolates with MIC values ranging between 0.125 and 1 μg/mL. It was found to possess bactericidal activity with MBC values 0.25 -1 μg/mL. When tested against the normal intestinal microbiota, **RW18** showed a significantly limited activity. In addition, when tested against Caco-2 cells, **RW18** showed a high safety index, indicating that it would not show harmful effects to the colon cells upon oral administration. Finally, the compound was highly stable in both LB culture medium and bacterial cell lysate. Overall, **RW18** presents a promising lead compound against *Clostridioides difficile.*

Keywords: MurA enzyme; bacterial resistance; peptidoglycan biosynthesis; 5-Nitrothiazole; *Clostridioides difficile*; Antimicrobial

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1. Introduction

Clostridioides difficile is an anaerobic, opportunistic bacterium and the infectious agent of *Clostridioides difficile* infection (CDI) [1]. The manifestations of the infection can array from a minor cases of diarrhea to a severe potentially life threatening complications such

as toxic megacolon [2]. In 2017 alone, *C. difficile* accounted for the loss of 12,800 infected patients in USA, imposing a substantial burden on the health sector compelling the United States Centers for Disease Control and Prevention (CDC) to classify *C. difficile* as a serious threat that require the rapid interference and management [1]. Furthermore, CDI is reflected as the primary cause of hospital acquired diarrhea worldwide as well as antibiotic concomitant diarrhea [3]. A major risk for CDI is the widespread use of antibiotics as they can disrupt the intestinal microbiota enabling the *C. difficile* colonization including the current repertoire therapy for CDI as vancomycin and fidaxomicin. Accordingly, vancomycin and fidaxomicin suffers from an increased risk of CDI recurrence accompanied with high treatment failure. Another candidate is metronidazole, once considered as the front line therapy for CDI, is now of restricted usage only in case no other options are available [4,5]. To add upon, *C. difficile* has developed antibiotic resistance hindering its eradication and clinical management [6]. Altogether, due to the emerging resistance, limited treatment options and high recurrence rates, dedicating efforts to introduce novel anti-CDI therapeutics have been fortified [5].

The present study is a part of our efforts on identifying novel MurA inhibitors targeting cell wall synthesis in bacteria in which compound **A** served as a lead for further optimization affording **RW18** (5-nitrothiazolyl pyrazolidinone derivative) after structural variations at position 4 of the pyrazolidinone scaffold (Figure 1). **RW18** showed significant MurA inhibitory activity, with IC_{50} value of 9.8 μM as well as exhibited equipotent inhibitory activity against MurA C115D mutant.

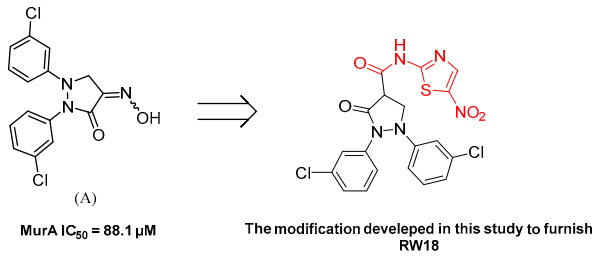


Figure 1. Optimization of compound (**A**) to obtain **RW18.**

In this report, we describe the discovery of **RW18**, obtained amongst our devoted efforts to develop MurA inhibitors, as a potent inhibitor of *C. difficile.* **RW18** was evaluated for its activity against a comprehensive panel of *C. difficile* clinical isolates and based on the obtained promising results, we investigated its influence on normal intestinal microbiota versus the known standard care antibiotics and studied its cytotoxicity against (Caco-2) cells.

2. Methods

2.1. Biology

2.1.1. MIC Determination against *S. aureus* (D2C NCTC 10833)

MIC values for *S. aureus* strain were measured as mentioned in Ref [7] with different RW18 concentrations (1.25 to 40 μM).

2.1.2. Determination of the MICs of RW18 against DIVERSE group of Gram-Positive Bacteria

The broth microdilution method was applied to ascertain the MICs of **RW18** against a panel of selected bacterial strains as described in Ref [8].

