

Identification of Highly Specific Diversity-Oriented Synthesis-Derived Inhibitors of *Clostridium difficile*

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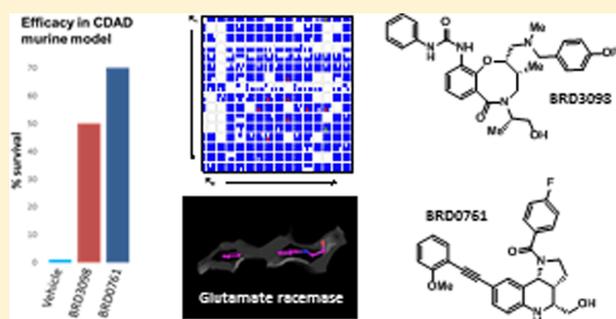
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 Supporting Information

**ABSTRACT:** In 2013, the Centers for Disease Control highlighted *Clostridium difficile* as an urgent threat for antibiotic-resistant infections, in part due to the emergence of highly virulent fluoroquinolone-resistant strains. Limited therapeutic options currently exist, many of which result in disease relapse. We sought to identify molecules specifically targeting *C. difficile* in high-throughput screens of our diversity-oriented synthesis compound collection. We identified two scaffolds with apparently novel mechanisms of action that selectively target *C. difficile* while having little to no activity against other intestinal anaerobes; preliminary evidence suggests that compounds from one of these scaffolds target the glutamate racemase. In vivo efficacy data suggest that both compound series may provide lead optimization candidates.

**KEYWORDS:** *C. difficile*, CDAD, glutamate racemase, diversity-oriented synthesis



*Clostridium difficile* is an anaerobic, spore-forming, opportunistic pathogen that is a major cause of antibiotic-associated diarrhea (AAD), often in hospitals and long-term care facilities. These infections occur when broad-spectrum antibiotics eliminate the normal intestinal flora, thus allowing *C. difficile* to colonize. *C. difficile*-associated diarrhea (CDAD) is a major cause of morbidity and mortality in the United States and Europe, with an estimated 450,000 cases and 29,000 deaths in 2011 in the United States.<sup>1</sup> Serious cases of *C. difficile* can progress to pseudomembranous colitis, a severe inflammation of the colon, and, rarely, to septicemia.<sup>2</sup> In recent years there has been an increase in both the incidence and severity of *C. difficile* infections, in part due to the emergence of more virulent strains (e.g., the NAP1 serotypes) and fluoroquinolone-resistant strains.<sup>3</sup> Clinical symptoms are due primarily to the activity of two toxins, TcdA and TcdB, which inactivate host GTPase proteins, leading to cell death and to the disruption of the intestinal epithelium.<sup>4</sup>

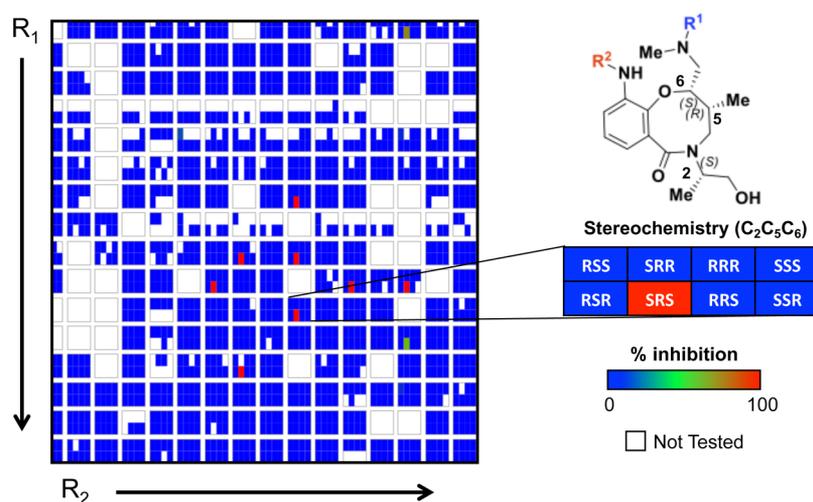
The standard treatment for CDAD is vancomycin antibiotic therapy, with some cases being treated by metronidazole. However, treatment with these relatively broad-spectrum antibiotics can recapitulate the disruption of normal intestinal flora that led to the initial *C. difficile* infection. Thus, recurrence of infection, either from spores remaining in the intestine or from spores in the environment, is a major challenge. Recently,

