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Ultrapotent Inhibitor of *Clostridioides difficile* Growth, which Suppresses Recurrence *in vivo*

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Abstract:

Clostridioides difficile (*C. difficile*) is the leading cause of healthcare-associated infection in the U.S. and considered an urgent threat by the Centers for Disease Control and Prevention (CDC). Only two antibiotics, vancomycin and fidaxomicin, are FDA-approved for the treatment of *C. difficile* infection (CDI) but these therapies still suffer from high treatment failure and recurrence. Therefore, new chemical entities to treat CDI are needed. Trifluoromethylthio containing *N*-(1,3,4-oxadiazol-2-yl)benzamides displayed very potent activities (sub- μ g/mL minimum inhibitory concentration (MIC) values) against Gram-positive bacteria. Here, we report remarkable antibacterial activity enhancement via halogen substitutions, which afforded new anti-*C. difficile* agents with ultrapotent activities (MICs as low as 0.003 μ g/mL (0.007 μ M)) that surpassed the activity of vancomycin against *C. difficile* clinical isolates. The most promising compound in the series, **HSGN-218** protected mice from CDI recurrence. Not only does this work provide a potential clinical lead for the development of *C. difficile* therapeutics but also it highlights dramatic drug potency enhancement via halogen substitution.

Introduction:

Clostridioides difficile (*C. difficile*) is a spore-forming Gram-positive anaerobic bacterium and the leading cause of nosocomial infections as well as antibiotic-associated diarrhea in the United States¹. In 2017, the Centers for Disease Control and Prevention (CDC) determined that in the U.S., 223,900 patients were hospitalized with *C. difficile* infection (CDI), resulting in 12,800 deaths and more than \$1 billion in healthcare costs². CDI causes severe diarrhea along with life-threatening complications such as toxic megacolon, pseudomembranous colitis, and systemic inflammatory response syndrome³. Manifestations of the disease are

credited to the toxin-mediated damage produced by two major toxins: toxin A (TcdA/enterotoxin) and toxin B (TcdB/cytotoxin), which catalyze the inactivation of Rho GTPases, ultimately causing intense inflammation of the gut, accompanied by necrosis and apoptosis of colonic mucosal cells⁴⁻⁵. Furthermore, *C. difficile*'s ability to produce spores hinders the clinical management of CDI because these spores are very resistant to environmental conditions, antibiotics, and disinfection processes. *C. difficile* spores can spread throughout the environment and once ingested by vulnerable hosts, they develop into vegetative cells that colonize the intestines, thereby producing toxins and establishing infection⁶⁻⁷. Therefore, *C. difficile* spores serve as the major cause of CDI circulation and recurrence.

CDI is typically caused from the use of antibiotics, which disrupts the reproduction of normal and protective gut microbiota, ultimately allowing C. difficile to grow in the colon and produce infectious toxins⁸. Although the overuse of antibiotics is one of the main reasons contributing to CDI, the management of CDI requires antibiotic treatment. Currently, there are only three drugs used to treat CDI: metronidazole, vancomycin, and fidaxomicin. Yet only vancomycin and fidaxomicin are approved by the FDA for treatment of CDI. Although, metronidazole was previously recommended as a first-line therapy for CDI, its use is now only limited to non-severe CDI cases when patients are unable to be treated with vancomycin or fidaxomicin⁹. Moreover, other limitations with metronidazole treatment are its potent activity against a wide spectrum of protective normal microbiota, as well as its high absorption (100% bioavailable) from the intestinal tract, restricting its concentrations in the colon¹⁰⁻¹¹. Although oral vancomycin is minimally absorbed into the systemic circulation¹², it has broad spectrum activity against Gram-positive bacteria, leading to a reduction in microbiome diversity¹³. Furthermore, both vancomycin and metronidazole treatments are inadequate due to high treatment failure (14% with vancomycin and 22% with metronidazole) and high recurrence rates (25% to 30%). This is because both antibiotics are ineffective against spores and also they cause disruption of the beneficial gut microbiota¹⁴⁻¹⁵. Fidaxomicin is the only new drug approved for CDI in the last 30 years. Fidaxomicin has lower recurrence rates compared to vancomycin and metronidazole because of its selectivity towards C. difficile; however, its high cost limits its use¹⁶⁻¹⁸. Even though vancomycin and fidaxomicin are FDA-approved therapies for CDI, emerging resistance or reduced susceptibility are evident to these antibiotics^{17, 19}. In addition, one emerging alternative non-antibiotic therapy for CDI is fecal microbiota transplant (FMT), which restores the disrupted normal microbiome, leading to renovation of the colonization resistance to C. difficile²⁰. While FMT appeared to be successful in the treatment of some CDI cases, it has many restrictions and poses a serious risk of transmitting infectious pathogens to the patients; especially immunocompromised and elderly patients²¹⁻²². Therefore, due to the increase in treatment failure and recurrence rates with the commonly used anti-CDI drugs, along with growths of CDI, efforts to develop novel anti-CDI therapeutics have intensified²³.

Our program focuses on the discovery of new *N*-(1,3,4-oxadiazol-2-yl)benzamides to combat the urgent threats of antibiotic-resistant bacteria²⁴⁻²⁵. We previously reported the trifluoromethylthio-containing (1,3,4-oxadiazol-2-yl)benzamide, **compound 12**, as a potent anti-MRSA agent²⁶. **Compound 12** was found to have bactericidal activity as well as being non-toxic to mammalian cells²⁶. **Compound 12** was however not evaluated *in vivo* as it was not deemed an

 ideal lead due to the presence of a potential thiophene toxicophore (Figure 1). In this report, we describe the generation of a new series of trifluoromethylthio containing (1,3,4-oxadiazol-2yl)benzamides, which leads to the identification of N-(5-(3,5-dichlorophenyl)-1,3,4-oxadiazol-2yl)-4-((trifluoromethyl)thio)benzamide (HSGN-218), which does not contain a thiophene (Figure 1). HSGN-218 was tested for its activity against a panel of clinical pathogenic C. difficile strains. Cytotoxicity against mammalian cells, bi-directional Caco-2 permeability and activity against normal gut microbiota were also investigated. Moreover, the activity of HSGN- treatment was evaluated in an *in vivo* CDI mouse model and its ability to prevent C. *difficile* recurrence in vivo was also investigated.