2.1.3. MICs and MBCs of RW18 against Diverse Range of *C. difficile* Strains

The MICs of **RW18** against *C. difficile* clinical isolates were measured, adopting the broth microdilution method. Briefly, in brain heart infusion supplemented (BHIS) broth,

a 0.5 McFarland solution of *C. difficile* was prepared to desired dilution (an inoculum size ~5 × 10^5 CFU/mL). Incubation of the prepared serial dilution of **RW18** with the bacteria for 48 h at 37 °C was performed, anaerobically, before MICs reading. MBC of **RW18** was attained by detecting the wells on BHIS agar plates that show no signs of growth.

2.1.4. Assessment of RW18 for Potential In Vitro Cytotoxicity against Caco-2 Cells

RW18 was evaluated for its potential cytotoxicity against (Caco-2) cell line, as reported in Ref [8].

2.1.5. Activity of RW18 against the Normal Intestinal Microbiota

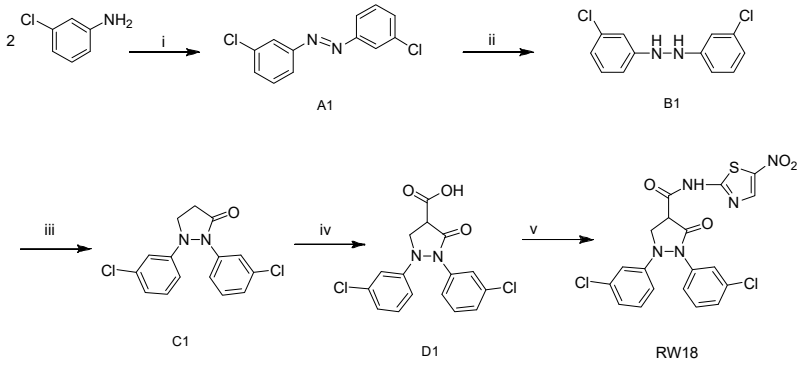
Briefly, a 5 × 10^5 CFU/mL bacterial concentration was attained in a bacterial solution that is equivalent to 0.5 McFarland standard either in BHIS broth or in MRS broth. Before MICs measurement, serial prepared dilutions of **RW18** were added afterwards with bacteria for incubation.

2.1.6. Stability in Bacterial Cell Lysate and in Culture Medium and

RW18's stability was assayed as described in Ref [9].

3. Results and discussion:

3.1. Chemistry. RW18 Was synthesized ACCORDING to the Steps Outlined in the Below Scheme



Scheme 1. Reagents used and conditions. (i) Cu(I)Br, toluene, pyridine, left to reflux for 24 h; (ii) Zn dust, saturated aq. NH_4Cl in acetone for 5 h at RT; (iii) acetone, K_2CO_3 then subsequent addition of 3-chloropropionyl chloride, left for 3 days at RT; (iv) 10 equiv of LDA in dry THF followed by excess CO_2, kept at −78 °C for 1 h then RT, HCl; (v) DMAP, intended amine in DCM, last EDC is added, left at RT overnight.

3.2. Biological Evaluation

3.2.1. Antibacterial Activity against Gram-Positive Bacteria

RW18 was tested against *S. aureus* (D2C NCTC 10833) and demonstrated a MIC value of 10 μM highlighting potent activity against the tested *S. aureus*.

Then, screening of **RW18's** antibacterial activity was investigated against numerous clinically important Gram-positive bacteria exhibiting multidrug-resistance. **RW18** was evaluated against methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), *Streptococcus pneumoniae,* vancomycin-resistant *Enterococcus faecalis* (VRE), vancomycin-resistant *S. aureus* (VRSA) as well as *C. difficile.* Remarkably, **RW18** displayed significant activity against *C. difficile (*MIC 0.25 μg/ mL) that notably surpassed the drug of choice, vancomycin. Thus, prompting us to screen **RW18s'** antibacterial activity against different clinical strains of *C. difficile* (Table 1).

Table 1. RW18 measured MICs in μg/ mL against important clinical Gram-positive bacterial strains.