the U.S. FDA approved fidaxomicin (Dificid). The activity of fidaxomicin was shown to be comparable to that of vancomycin, but the rate of infection recurrence is lower.<sup>5</sup> The recurrence rates after fidaxomicin treatment are still notable and leave a need for improved therapeutic options. It has become clear that the key to preventing recurrence is to specifically target *C. difficile* while minimally affecting other members of the normal gut flora, which can out-compete the pathogenic Clostridia.<sup>6</sup> Most recently, fecal microbiota transplant (FMT) has been shown to be successful for difficult-to-treat *C. difficile* infections.<sup>7,8</sup> A 2013 *Guideline for Industry* supports the use of FMT in recurrent CDAD cases that are not responsive to standard treatment, although patients are required to provide informed consent.<sup>9</sup> Despite the promise of FMT, we believe that there is still a need for new *C. difficile* chemotherapeutics, provided that they are more selective and effective at preventing relapse than the current standard of care.

The drug discovery community has recognized the limitation of current compound collections for yielding the novel compounds required to modulate challenging targets, particularly antibacterial targets.<sup>10</sup> Compounds in most screening collections are, in general, flat, aromatic, and heteroaromatic

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**Figure 1.** Series 1 compounds inhibit *C. difficile* growth in a stereoselective manner. High-throughput screening data is depicted in a stereostructure–activity relationship (SSAR) viewer, showing percent inhibition of growth of *C. difficile* cultures in the presence of 16  $\mu\text{g/mL}$  of test compounds. R group combinations are arrayed on the X and Y axes. Each square shows the activity of eight separate stereoisomers, with the stereochemistry identified to the right. Degree of inhibition of *C. difficile* growth is indicated by the color gradient, with red indicating 90–100% inhibition. Only compounds with the SRS configuration inhibited *C. difficile* growth.

**Table 1.**  $\text{EC}_{90}$  ( $\mu\text{g/mL}$ ) Values of Select Compounds Showing Selective Inhibition of *C. difficile* Growth<sup>a</sup>

	Series 1			Series 2	
Strain	BRD3098	BRD6029	BRD2648	BRD3141	BRD0761
<i>C. difficile</i> (BAA-1382)	4	2	1	0.25	0.5
<i>E. faecium</i>	>64	>64	>64	>64	>64
<i>S. aureus</i>	>64	>64	>64	>64	>64
<i>S. pneumoniae</i>	>64	>64	>64	>64	>64
<i>A. baumannii</i>	>64	>64	>64	>64	>64
<i>E. coli</i>	>64	>64	>64	>64	>64
<i>K. pneumoniae</i>	>64	>64	>64	>64	>64
<i>P. aeruginosa</i>	>64	>64	>64	>64	>64

<sup>a</sup>*C. difficile* strains were grown anaerobically, whereas the remaining strains were grown aerobically.  $\text{EC}_{90}$  values were measured in high throughput as described under Materials and Methods.

structures, depleted in  $\text{sp}^3$ -hybridized atoms that facilitate stereochemical and skeletal diversity. The Broad Institute has synthesized a collection of approximately 100,000 novel diversity-oriented synthesis (DOS) derived small molecules, representing more than 250 unique scaffolds not available elsewhere.<sup>11</sup> The small molecules in this collection are similar to natural products in that they contain a higher ratio of  $\text{sp}^3$ -hybridized atoms and stereocenters relative to compounds found in conventional screening collections.<sup>12</sup> In addition, all available stereoisomers are present in the screening collection as unique entities, providing rich stereostructure–activity relationship (SSAR) data (in addition to traditional structure–activity relationship data) directly from primary screens. As the DOS collection has previously yielded unique mechanism-of-action hits for pathogenic agents including *Plasmodium falciparum*<sup>13,14</sup> and *Trypanosoma cruzi*,<sup>15</sup> we set out to screen the library against a toxin-producing strain of

*C. difficile*. We describe here the identification and characterization of two novel DOS-derived compound series that are potent in vitro inhibitors of *C. difficile* and are more selective than either vancomycin or fidoxamicin. These compounds appear to have unique mechanisms of action compared to current *C. difficile* therapeutics and may provide new paths forward for the treatment and prophylaxis of CDAD.