Figure 1. Compound 12 contains a potential thiophene toxicophore but was found to be potent against C. difficile. Utilization of halogen substitution led to the discovery of an ultrapotent anti-C. difficile agent (HSGN-218) with a 70-times imporvement in potency (from 0.5 μ g/mL (1.4 μ M) to 0.007 μ g/mL (0.02 μM).

Results and Discussion:

Halogenation, a High-Level Medicinal Chemistry Design Strategy

Halogens (X = F, Cl, Br, and I) are commonly used substituents in medicinal chemistry and drug discovery²⁷⁻³⁰. For instance, around 40% of the drugs currently FDA-approved or in clinical trials are halogenated and about 25% of the published medicinal chemistry papers and patents contain the late stage addition of halogen atoms²⁸. Likewise, 35% of the top-15 selling drugs from 2010 to 2016 are halogenated³¹ (Figure 2A). Of the halogenated drugs, 57% contain fluorine, 38% contain chlorine, 4% contain bromine, and only 1% contain iodine²⁸. The addition of halogen substitutents has been shown to have a major effect on a drug's potency and pharmacological properties. Regarding pharmacological properties, addition of halogen substituents to lead compounds has been shown to increase lipophilicity, permeability, membrane binding and metabolic stability³²⁻³³. Likewise, insertion of halogen atoms into leadlike compounds also showed enhanced drug metabolism because the carbon-halogen bond is not easily metabolized by cytochrome P450²⁸. Concerning potency, halogen atom substitution's effect has been documented. For example, L86-8276, a cyclin-dependent kinase 2 (CDK2) inhibitor was shown to have an IC₅₀ value of 2.4 μ M (**Figure 2B**)³⁴. Yet, the addition of a chlorophenyl group to give **Flavopiridol** showed a six-fold improvement in potency to give an IC₅₀ of 0.4 μ M against CDK2 (**Figure 2B**)³⁴.



Figure 2. Importance of the addition of halogen substituents to lead compounds. **A.** Examples of the top-15 selling drugs that are halogenated. **B.** Addition of chlorophenyl to CDK2 inhibitors led to a six-fold enhancement in potency.

Synthesis and Anti-*C. difficile* Activity of Trifluoromethylthio Containing (1,3,4-oxadiazol-2-yl)Benzamides

We previously reported that **compound 12** was potent against a panel of clinically important Gram-positive bacteria²⁶. Based on its broad-spectrum Gram-positive activity, we wondered if it would be active against *C. difficile*. **Compound 12** inhibited *C. difficile* ATCC BAA 1801 with an MIC of 0.5 μ g/mL (1.4 μ M) (see **Figure 1** and **Table 1**), which is comparable to vancomycin. However, **compound 12** contains an unsubstituted thiophene moiety, which can lead to toxicity concerns (**Figure 1**). For instance, thiophene metabolism, caused by cytochrome P450 mediated oxidation, can lead to the formation of reactive metabolites, thiophene-S oxides³⁵⁻³⁶, thiophene epoxides³⁶, and sulphenic acids³⁷, which have a high propensity to react with nucleophiles such as water and glutathione³⁸. We were however encouraged that **compound 12** showed good activity against *C. difficile*, so we proceeded to make new analogs, which did not contain thiophene but instead substituted phenyl groups.





*Reagents and Conditions: (a) Semicarbazide hydrochloride, NaOAc, MeOH:H₂O (1:1), rt, 30 min, 95% (b) Bromine, NaOAc, AcOH, 60 °C, 1 h, 40% - 70% (c) BOP Reagent, DIPEA, DMF, rt, 12 h, 16% - 33%.

In our previous report²⁶, we determined that the 4-(trifluoromethylthio)phenyl group is vital for optimal activity so we kept this constant. The synthesis of the compounds began with a substituted benzaldehyde followed by the addition of semicarbazide and sodium acetate to give the corresponding semicarbazone. Then, using bromine and sodium acetate, the semicarbazone was cyclized into the subsequent 1,3,4-oxadizol-2-amine (**Scheme 1**). Amide coupling between the 1,3,4-oxadiazol-2-amine and 4-trifluoromethylthio benzoic acid using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent gave the desired trifluoromethylthio containing (1,3,4-oxadiazol-2-yl)benzamides (**Scheme 1**).

With the compounds in hand (see **Table 1**), we proceeded to evaluate them against *C*. *difficile*. Halogen substitutions (especially the Cl, F or CF₃ groups) resulted in the most active compounds. Substitution with OMe, Me, and *i*-propy groups showed only moderate to no activity (see **Table 1** for MICs of compounds **6**, **11**, **12**, and **13** against *C*. *difficile* ATCC BAA 1801). For halogen substituents, the position on the phenyl ring was also important. For example, the MIC for *meta*-Cl (**5**) was 0.03 µg/mL (0.08 µM), whereas that for the *ortho*- (**3**) and *para*- (**9**) analogs were 4 µg/mL (10.0 µM) and 2 µg/mL (5.0 µM) respectively against *C*. *difficile* ATCC BAA 1801 (**Table 1**). Additionally, for di-substituted halogen containing compounds, the position of the halogens affected activity. For instance, the 3,5-dichlorophenyl analog (**15**, **HSGN-218**) was more than four times more potent than the 2,4-dichlorophenyl (**14**) analog (MIC = 0.06 µg/mL and 0.007 µg/mL for **14** and **15** respectively). We also proceeded to investigate subsitution of the phenyl group with heteroaromatics, such as pyridinyl (**17**) which had only moderate activity (**Table 1**) allowing us to conclude that the phenyl ring is needed for optimal activity.

Table 1. MICs in μ g/mL (μ M) of HSGN-218, analogs, and vancomycin, against *C. difficle* ATCC BAA 1801.