Compound/Control Antibiotics	MSSA NRS107	MRSA NRS119	MRSA USA300	VRSA 10	*S. pneumoniae* ATCC 700677	*E. faecalis* ATCC 51299	*C. difficile* ATCC BAA 1870
RW18	2	1	4	4	4	>64	0.25
Linezolid	0.5	32	1	0.5	1	1	1
Vancomycin	1	1	1	32	2	64	1

3.2.2. Comprehensive Antibacterial Profile of RW18 against Numerous Clinical Strains of *C. difficile*

After the initial screening, the antibacterial profile **of RW18** against a panel of *C. difficile* strains was evaluated. **RW18** elicited high potency against the targeted *C. difficile* strains (MIC values 0.125–1 μg/ mL). (Table 2) **RW18** presented closely comparable activity to vancomycin, the standard therapeutic drug for CDI or even presented slightly improved activity against some of the tested strains. Of note, **RW18** and the reported anticlostridial agents as nitazoxanide and related analogs share the nitrothiazole motif, which accounts for their action. Thus, the high potency of **RW18** observed against the tested *C. difficile* strains may be therefore attributed to this effect along with MurA inhibition.

Table 2. MICs (μg/mL) of **RW18** against clinical isolates of *C. difficile.*

C. difficile strains	Compounds/Control Antibiotics			*C. difficile* strains	Compounds/Control Antibiotics		
	RW18	**Vancomycin**	**Fidaxomicin**		**RW18**	**Vancomycin**	**Fidaxomicin**
NR-32895	0.5	2	0.03	NR-49283	0.5	0.5	0.06
NR-32889	0.5	2	0.03	NR-49284	0.5	0.5	0.06
NR-32903	0.5	0.5	0.06	NR-49285	0.5	0.5	0.06
NR-32904	0.5	0.5	0.06	NR-49286	0.5	0.25	0.125
NR-13427	0.5	2	0.06	NR-49287	0.5	1	0.125
NR-13436	0.5	0.5	0.06	NR-49288	0.25	0.5	0.03
NR-49277	0.5	0.5	0.06	NR-49291	1	0.5	0.125
NR-49278	0.5	0.5	0.06	NR-49294	0.5	0.25	0.06
NR-49279	0.25	0.25	0.125	ATCC 43255	0.125	0.5	0.03
NR-49282	0.5	0.25	0.06	HM-88	0.5	0.5	0.015
NR-32895	0.5	2	0.03	NR-49283	0.5	0.5	0.06
NR-32889	0.5	2	0.03	NR-49284	0.5	0.5	0.06

3.2.3. Minimum Bactericidal Concentrations

MBCs assay was performed to determine whether **RW18** retains a bactericidal or bacteriostatic activity against *C. difficile*. The assay was performed against 6 representative strains of the tested isolates. As compared to the 6 tested isolates MICs' values, MBC values determined were as equal or 2 times higher than their corresponding MIC values (Table 3) signifying that **RW18** retains bactericidal activity against *C. difficile* parallel to the bactericidal activity exerted by the standard anticlostridial agents, vancomycin and fidaxomicin.

Table 3. MBCs (μg/mL) of **RW18** against clinical isolates of *C. difficile.*

C. difficile Strains	Compounds/Control Antibiotics			*C. difficile* Strains	Compounds/Control Antibiotics		
	RW18	**Vancomycin**	**Fidaxomicin**		**RW18**	**Vancomycin**	**Fidaxomicin**
NR-32895	1	2	0.06	NR-49284	0.5	0.5	0.06
NR-32904	1	0.5	0.06	ATCC BAA 1870	0.25	2	0.25

NR-49278	0.5	0.5	0.125	ATCC 43255	0.25	1	0.06

3.2.4. Activity of RW18 against the Human Gut Microbiota

One of the restrictions of antibiotics administration, particularly broad-spectrum agents, is the disruption of the normal gut microbial community composition facilitating the road for opportunistic pathogens as vancomycin resistant *Enterococci* (VRE) and *C. difficile* to colonize the gut [10,11]. Accordingly, it was compelling to evaluate the impact of **RW18** on representative bacteria that comprise the intestinal microbiota. Consequently, we proceeded to test the antibacterial activity of **RW18** against commensal organisms such as species of *Bacteroides, Lactobacillus* and *Bifidobacterium*. As shown in Table 4, the antibacterial activity of **RW18** against the tested strains of *B. fragilis* was relatively modest (MICs range from 4 to 8 μg/mL) compared to the limited activity of vancomycin and fidaxomicin.

Activity of **RW18** against *Bifidobacterium* species was limited (MICs 32-64 μg/mL) (excluding activity against *B. longum* HM 846). However, standard anticlostridial agents, vancomycin and fidaxomicin, furnished potent activity against *Bifidobacterium spp.* Moreover, **RW18** did not inhibit the growth of *Lactobacillus spp* at any tested concentration up to the highest concentration tested of 64 μg/mL. Whereas, vancomycin and fidaxomicin, inhibited the *Lactobacillus* tested strains at much reduced concentrations (0.5 to 4 μg/mL). Compared with vancomycin and fidaxomicin, the reference antibiotics, **RW18** exhibits restricted activity against the tested normal gut microbiota.