## RESULTS AND DISCUSSION

The inhibitory activity of the Broad Institute's DOS small molecule collection (~100,000 compounds) was evaluated under anaerobic growth conditions against *C. difficile* BAA-1382 as part of a larger screen of the entire DOS compound collection against eight bacterial strains. Hits were defined as inhibiting the growth of *C. difficile* at >80% at the screening concentration of 16  $\mu\text{g/mL}$  (220 compounds; hit rate = 0.2%). Examination of the high-throughput screening (HTS) data

**Table 2. Minimum Inhibitory Concentrations ( $\mu\text{g}/\text{mL}$ ) Required for Complete Inhibition of Growth of BRD3098, BRD0761, and Vancomycin against a Panel of Clinical *C. difficile* Isolates and Other Intestinal Species Grown under Anaerobic Conditions<sup>a</sup>**

organism	MMX no.	phenotype/ATCC no.	MIC ( $\mu\text{g}/\text{mL}$ )		
			BRD3098	BRD0761	vancomycin
<i>C. difficile</i>	5667	NAP1; BAA-1803	1	0.5	2
<i>C. difficile</i>	4994	NAP1; BAA-1805	0.5	0.12	1
<i>C. difficile</i>	5668	NAP1; BAA-1805	1	0.5	2
<i>C. difficile</i>	4820	BAA-1382	2	0.12	2
<i>C. difficile</i>	4821	ATCC 43255	1	1	1
<i>C. difficile</i>	4381	ATCC 700057	1	0.12	1
<i>C. difficile</i>	5680		0.5	0.25	0.5
<i>C. difficile</i>	3590		1	0.12	0.5
<i>C. difficile</i>	3591		2	0.25	1
<i>C. difficile</i>	3593		1	<0.06	0.5
<i>C. difficile</i>	3594		1	0.25	0.5
<i>C. difficile</i>	5921		1	0.12	0.5
<i>C. coccoides</i>	5927	ATCC 29236	>64	8	4
<i>C. perfringens</i>	0124	ATCC 13124	>64	>64	0.5
<i>E. lentum</i>	1274	ATCC 43055	>64	>64	1
<i>B. fragilis</i>	0123	ATCC 25285	>64	>64	16
<i>B. fragilis</i>	3374		>64	>64	16
<i>B. thetaiotaomicron</i>	3496		>64	>64	>64
<i>P. melaninogenica</i>	3437		8	16	32
<i>P. melaninogenica</i>	3442		>64	>64	16
<i>B. bifidum</i>	3965	ATCC 15696	>64	>64	1
<i>B. infantis</i>	3966	ATCC 15702	8	>64	0.5
<i>B. longum</i>	3968	ATCC 15707	>64	>64	0.5
<i>P. anaerobius</i>	3532	ATCC 27337	>64	>64	0.25
<i>P. asaccharolyticus</i>	5930	ATCC 29743	>64	>64	0.12
<i>P. anaerobius</i>	3542		>64	>64	0.25
<i>L. acidophilus</i>	5710	ATCC 4356	>64	>64	>64
<i>L. gasseri</i>	4148	ATCC 33323	>64	8	1
<i>L. rhamnosus</i>	5711	ATCC 7469	>64	>64	>64
<i>E. coli</i>	0102	ATCC 25922	>64	>64	>64
<i>E. coli</i>	2501	ESBL	>64	>64	>64
<i>E. faecalis</i>	0101	ATCC 29212	>64	>64	4
<i>E. faecium</i>	4202	VSE	>64	>64	1