Compound/Control	- R ₁	-R ₂	- R ₃	-R ₄	X	MICs in
Antibiotic						μg/mL
						(μΜ)
Compound 12	-	-	-	-	-	0.5 (1.4)
1	Н	Н	Н	Н	СН	2 (5.5)
2	F	Н	Н	Н	СН	4 (10.4)
3	Cl	Н	Н	Н	СН	4 (10.0)
4	Н	F	Н	Н	СН	0.03
						(0.08)
5	Н	Cl	Н	Н	СН	0.03
						(0.08)
6	Н	OMe	Н	Н	СН	4 (10.1)
7	Н	CF ₃	Н	Н	СН	0.015
						(0.04)
8	Н	Н	F	H	СН	4 (10.4)
9	Н	Н	Cl	Н	СН	2 (5.0)
10	Н	H	CF ₃	Н	СН	0.125
						(0.29)
11	Н	Н	OMe	Н	СН	4 (10.1)
12	Н	Н	CH ₃	Н	СН	4 (10.5)
13	Н	Н	<i>i</i> -Propyl	Н	СН	128
						(314.2)
14	Cl	H	Cl	Н	СН	0.03
						(0.07)
15; HSGN-218	Н	Cl	Н	Cl	СН	0.007
						(0.02)
16	OMe	Н	Н	Cl	СН	2 (4.7)
17	Н	Н	Н	Н	N	8 (21.8)
Vancomycin	-	-	-	-	-	1 (0.7)
Metronidazole	-	-	-	-	-	0.25
						(1.46)
Fidaxomicin	-	-	-	-	-	0.06
						(0.06)

Comprehensive antibacterial profile of HSGN-218 against various *C. difficile* clinical isolates

After the initial screening of **HSGN-218**, we assessed its antibacterial profile against a panel of *C. difficile* clinical isolates. As depicted in **Table 2**, **HSGN-218** exhibited exceptional actvity against *C. difficile* clinical isolates with MICs ranging from 0.003 μ g/mL (0.007 μ M) to 0.03 μ g/mL (0.07 μ M). Vancomycin displayed MICs ranging from 0.25 μ g/mL (0.2 μ M) to 1 μ g/mL (0.7 μ M) against all the tested strains (**Table 2**). With regard to micromolar concentrations, **HSGN-218** is between 2.5 to 100 times more potent than vancomycin in inhibiting clinically relevant *C. difficile* growth *in vitro*. Metronidazole inhibited the growth of the tested *C. difficile* strains at concentrations ranging from 0.125 μ g/mL (0.7 μ M) to 0.25 μ g/mL (1.46 μ M). Fidaxomicin displayed MIC values ranging from 0.015 μ g/mL (0.01 μ M) to 0.06 μ g/mL (0.06 μ M).

Table 2. MICs in μ g/mL (μ M) of **HSGN-218** and vancomycin against various *C. difficile* clinical isolates.

Compound /Control Antibiotic	C. difficile NR- 13432 (isolate 6)	C. difficile NR- 13435 (isolate 9)	C. difficile NR- 32883 (P2)	C. difficile NR- 32891 (P13)	C. difficile NR- 32895 (P19)	C. difficile NR- 32904 (P30)	C. difficile ATCC 43255
HSGN-218	0.03	0.003	0.007	0.007	0.007	0.007	0.015
	(0.07)	(0.007)	(0.02)	(0.02)	(0.02)	(0.02)	(0.04)
Vancomycin	0.25 (0.2)	1 (0.7)	0.5 (0.4)	0.5 (0.4)	1 (0.7)	1 (0.7)	1 (0.7)
Metronidazole	0.25	0.125	0.125	0.125	0.25	0.25	0.25
	(1.46)	(0.7)	(0.7)	(0.7)	(1.46)	(1.46)	(1.46)
Fidaxomicin	0.06	0.06	0.03	0.015	0.03	0.015	0.015
	(0.06)	(0.06)	(0.03)	(0.01)	(0.03)	(0.01)	(0.01)

Antibacterial profile of HSGN-218 against vancomycin-resistant enterococci and Gramnegative bacteria

Next, the antibacterial activity of **HSGN-218** was assessed against vancomycin-resistant enterococci (VRE) and *Escherichia coli* that are highly common bacteria in the gut. The overgrowth of VRE and colonization of the gut are one of the major issues associated with the vancomycin and metronidazole treatment of CDI³⁹⁻⁴⁰. Thus, anticlostridial agents capable of inhibiting the growth of VRE are highly desirable. On the other hand, *E. coli* is the predominant aerobic bacteria colonizing in the gut which remains resident throughout the life of the host⁴¹. As depicted in **Table 3**, **HSGN-218** exhibited potent activity against VRE clinical isolates with MICs ranging from 0.06 μ g/mL (0.14 μ M) to 0.125 μ g/mL (0.29 μ M) outperforming vancomycin and metronidazole. When tested against *E. Coli*, **HSGN-218** was found to be inactive against *E. coli* BW25113 (wild-type strain). Conversely, the compound showed moderate activity (MIC = 4 μ g/mL (9.2 μ M)) against *E. coli* JW55031 which is deficient in AcrAB-TolC efflux pump. Thus, the lack of activity against the wild-type *E. coli* could be attributed to that **HSGN-218** may be a substrate for AcrAB-TolC efflux pump.

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Table 3. MICs in μ g/mL (μ M) of **HSGN-218** and control antibiotics against vancomycinresistant enterococci (VRE) and *Escherichia coli* isolates.

Compound /Control Antibiotic	<i>E. faecium</i> ATCC 700221	<i>E. faecalis</i> ATCC 51299	<i>E. coli</i> JW55031 (TolC Mutant	<i>E. coli</i> BW25113 (wild-type strain)
HSGN-218	0.125 (0.29)	0.06 (0.14)	4 (9.2)	>16 (>36.8)
Vancomycin	32 (22.1)	>64 (>44.2)	>64 (>44.2)	>64 (>44.2)
Metronidazole	>64 (>373.9)	>64 (>373.9)	NT ¹	NT
Linezolid	1 (3.0)	1 (3.0)	16 (47.4)	>64 (>189,7)
Gentamicin	NT	NT	0.25 (0.52)	0.25 (0.52)

NT¹, not tested

HSGN-218 is highly tolerable to human cell lines

Prokaryotic cell selectivity is a vital attribute for any antibiotic candidate. Thus, **HSGN-218** was assessed for toxicity to mammalian cells. **HSGN-218** showed an excellent safety profile against human colorectal cells (Caco-2) (**Figure 3**). It was highly tolerable to Caco-2 cells at concentrations higher than 64 μ g/mL. This concentration is more than 9,000-times higher than the compound's corresponding MIC value against *C. difficile* ATCC BAA 1801 used in the initial screening.



Figure 3. *In vitro* cytotoxicity assessment of **HSGN-218** (tested in triplicate) against human colorectal cells (Caco-2) using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Results are presented as percent viable cells relative to DMSO (negative control). Error bars represent standard deviation values. A one-way ANOVA, with post hoc Dunnet's multiple comparisons test, determined statistical difference between the values obtained for the compound and DMSO (denoted by the asterisk) (P < 0.05).