Table 4. RW18 measured MICs in μg/mL against chosen representatives of the normal gut microbiota.

Bacterial Strains	Compounds/Control Antibiotics			Bacterial Strains	Compounds/Control Antibiotics		
	RW18	**Vancomycin**	**Fidaxomicin**		**RW18**	**Vancomycin**	**Fidaxomicin**
B. fragilis HM 20	4	64	>64	*B. breve HM 1120*	64	1	≤0.06
B. fragilis HM 709	4	64	>64	*B. angulatum HM 1189*	32	1	≤0.06
B. fragilis HM 718	8	>64	>64	*L. gasseri HM 403*	>64	2	1
B. fragilis HM 719	8	>64	>64	*L. gasseri HM 407*	>64	4	1
B. breve HM 412	64	1	≤0.06	*L. crispatus HM 103*	>64	1	0.5
B. longum HM 846	16	0.5	≤0.06	*L. crispatus HM 420*	>64	1	2

3.2.5. In Vitro Cytotoxicity Assessment of RW18

Selectivity to prokaryotic cell is a crucial asset for prospective antibiotic therapeutics. Therefore, we investigated **RW18** toxicity against mammalian cells. **RW18** presented outstanding tolerability to human colorectal adenocarcinoma (Caco-2) after exposure for 24 hours at high concentrations as 64 μg/mL (Figure 2). Compared to the compound's MICs against *C. difficile*, the obtained concentration were 64 to 512 folds higher highlighting that **RW18** has high tolerability and potential safety to the colon cells upon oral administration and to mammalian cells in general.

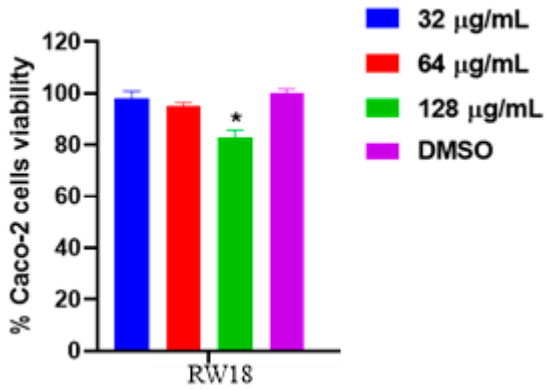


Figure 2. In vitro cytotoxicity of **RW18** was assessed against Caco-2 cells. **RW18** was tested as triplicates at 3 concentrations (32, 64, and 128 µg/mL). Results are shown as viable cells percent relative to cells treated with DMSO, with error bars presenting the values of standard deviation. Statistical analysis was performed using a two-way ANOVA with post hoc Dunnett's test for multiple comparisons. Obtained statistical difference ($p < 0.05$) between each test agent values compared to the DMSO-treated cells is denoted by an asterisk (*).

3.2.6. Stability Assessment of RW18 in Both Culture Medium and Bacterial Cell Lysate

The stability of **RW18** in LB culture medium and bacterial cell lysate (*S. aureus Newman*) was evaluated using LC-MS/MS. Succeeding incubation of **RW18** at 37 °C overnight, **RW18** persisted unchanged highlighting its stability. This finding demonstrates that its antibacterial activity is likely due to its intact form.

4. Conclusions

An increasingly growing need nowadays is to develop new chemical entities to overcome the major burden of antibiotic resistant bacteria, among which is *C. Difficile*, the leading causative agent of nosocomial diarrhea. This antibiotic resistance of *C. difficile* in addition to its increasing rates of recurrence have made CDI as a global challenge, hindering its effective treatment. This study reports the discovery of novel 5-nitrothiazolyl pyrazolidinone derivative **(RW18)** as a highly potent inhibitor of *C. difficile* growth with MIC values ranging between 0.125- 1 µg/mL. The compound is also stable in LB culture medium in addition to bacterial cell lysate and revealed confined activity when assessed against human normal gut microbiota. Taken together, **RW18** presents a prominent lead for the development *C. difficile* therapeutics.

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