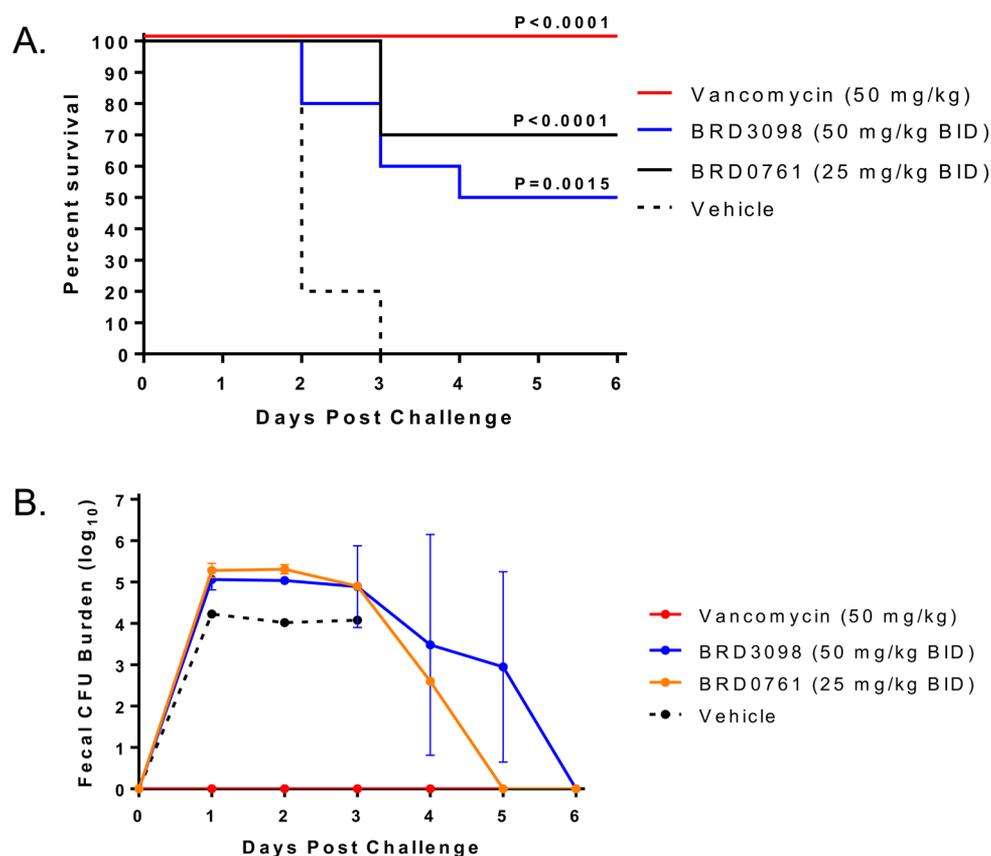
<sup>a</sup>All strains were grown anaerobically in the presence of increasing doses of test compounds for 48 h. MICs were determined by visual inspection of cultures. MMX refers to the Micromyx strain ID.

using a custom viewer for comparing the relationship between structure and activity revealed a number of hit clusters with preliminary stereochemical and appendage SAR, exemplified by series 1, which exhibited striking SSAR, as only one of eight possible stereoisomers for each compound inhibited the growth of *C. difficile* (Figure 1).

Subsequently, all hits were retested in a dose–response format for their effect on *C. difficile* growth, as well as the other seven bacterial strains assessed in the larger screen. On the basis of these results, two series, represented by BRD3098 (series 1) and BRD0761 (series 2) with potent and selective *C. difficile* growth inhibition activity, were prioritized for follow-up studies (Table 1). The profile of these hit compounds is more selective than that of fidoxamicin, which, like vancomycin, inhibits both *Enterococcus faecium* and *Staphylococcus aureus*.<sup>16</sup> However, because the initial specificity panel included only aerobically grown strains, the minimum inhibitory concentrations (MIC) of BRD3098 and BRD0761 were measured against a panel of *C. difficile* clinical isolates and other intestinal anaerobes, with vancomycin as the comparator. Similar to vancomycin, BRD3098 and BRD0761 inhibited all of the *C. difficile* isolates,

with BRD0761 showing greater potency than vancomycin (Table 2). Importantly, the selectivity profiles of BRD3098 and BRD0761 were superior to that of vancomycin, which had a broader range of activity, notably against Bifidobacteria and Peptostreptococci.

Initial in vitro profiling of BRD3098 and BRD0761 for solubility, permeability (as assessed in Caco-2 cells), and stability in simulated gastric fluids suggested that these compounds would not have good systemic bioavailability and thus might be suitable for oral dosing for *C. difficile* infections (Supplementary Table 1). Proof-of-concept efficacy studies were therefore performed using a murine CDAD model,<sup>17</sup> with twice daily oral dosing at either 50 mg/kg (BRD3098) or 25 mg/kg (BRD0761). As shown in Figure 2A, both compounds exhibited significant delays in mortality compared to the vehicle control, although neither compound reduced mortality over the course of the study as effectively as vancomycin (once daily dose of 50 mg/kg). In addition, both compounds resulted in a decrease in fecal shedding of *C. difficile* (Figure 2B), although this effect was significantly delayed compared to that of vancomycin.