HSGN-218 demonstrates low Caco-2 permeability:

In order to treat CDI, it's vital that a compound does not cross the gastrointestinal tract but instead stays localized in the gut. Thus, we assessed whether **HSGN-218** would permeate across the gastrointestinal tract via a Caco-2 bidirectional permeability assay⁴². The assay

(performed as a service at Eurofins Panlabs (MO, USA) demonstrated that **HSGN-218** showed limited ability to permeate across Caco-2 bilayers ($P_{app} = 0.2 \times 10^{-6} \text{ cm s}^{-1}$ from the apical to basolateral and $P_{app} = 0.1 \times 10^{-6} \text{ cm s}^{-1}$ from the basolateral to apical, see **Table 4**). This permeability is comparable to rinitidine ($P_{app} = 0.5 \times 10^{-6} \text{ cm s}^{-1}$ from the apical to basolateral and $P_{app} = 1.3 \times 10^{-6} \text{ cm s}^{-1}$ from the basolateral to apical, see **Table 4**), a drug that is known to have low permeability across Caco-2 bilayers. Propranolol was used as a high permeability control as its $P_{app} = 37.2 \times 10^{-6} \text{ cm s}^{-1}$ from the apical to basolateral and $P_{app} = 22.7 \times 10^{-6} \text{ cm s}^{-1}$ from the basolateral to apical (**Table 4**). Therefore, the Caco-2 permeability results indicate that **HSGN-218** will not cross the gastrointestinal tract and instead concentrate in the gut, the site for *C. difficile* infections.

Compound/Control Drug	Mean A \rightarrow B P _{app}	Mean $B \rightarrow A P_{app}$	Notes
	(cm s ⁻¹)	(cm s ⁻¹)	
HSGN-218	0.2 x 10 ⁻⁶	0.1 x 10 ⁻⁶	Low Permeability
Ranitidine	0.5 x 10 ⁻⁶	1.3 x 10 ⁻⁶	Low Permeability
			Control
Propranolol	37.2 x 10 ⁻⁶	22.7 x 10 ⁻⁶	High Permeability
			Control

Table 4. Caco-2 Permeability Analysis for HSGN-218 and Control Drugs.

In vitro antibacterial evaluation of HSGN-218 against normal microflora.

Antibiotics administration (especially broad-spectrum ones) causes alteration of the normal intestinal microbial composition, resulting in gut colonization by opportunistic pathogens like C. difficile⁴³. Consequently, we investigated whether HSGN-218 has a deleterious effect on important representative members of the normal gut microbiota such as Lactobacillus spp and Bacteroides spp. Bacteroides spp comprise a large proportion of the intestinal microbiota, which were reported to contribute to bile acid-mediated inhibition of C. difficile and prevent CDI in mouse model⁴⁴⁻⁴⁵. Additionally, lactobacilli were reported to interfere with C. difficile both in vitro and in vivo⁴⁶⁻⁴⁷. As depicted in Table 5, HSGN-218 exhibited weak antibacterial activity against *Lactobacillus* strains (MIC = 16 μ g/mL (36.8 μ M)) and inhibited growth of species of Bacteroides (MIC=1-2 µg/mL (2.3-4.6 µM)). Similarly, vancomycin inhibited Lactobacillus strains (MICs = $\leq 1-2 \mu g/mL (0.7-1.4 \mu M)$) and exhibited weak activity against *Bacteroides* spp (MICs = $32-64 \ \mu g/mL$ (22.1-44.2 μ M)). Although **HSGN-218** was similar to vancomycin, the anti-CDI drug of choice, in inhibiting the growth of certain species of the normal microbiota, it must be noted that **HSGN-218** inhibits C. difficile at concentrations that are 100-times less than what is needed to inhibit *Bacteroides* (compare **Table 2** with **Table 5**). On the other hand, vancomycin inhibited both C. difficile and Lactobacillus strains with comparable MIC values of $1-2 \mu g/mL$. Metronidazole and fidaxomicin (to a lesser extent) also inhibit certain members of the normal intestinal microbiota⁴⁸⁻⁵⁰.

Bacterial strains	HSGN-218	Vancomycin	Metronidazole	Fidaxomicin
Lactobacillus	16 (36.8)	≤1 (≤0.7)	>64 (>373.9)	>64 (>60.5)
gasseri HM-400				
Lactobacillus	16 (36.8)	2 (1.4)	>64 (>373.9)	>64 (>60.5)
crispatus HM-103				
Lactobacillus	16 (36.8)	2 (1.4)	>64 (>373.9)	>64 (>60.5)
crispatus HM-371				
Bacteroides fragilis	2 (4.6)	64 (44.2)	≤1 (5.84)	>64 (>60.5)
HM-711				
Bacteroides fragilis	1 (2.3)	32 (22.1)	2 (11.68)	>64 (>60.5)
HM-709				
Bacteroides dorei	2 (4.6)	64 (44.2)	≤1 (5.84)	>64 (>60.5)
HM-719				

Table 5. MICs in $\mu g/mL$ (μM) of HSGN-218 and vancomycin against human normal gut microbiota

Frequency of mutation.

HSGN-218

Vancomycin

The promising results of **HSGN-218** led us to investigate the likelihood of *C. difficile* to develop resistance to HSGN-218. No resistant mutants were isolated at a concentration of $15 \times$ MIC and $20 \times MIC$ in the presence of a high inoculum of C. difficile (Table 6), indicating that C. difficle is unlikely to form rapid resistance to HSGN-218. Likewise, vancomycin exhibited low frequency of mutation ($<1.1 \times 10^{-9}$) and no resistant mutants were isolated, in agreement with a previous report⁵¹.

Table 6. Frequency of mutation of HSGN-218 against C. <i>alyficue</i> ATCC 43235			
Test agent	Frequency of mutation		
	$15 \times MIC$	$20 \times MIC$	

<1.1×10⁻⁹

<1.1×10-9

<1.1×10⁻⁹

<1.1×10-9

Table	6.	Frequency	of mu	tation	of HS	SGN	-218	against	С.	difficile	ATCC	43255

<i>In vivo</i> efficacy of HSGN-218 in a CDI mouse model ⁵²	

The potent antibacterial activities of HSGN-218 against C. difficile prompted us to investigate its efficacy in a CDI mouse model and its potential to protect mice from CDI recurrence, as described before. As shown in Figure 4, vancomycin (10 mg/kg) protected 100% of mice up to 5 days, as previously reported⁵³⁻⁵⁴. HSGN-218 (50 mg/kg), was able to significantly protect 66.7% of the mice against C. difficile during the 5-days treatment period.



Figure 4. *In vivo* efficacy of **HSGN-218** in a CDI mouse model. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) test. Asterisks (*) denote statistically significant difference between mice treated with either **HSGN-218**, or vancomycin in comparison with the vehicle-treated mice.

After testing the efficacy of **HSGN-218** in the CDI mouse model, we sought to investigate this promising activity of **HSGN-218** in preventing *C. difficile* recurrence. *C. difficile* recurrence is challenging to treat. In addition to the subsequent prolongation of *C. difficile* shedding and transmission, 1 out of every 5 patients experienced *C. difficile* recurrence episode died within 30 days of diagnosis⁵⁵. Therefore, we sought to investigate this promising activity of **HSGN-218** in preventing *C. difficile* recurrence. Mice were infected and treated for 5 days and then they were monitored for survival and possible *C. difficile* recurrence until the 21st day. Vancomycin-treated mice survived the first 5 days (similar to prior reports)⁵³, but in accordance with previous studies^{52, 54}, mice treated with vancomycin were susceptible to *C. difficile* recurrence and 83.3% of vancomycin-treated mice died after stopping vancomycin treatment. In contrast, **HSGN-218** (50 mg/kg), significantly protected mice from CDI recurrence with 100% survival after 5- days treatment period (**Figure 5**).



Figure 5. *In vivo* efficacy of **HSGN-218** against CDI recurrence. Mice were treated with **HSGN-218** (50 mg/kg), vancomycin (10 mg/kg) or the vehicle for 5 days and treatments were stopped thereafter. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) test. Asterisks (*) denote statistically significant difference between mice treated with either **HSGN-218**, or vancomycin in comparison with the vehicle-treated mice. Pound (#) denotes statistically significant difference between mice treated mice statistically significant difference between mice treated mice.

Conclusion:

In conclusion, we have identified **HSGN-218** as a highly potent small molecule inhibitor of *C. difficile* growth. **HSGN-218** is up to 100-times more active (MICs ranging from 0.003 μ g/mL (0.007 μ M) to 0.03 μ g/mL (0.07 μ M)) against *C. difficile* clinical isolates than vancomycin, the drug of choice for CDI. The compound is also non-toxic to mammalian cells as well as demonstrates low Caco-2 bidirectional permeability, indicating that **HSGN-218** would have minimal systemic absorption. Even though **HSGN-218** inhibited the growth of certain representative members of normal microbiota, excitingly, **HSGN-218** protected mice from CDI as well as it showed significant efficacy against *C. difficile* recurrence. Therefore, compound **HSGN-218** is considered as a lead compound to develop as anti- *C. difficile* therapeutic and deserves serious consideration.

Experimental Section:

Chemistry:

General Information: unless noted otherwise, all reagents and solvents were purchased from commercial sources and used as received. The ¹H, ¹³C, and ¹⁹F NMR spectra were obtained in DMSO- d_6 as solvent using a 500 MHz spectrometer with Me₄Si as an internal standard. Chemical shifts are reported in parts per million (δ) and are calibrated using residual undeuterated solvent as an internal reference. Data for ¹H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, or combinations thereof. High resolution mass spectra (HRMS) were obtained using electron spray ionization (ESI) technique and as TOF mass analyzer. Compounds were characterized by ¹H

NMR, ¹³C NMR, ¹⁹F NMR, and HRMS data. The purity of compounds was determined to be greater than 95% by measuring the absorbance at 260 nm with high performance liquid chromatography (HPLC) (See supprting information). HPLC spectra were recorded on an Agilent 1260 Infinity system using a ZORBAX RR Eclipse Plus C18 column. The mobile phase gradient went from 50% H_2O : 50% MeOH over 5 minutes and then 40% H_2O : 60% MeOH for 5 minutes, followed by 10% H_2O : 90% MeOH for 2 minutes and lastly 50% H_2O : 50% MeOH for 3 minutes at a 1 mL/min flow rate.

Synthesis of 1,3,4-oxadiazol-2-amines [I.1 – I.17]:

The synthesis of **I.1-I.17** was performed using a literature reported procedure⁵⁶. Obtained ¹H, ¹³C, and ¹⁹F spectra were in agreement with literature reported data.

Amide Coupling Procedure for the Synthesis of Compounds 1-17:

A 20 mL screw caped vial, charged with the corresponding acid (1 eq.), amine (1 eq.), BOP reagent (2.7 eq.) and diisopropylethylamine (1.5 mL) in DMF solvent (5 mL) was stirred at room temperature for 16 h. After completion, the reaction mixture was concentrated under reduced pressure, followed by flash column chromatography (hexanes:ethyl acetate 90:10 to 70:30) to give the desired product.

N-(5-Phenyl-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (1):

Off-white solid (46 mg, 28%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 8.0 – 7.9 (m, 2H), 7.9 (m, 2H), 7.6 (m, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.6, 161.1, 158.6, 136.0, 135.8, 132.2, 131.1 (q, *J* = 308.7 Hz), 130.1, 129.8, 128.6, 126.6, 123.8. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₆H₁₁F₃N₃O₂S [M + H]⁺ 366.0524, found 366.0522. Purity by HPLC was found to be 96%.

N-(5-(2-Fluorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (2):

Off-white solid (38 mg, 22%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.1 (m, 2H), 8.0 (td, *J* = 7.6, 1.8 Hz, 1H), 7.9 (m, 2H), 7.7 (tdd, *J* = 7.4, 5.1, 1.8 Hz, 1H), 7.5 – 7.4 (m, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.6, 160.6 (d, *J* = 257.0 Hz), 158.7, 157.7, 136.1, 135.7, 134.5 (d, *J* = 8.82 Hz), 131.1 (q, *J* = 308.7 Hz), 130.1, 129.7, 128.6, 125.8, 117.6 (d, *J* = 20.2 Hz), 112.2 (d, *J* = 11.3 Hz). ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F), -112.0 (d, *J* = 5.7 Hz, 1F). HRMS (ESI) m/z calcd for C₁₆H₁₀F₄N₃O₂S [M + H]⁺ 384.0430, found 384.0429. Purity by HPLC was found to be 96%.