**Figure 2.** BRD3098 and BRD0761 are efficacious in a murine model of *C. difficile* associated disease (CDAD). (A) Female C57/Bl6 mice were treated with an antibiotic cocktail on days  $-14$  to  $-5$  of the study prior to receiving a single oral dose of clindamycin (10 mg/kg) on day  $-3$ . On day 0, animals were challenged with approximately  $4.5 \log_{10}$  spores/0.5 mL in PBS. Compounds formulated in 15% vitamin E-TPGS were dosed orally 2 h after infection, and treatment continued twice daily through day 4. Animals were observed at least twice daily for signs of morbidity and mortality. Clinical signs on days 0–6 including lethargy, hunched posture, ruffled coat, wet tail/abdomen, and hypothermia were recorded and scored from 0 to 4 as described under [Materials and Methods](#). Animals with  $\geq 25\%$  weight loss were humanely euthanized. All groups contained 10 animals. (B) BRD3098 and BRD0761 treatment reduced the shedding of *C. difficile* CFU in feces. Feces were collected daily, and fecal pellets were suspended in 50% EtOH and PBS and vortexed. Serial dilutions of the samples were plated on TCCFA agar and grown in an anaerobic atmosphere at  $37^\circ\text{C}$  overnight to enumerate CFU. Data are plotted as an average CFU from each study group on each day; error bars represent the standard deviation.

Preliminary SARs for both series were also evaluated using relevant compounds available from the screening library. The SAR for series 2, which originated from a library<sup>18</sup> synthesized with a sparse-matrix design,<sup>19</sup> was limited to two stereoisomers (BRD0761 and BRD3141). As a result of the library design, only a handful of structurally related analogues were available, which upon testing exhibited weaker activity against *C. difficile* (data not shown). Closely related analogues for series 1 from the screening library were more prevalent and showed various levels of activity against *C. difficile* when retested at dose. These data are shown in [Table 3](#) as the concentration (in  $\mu\text{g}/\text{mL}$ ) required for at least 90% inhibition of growth ( $\text{EC}_{90}$ ). Compounds with a range of tertiary amines substituted at the  $\text{R}_1$  position, including saturated rings and substituted benzylic groups, showed various activities against *C. difficile*. Para substituents on the benzylic amine show potent activity, although ortho- and meta-substituents were not available from the collection. The corresponding secondary amine (where  $\text{R}_1$  is H) shows limited activity, and changing the functionality of the nitrogen to a sulfonamide or urea resulted in a loss of activity. Substitutions at the second site ( $\text{R}_2$ ) show a clear requirement for a urea functional group for inhibition of *C. difficile*, and preliminary SAR shows that a para substitution

on the aromatic ring is tolerated to a limited degree, with the  $p\text{-CF}_3$  group showing the best inhibitory activity. Other functional groups, such as sulfonamides and amides (not shown), lead to a loss in activity.

With satisfactory results from the *in vitro* profiling and *in vivo* efficacy studies, along with good preliminary SAR, hit-to-lead efforts were focused on the general structure of series 1 in [Figure 1](#). [Table 4](#) shows the optimization of changes in the  $\text{R}_1$  and  $\text{R}_2$  regions of the molecule. The  $p\text{-CF}_3$ -phenyl urea combined with diaryl  $\text{R}_1$  groups leads to compounds with good activity in the *C. difficile* antibacterial assays, with the best compound having an MIC of  $0.25 \mu\text{g}/\text{mL}$ . We next explored replacements for the urea such as carbonates, sulfonamides (inactive, not shown) and isosteric amides. This led to improved antibacterial activity, with one of the best compounds having a  $m\text{-CF}_3$ -phenyl-acetylamine as an  $\text{R}_2$  group and a phenylthio benzyl group in the  $\text{R}_1$  position (e.g., WXL1631, [Table 4](#)). Five of the most potent compounds, including WXL1631, were tested in the *in vivo* model; unfortunately, none of these compounds were superior to the original urea series of inhibitors, possibly due to very high protein binding and poor intestinal microsomal stability as measured *in vitro* ([Supplementary Table 1](#)).

Table 3. Structure–Activity Relationships of the High-Throughput Screening Hits from Series 1<sup>a</sup>

R2	R1									
	--H	Cyclohexyl	Phenyl	4-Methoxyphenyl	4-Phenylphenyl	4-(Trifluoromethyl)phenyl	4-Phenylphenyl	4-(Phenylsulfonyl)phenyl	4-(4-Chlorophenyl)phenyl	4-(4-Fluorophenyl)phenyl
H---	>64									
	NT	NT	NT	NT	4*	32	8	NT	NT	
	NT	NT	NT	NT		8	2**	NT	NT	
	64	2	32	1	1		1***	>64	>64	
	NT	NT	NT	NT	4	>64	>64	NT	NT	
	NT	NT	NT	NT	>64	NT	>64	NT	NT	

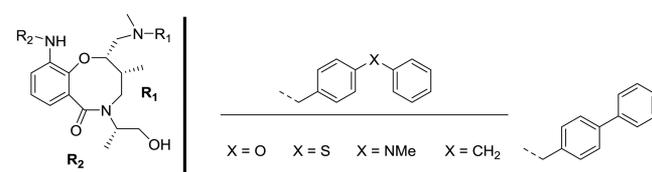
<sup>a</sup>The EC<sub>90</sub> concentrations ( $\mu\text{g}/\text{mL}$ ) of compounds with the indicated R1 and R2 groups are indicated; all compounds have the SRS stereochemistry. “NT” indicates compounds that were inactive in the primary screen and not tested at dose. Blank cells indicate compounds were unavailable for testing. \*, \*\*, and \*\*\* denote the data and structure corresponding to BRD3098, BRD6029, and BRD2648, respectively.

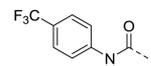
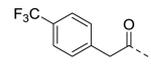
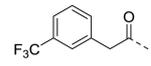
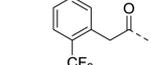
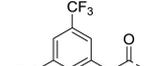
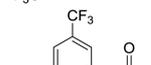
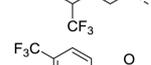
In parallel to the medicinal chemistry efforts, the modes of action for both series were investigated. On the basis of the similarly specific activity profiles against *C. difficile* and other gut anaerobes, it was possible that the two compound series were targeting the same bacterial process. To investigate the mechanisms of action, BRD3098 and BRD0761 were assessed in time–kill kinetics and macromolecular synthesis inhibition studies (Figure 3). Despite a transient drop in viable bacterial counts, BRD3098 did not significantly reduce the viable CFU over a 24 h period when tested at 4 $\times$  and 8 $\times$  the MIC against *C. difficile* BAA-1382 (Figure 3A), suggesting that the compound inhibits *C. difficile* growth but is not bactericidal. In contrast, BRD0761 resulted in a 3 log decrease in viable CFU over a 24 h period in the same *C. difficile* strain (Figure 3B), suggesting that the compound is bactericidal. Macromolecular synthesis inhibition studies for cell wall biosynthesis and protein synthesis were also performed for both compounds. BRD3098 did not show an obvious dose-dependent inhibition of either cell wall biosynthesis or protein synthesis (Figure 3C); rather, both pathways were inhibited at higher concentrations, whereas protein synthesis was enhanced at lower concentrations. Conversely, BRD0761 exhibited a dose-dependent reduction of [<sup>14</sup>C]N-acetylglucosamine uptake in treated cultures, suggesting that the compound targets cell wall biosynthesis (Figure 3D).

To further investigate the mechanism of action of the two compound series, we set out to identify resistance-inducing mutations. BRD3098 shows very weak inhibition of *C. difficile* growth when tested on agar plates, and no stable resistant mutants could be identified via serial passage; as a result, the putative target could not be identified. Agar selection was utilized to generate colonies spontaneously resistant to

BRD0761 in both BAA-1382 and 700057 *C. difficile* strains. The spontaneous mutation frequency ranged from 1.6–4.8  $\times 10^{-8}$ , consistent with a single target molecule. Whole genome sequencing of four individual clones (three from parental strain BAA-1382 and one from parental strain 700057) revealed that all four clones contained point mutations within the *murI* gene, which encodes glutamate racemase (Figure 4a). Glutamate racemase, an essential protein, converts L-glutamate to D-glutamate, which is incorporated into the peptidoglycan layer during cell wall biosynthesis.<sup>20,21</sup> Some bacterial species have been shown to have two homologues of glutamate racemase (e.g., *Bacillus* species, see review in ref 21); however, we were unable to identify any apparent homologues within the *C. difficile* genome (data not shown), consistent with the spontaneous mutation frequency of  $\sim 10^{-8}$ .