N-(5-(2-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (3):

Off-white solid (41 mg, 23%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 8.0 – 7.9 (m, 1H), 7.9 (m, 2H), 7.7 (m, 1H), 7.6 (td, J = 7.8, 1.6 Hz, 1H), 7.6 (t, J = 7.6 Hz, 1H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.2, 159.2, 158.8, 136.1, 135.6, 133.5, 132.3, 131.6, 131.5, 131.1 (q, J = 308.7 Hz), 130.1, 128.7, 128.3, 123.0. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₆H₁₀ClF₃N₃O₂S [M + H]⁺ 400.0134, found 400.0135. Purity by HPLC was found to be 96%.

N-(5-(3-Fluorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (4):

Off-white solid (55 mg, 32%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 7.9 (m, 2H), 7.8 (m, 1H), 7.7 – 7.6 (m, 2H), 7.5 (td, J = 8.5, 2.7 Hz, 1H). ¹³C NMR (126 MHz, DMSO- d_6) δ

165.3, 163.7 (d, J = 245.7 Hz), 160.2, 158.7, 136.0, 135.5, 132.4 (d, J = 8.82 Hz), 131.1 (q, J = 308.7 Hz), 130.2, 128.7, 125.9 (d, J = 8.82 Hz), 122.8, 119.3 (d, J = 21.4 Hz), 113.3 (d, J = 25.2 Hz). ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F), -112.5 (q, J = 8.5 Hz, 1F). HRMS (ESI) m/z calcd for C₁₆H₁₀F₄N₃O₂S [M + H]⁺ 384.0430, found 384.0429. Purity by HPLC was found to be 98%.

N-(5-(3-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (5):

Off-white solid (35 mg, 19%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 7.9 – 7.8 (m, 4H), 7.7 (dd, J = 23.9, 7.9 Hz, 2H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.4, 159.8, 159.0, 136.1, 134.6, 132.0, 132.0, 131.1 (q, J = 308.7 Hz), 130.2, 128.9, 128.5, 126.0, 125.8, 125.2. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₆H₁₀ClF₃N₃O₂S [M + H]⁺ 400.0134, found 400.0135. Purity by HPLC was found to be 96%.

N-(5-(3-Methoxyphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (6):

Off-white solid (42 mg, 24%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 7.9 (m, 2H), 7.6 – 7.5 (m, 2H), 7.4 (s, 1H), 7.2 (d, J = 7.9 Hz, 1H), 3.8 (s, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.5, 160.9, 160.2, 158.6, 136.0, 135.7, 131.2 (q, J = 308.7 Hz), 130.1, 128.7, 128.6, 125.0, 118.9, 118.3, 111.5, 55.9. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₃F₃N₃O₂S [M + H]⁺ 396.0630, found 396.0632. Purity by HPLC was found to be 98%.

N-(5-(3-Trifluoromethylphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (7):

Off-white solid (53 mg, 27%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.2 (d, *J* = 7.9 Hz, 1H), 8.2 – 8.1 (m, 3H), 8.0 (m, 1H), 7.9 (m, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.4, 159.8, 159.1, 136.1, 135.8, 131.4, 131.1 (q, *J* = 308.7 Hz), 130.8 (q, *J* = 31.5 Hz), 130.5, 130.4, 130.1, 128.6, 125.2 (q, *J* = 272.2 Hz), 125.0, 122.8, ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F), -62.8 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₀F₆N₃O₂S [M + H]⁺ 434.0398, found 434.0399. Purity by HPLC was found to be 98%.

N-(5-(4-Fluorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (8):

Off-white solid (43 mg, 24%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.1 (m, 2H), 8.0 (dd, J = 8.7, 5.5 Hz, 2H), 7.9 (m, 2H), 7.4 (t, J = 8.7 Hz, 2H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.5 (d, J = 250.7 Hz), 160.4, 158.6, 136.1, 135.7, 131.1 (q, J = 308.7 Hz), 130.1, 129.3 (d, J = 8.82 Hz), 128.6, 120.5, 117.2 (d, J = 22.7 Hz). ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F), -108.8. (s, 1F). HRMS (ESI) m/z calcd for C₁₆H₁₀F₄N₃O₂S [M + H]⁺ 384.0430, found 384.0431. Purity by HPLC was found to be 96%.

N-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (9):

Off-white solid (36 mg, 20%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 8.0 – 7.9 (m, 2H), 7.9 (m, 2H), 7.7 – 7.6 (m, 2H). ¹³C NMR (126 MHz, DMSO- d_6) δ 164.5, 160.4, 158.6, 137.0, 136.1, 135.6, 131.1 (q, J = 308.7 Hz), 130.1, 128.6, 128.4, 127.2, 122.7. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₆H₁₀ClF₃N₃O₂S [M + H]⁺ 400.0134, found 400.0132. Purity by HPLC was found to be 97%.

N-(5-(4-Trifluoromethylphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (10):

Off-white solid (42 mg, 22%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (dd, J = 14.0, 8.0 Hz, 4H), 8.0 (m, 2H), 7.9 (m, 2H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.2, 160.0, 159.0, 136.1, 135.6, 132.0 (q, J = 31.5 Hz), 131.1 (q, J = 308.7 Hz), 130.1, 128.7, 127.6, 127.4, 126.9, 125.3 (q, J = 272.2 Hz).¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F), -62.8 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₀F₆N₃O₂S [M + H]⁺ 434.0398, found 434.0397. Purity by HPLC was found to be 99%.

N-(5-(4-Methoxyphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (11):

Off-white solid (48 mg, 27%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (d, J = 8.0 Hz, 2H), 7.9 – 7.8 (m, 4H), 7.1 (m, 2H), 3.8 (s, 3H). ¹³C NMR (126 MHz, DMSO- d_6) δ 164.0, 162.5, 161.4, 158.1, 136.1, 131.1 (q, J = 308.7 Hz), 130.1, 128.4, 127.3, 116.1, 115.4, 115.1, 56.0. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₃F₃N₃O₂S [M + H]⁺ 396.0630, found 396.0631. Purity by HPLC was found to be 99%.