Molecular modeling was utilized to investigate the potential interactions of BRD0761 within the inhibitor binding pocket of glutamate racemase. Analysis of the Protein Data Bank collection indicated that *C. difficile* glutamate racemase was not available, but a structure of the protein from *Helicobacter pylori* had been solved with a pyridodiazepine amine inhibitor bound at the dimer interface.<sup>22</sup> Sequence comparison between *C. difficile* and *H. pylori*, in conjunction with the inhibitor binding site, identified four amino acid modifications necessary to produce a *C. difficile*-like Glu racemase inhibitor binding pocket. A model of BRD0761 docked into this binding pocket indicated that our ligand contains features similar to the pyridodiazepine inhibitor of the crystal structure, as illustrated in Figure 4B. The fluorophenyl of BRD0761 occupies the phenyl ring binding site, whereas the fused aromatic of our compound sits in the thiophene binding site. In this proposed binding mode, the terminal methoxyphenyl group of BRD0761

**Table 4. Optimization of Urea and Amide Analogues of Lead Compounds<sup>a</sup>**


	X = O	X = S	X = NMe	X = CH <sub>2</sub>	
	1	0.25	0.5	1.0	2
	0.25	0.03	0.5	0.125	0.5
	0.125	0.03*	NA	NA	1.0
	4	1	NA	NA	>16
	0.5	0.06	NA	NA	2.0
	0.125	0.06	0.125	NA	0.25
	<0.016	0.03	NA	NA	0.06

<sup>a</sup>Structure–activity relationships of amide analogues of lead urea compound 4. The MICs ( $\mu\text{g/mL}$ ) against *C. difficile* of compounds with the indicated R<sub>1</sub> and R<sub>2</sub> groups are indicated; all compounds have the SRS stereochemistry. “NA” indicates compounds were not available. \* indicates compound WXL1631 (see [Supplementary Table 1](#) for additional data).

occupies part of the glutamate binding pocket. Access to this site is via a somewhat constricted region of the protein, thus necessitating the acetylene linker ([Figure 4C](#)). Mapping of the resistance-inducing mutations to this binding model revealed that serine 44 is within the binding pocket, with the OH group from the serine predicted to interact with the carbonyl O of BRD0761 ( $\sim 2.89$  Å). The other three identified mutations are farther removed from the binding pocket ( $>10$  Å) but could have distal effects upon the binding pocket structure. To further validate the resistance-inducing mutations and their potential effects on inhibitor binding, future studies could include crystallography of the wild type and mutant *C. difficile* MurI enzyme (with and without the inhibitor) and complementation of the mutations.

In summary, we have identified two very selective series of inhibitors of *C. difficile*, one of which appears to disrupt cell wall biosynthesis via inhibition of glutamate racemase. Glutamate racemase has been well validated as a target in other Gram-positive bacteria, including *H. pylori*, *S. aureus*, and *Mycobacterium tuberculosis*.<sup>22–25</sup> Interestingly, studies from AstraZeneca have shown that glutamate racemases show species specificity with respect to structural and enzymatic properties. Their group was able to exploit these differences to identify selective inhibitors of *H. pylori*.<sup>22,23</sup> Our findings suggest that glutamate racemase may also be a suitable target for the identification of *C. difficile*-specific antibiotics, but