N-(5-(4-Methylphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (12):

Off-white solid (35 mg, 21%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.1 (m, 2H), 7.9 (m, 4H), 7.4 (m, 2H), 2.4 (s, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.7, 161.3, 158.4, 142.5, 136.1, 135.8, 131.1 (q, *J* = 308.7 Hz), 130.4, 130.1, 128.5, 126.6, 121.1, 21.6. ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₃F₃N₃O₂S [M + H]⁺ 380.0681, found 380.0682. Purity by HPLC was found to be 98%.

N-(5-(4-Isopropylphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (13):

Off-white solid (39 mg, 21%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.2 – 8.1 (m, 2H), 7.9 (dd, J = 8.3, 2.5 Hz, 4H), 7.5 (m, 2H), 3.0 (h, J = 6.9 Hz, 1H), 1.2 (d, J = 6.9 Hz, 6H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.5, 161.2, 158.4, 153.1, 136.1, 135.8, 131.2 (q, J = 308.7 Hz), 130.1, 128.5, 127.9, 126.7, 121.4, 33.9, 23.9. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₉H₁₇F₃N₃O₂S [M + H]⁺ 408.0994, found 408.0993. Purity by HPLC was found to be 97%.

N-(5-(2,4-Dichlorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (14):

Off-white solid (35 mg, 18%). ¹H NMR (500 MHz, DMSO- d_6) δ 8.1 (m, 2H), 8.0 (m, 1H), 7.9 – 7.8 (m, 3H), 7.7 (dd, J = 8.5, 2.1 Hz, 1H). ¹³C NMR (126 MHz, DMSO- d_6) δ 165.1, 158.8, 158.5, 137.5, 136.1, 135.5, 133.3, 132.6, 131.2, 131.1 (q, J = 308.7 Hz), 130.1, 128.7, 122.0. ¹⁹F NMR (471 MHz, DMSO- d_6) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₆H₉Cl₂F₃N₃O₂S [M + H]⁺ 433.9745, found 433.9747. Purity by HPLC was found to be 99%.

N-(5-(3,5-Dichlorophenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (15, HSGN-218):

Off-white solid (37 mg, 19%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.1 (m, 2H), 7.9 (m, 5H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.4, 159.7, 158.7, 136.1, 135.7, 131.4, 131.2 (q, *J* = 308.7 Hz), 130.1, 128.4, 127.2, 126.3, 124.9. ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F). HRMS (ESI)

m/z calcd for $C_{16}H_9Cl_2F_3N_3O_2S$ [M + H]⁺ 433.9745, found 433.9744. Purity by HPLC was found to be 99%.

N-(5-(5-Chloro-2-methoxyphenyl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (16):

Off-white solid (46 mg, 24%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.1 (m, 2H), 7.9 (m, 2H), 7.8 (d, *J* = 2.7 Hz, 1H), 7.6 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.3 (m, 1H), 3.9 (s, 3H).¹³C NMR (126 MHz, DMSO-*d*₆) δ 165.8, 158.7, 156.8, 136.1, 135.8, 133.3, 131.2 (q, *J* = 308.7 Hz), 130.1, 129.4, 128.6, 126.3, 124.9, 115.4, 114.4, 57.1. ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₇H₁₂ClF₃N₃O₃S [M + H]⁺ 430.0240, found 430.0241. Purity by HPLC was found to be 97%.

N-(5-(Pyridin-2-yl)-1,3,4-oxadiazol-2-yl)-4-((trifluoromethyl)thio)benzamide (17):

Off-white solid (26 mg, 16%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.8 (d, *J* = 4.7 Hz, 1H), 8.1 (dd, *J* = 8.2, 2.7 Hz, 3H), 8.0 (td, *J* = 7.8, 1.8 Hz, 1H), 7.9 (m, 2H), 7.6 (ddd, *J* = 7.6, 4.8, 1.2 Hz, 1H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 164.9, 160.8, 159.0, 150.7, 143.2, 138.3, 136.1, 135.5, 131.1 (q, *J* = 308.7 Hz), 130.1, 128.7, 126.6, 122.9. ¹⁹F NMR (471 MHz, DMSO-*d*₆) δ -42.4 (s, 3F). HRMS (ESI) m/z calcd for C₁₅H₁₀F₃N₄O₂S [M + H]⁺ 367.0477, found 367.0475. Purity by HPLC was found to be 98%.

Bacterial strains media, cell lines and reagents

Bacterial strains used in this study (**Table 1S**) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and the American Type Culture Collection (ATCC). E. coli BW25113 and JW25113 were obtained from the Coli Genetic Stock Center (CGSC), Yale University, USA. Brain heart infusion broth was purchased from Becton, Dickinson and Company (Cockeysville, MD, USA) and was purchased from Fisher Scientific. Yeast extract, L-cysteine, vitamin K, hemin and phosphate buffered saline (PBS) were all obtained from commercial vendors. Human colorectal adenocarcinoma epithelial cells (Caco-2) (ATCC HTB-37) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) was purchased from Corning (Manassas, VA, USA). Vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO, USA), linezolid and gentamicin sulfate (Chem-Impex International, Wood Dale, IL, USA), metronidazole (Alfa Aesar, Ward Hill, MA, USA), and fidaxomicin (Cayman Chemical, Ann Arbor, MI, USA) were purchased commercially. Compounds were synthesized from commercial sources in our laboratory.

Determination of the MICs against *C. difficile* clinical isolates:

The minimum inhibitory concentrations (MICs) of tested compounds and control drug; vancomycin, were determined using the broth microdilution method, as previously described⁵⁷⁻⁶⁰ against *C. difficile* clinical isolates. Briefly, 0.5 McFarland bacterial solution was prepared and diluted in brain heart infusion supplemented (BHIS) broth (to an inoculum size $\sim 5 \times 10^5$

CFU/mL). Test agents were added and serially diluted before plates were incubated anaerobically at 37°C for 48 hours. MICs reported are the lowest drug concentration that completely suppressed the growth of bacteria, as observed visually.

Determination of the MICs against vancomycin-resistant enterococci (VRE) and *Escherichia coli* strains

The MICs of **HSGN-218** and control drugs were determined using the broth microdilution method, according to guidelines outlined by the Clinical and Laboratory Standards Institute ($CLSI^{61}$) against *Enterococcus faecium*, *Enterococcus faecalis* and *Escherichia coli* strains. Bacterial strains were grown aerobically overnight on tryptone soy agar (TSA) plates at 37° C. Afterwards, a bacterial solution equivalent to 0.5 McFarland standard was prepared and diluted in cation-adjusted Mueller-Hinton broth (CAMHB) (for *E. coli*) or tryptone soy broth (TSB) (for enterococcul strains), to achieve a bacterial concentration of about 5×10^5 CFU/mL. Test agents were added in the first row of the 96-well plates and serially diluted along the plates. Plates were then, incubated as previously described. MICs reported in Table 3 are the minimum concentrations of the test agents that completely inhibited the visual growth of bacteria.

In vitro cytotoxicity analysis of HSGN-218 against human colorectal cells.

Compounds were assayed for potential cytotoxicity against a human colorectal adenocarcinoma (Caco-2) cell line, as described previously⁶²⁻⁶³. Briefly, tested compounds were incubated with Caco-2 cells for 2 hours. Then, cells were incubated with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent for 4 hours before measuring absorbance values (OD₄₉₀).

Caco-2 permeability assay

Assay and data analysis were performed by Eurofins Panlabs (MO, USA) according to a previously reported protocol⁶⁴⁻⁶⁵. The apparent permeability coefficient (Papp) of the tested agents was calculated using the equation below:

$$P_{app}(cm/s) = \frac{V_{R} * C_{R,end}}{\Delta t} * \frac{1}{A^{*}(C_{D,mid} + C_{R,mid})}$$

where V_R is the volume of the receiver chamber. $C_{R,end}$ is the concentration of the test compound in the receiver chamber at the end time point, Δt is the incubation time and A is the surface area of the cell monolayer. $C_{D,mid}$ is the calculated mid-point concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minute and the donor concentration at the end time point. $C_{R,mid}$ is the mid-point concentration of the test compound in the receiver side, which is one half of the receiver concentration at the end time point. Concentrations of the test compound were expressed as peak areas of the test compound.

In vitro antibacterial evaluation of HSGN-218 against normal microflora.

The broth microdilution assay was utilized to determine the MICs of **HSGN-218** against commensal organisms that compose the human gut microflora, as described elsewhere^{48, 61, 66}. A bacterial solution equivalent to 0.5 McFarland standard was prepared and diluted in BHIS broth (for *Bacteroides*) or in MRS broth (for *Lactobacillus*) to achieve a bacterial concentration of

about 5×10^5 CFU/mL. Test agents were added and serially diluted along the plates. Plates were incubated for 48 hours at 37°C before recording the MIC by visual inspection of growth.

Frequency of spontaneous mutation.

HSGN-218 was tested against *C. difficile* to determine the likelihood of development of spontaneous mutation as previously described^{51, 67}. Briefly, **HSGN-218** and vancomycin were added to BHIS agar to achieve a final concentration of $15 \times \text{MIC}$ and $20 \times \text{MIC}$ and poured in plates and left to dry out. An inoculum of ~ 10^9 CFU/mL of *C. difficile* ATCC 43255 was spread over the plates and incubated anaerobically at 37°C for 48 hours before plates were checked for the possible bacterial growth.

Preparation of C. difficile spores for mice infection

C. difficile spores were prepared as described earlier ^{68,52}. Briefly, *C. difficile* ATCC 43255 was inoculated onto BHIS agar and incubated anaerobically for 5 days. Spores were collected anaerobically using PBS containing 10% bovine serum albumin, heated at 70°C for 20 minutes to get rid of vegetative cells and counted by dilution and plating onto BHIS supplemented with 0.1% taurocholic acid. Spores were then, stored at 4°C overnight before infecting mice.

C. difficile infection (CDI) mouse model

The study was reviewed, approved and performed following the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in individually ventilated autoclaved cages and received sterile food and water ad libitum throughout the duration of the experiment. CDI mouse model was performed as described previously⁵². Eight-week-old female pathogen-free C57BL/6 mice (Jackson, ME, USA) were pre-treated with an antibiotic cocktail in sterile drinking water to disrupt the mice normal intestinal microflora, reducing the colonization resistance and facilitating infection with the toxigenic strain of C. difficile. Afterwards, mice were switched to regular autoclaved water for 2 days and they received a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day prior to C. difficile challenge. For infection, mice were restrained and infected via oral gavage with 1.3 x 10⁶ spores of C. difficile ATCC 43255. Following infection, mice were randomly allocated into groups (n=6) for treatment. Two hours post-infection, one groups were treated orally with HSGN-218 (50 mg/kg), one group was treated with vancomycin (10 mg/kg) via oral gavage, and one group was treated orally with the vehicle (10% DMSO, 10% tween 80, 80% PBS). Treatments were continued once daily for five days and mice were closely monitored for disease signs (including weight loss, behavioral changes, hunched posture, decreased activity, wet tail and diarrhea).

In vivo efficacy of HSGN-218 in C. difficile recurrence

In order to investigate the activity of **HSGN-218** in preventing *C. difficile* recurrence, mice were infected, as described above and one group was treated orally with **HSGN-218** (50 mg/kg), one group was treated with vancomycin (10 mg/kg) via oral gavage, and one group was treated orally with the vehicle for 5 days. Treatments were stopped after 5 days, and mice were monitored for

disease signs and recurrence of infection till the 21st day. Then, mice were humanely euthanized using CO₂ asphyxiation.

Statistical analyses

The survival data were analyzed by Log-rank (Mantel-Cox) test utilizing GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

In Silico PAINS Analysis

All synthesized analogs were subjected to PAINS filters by using the SwissADME program⁶⁹. Molecular formula strings of analogs were manually entered into the program, which indicated no PAINS were found.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.xxxxxx.

- Various bacterial strains used in this study; and ¹H NMR, ¹³C NMR, and ¹⁹F NMR spectra of analogs
- Molecular SMILES strings and MIC values

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Abbreviations:

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ATCC	American type culture collection
BHIS	brain heart infusion supplemented
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
CDI	Clostridium difficile infection
CLSI	clinical and laboratory standards institute
DIPEA	diisopropylethylamine
DMEM	dulbecco's modified eagle medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
H ₂ O	water
MeOH	methanol
MIC	minimum inhibitory concentration

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaOAc	sodium acetate
PAINS	pan assay interference compounds
PBS	phosphate buffered saline
RT	room temperature
TSA	tryptic soy agar
TSB	tryptic soy broth
	ACS Paragon Plus Environment

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SCF₃) MIC = 0.003 µg/mL Gut-restricted /preventsrC. difficile recurrence in mice