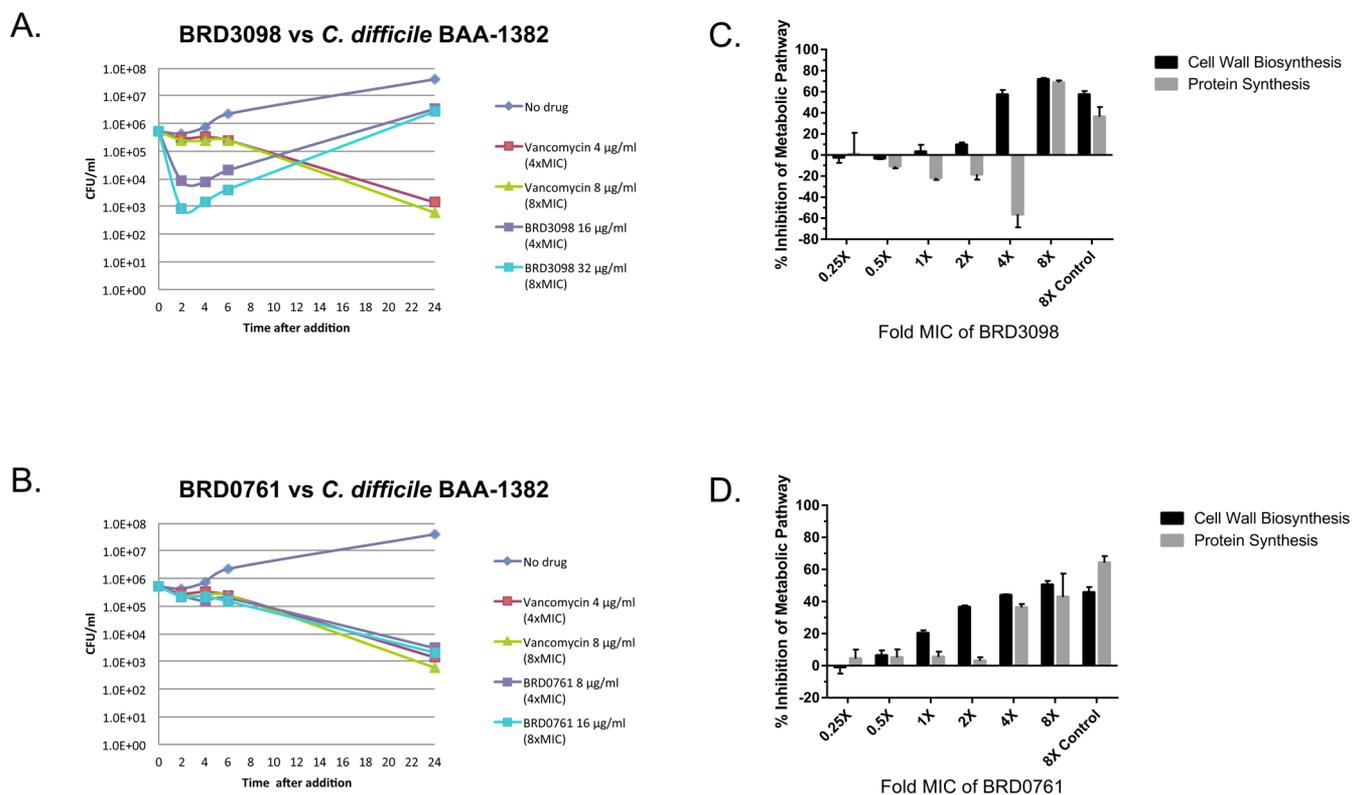
additional experiments are likely required to confirm the mechanism of action of BRD0761.

Although the target is known for only series 2 (represented by BRD0761), strengths and weaknesses exist and should be assessed in determining the progressability of each series. Ideal development candidates would have low absorption and systemic exposure, good stability in the intestines, good solubility, and limited toxicity ([Supplemental Table 1](#)). In addition, compounds with novel mechanisms of action are ideal. Although series 1 (BRD3098) does not have an identified target, the compounds appear to have a novel mechanism of action, and resistant bacteria could also not be identified, which may be advantageous in the clinic. From a medicinal chemistry standpoint, optimization of series 1 likely requires reduction of protein binding and in vitro cytotoxicity to further advance the series ([Supplemental Table 1](#)). Conversely, the apparent target of series 2 is the MurI enzyme; single-step resistant mutants could be relatively easily obtained (frequency  $\sim 10^{-8}$ ), which may be problematic for clinical applications. This could be circumvented either by using MurI inhibitors in combination with other antibiotics or at high dosages if the compounds are tolerated. In addition, identification of the target enables structure-guided medicinal chemistry optimization of BRD0761, which requires reduction of plasma protein binding and in vitro cytotoxicity as well as increased solubility to advance the molecules. Structure-guided medicinal chemistry will also most likely be a more efficient approach than that of optimizing BRD3098, which to date has not identified a more superior compound in in vivo efficacy studies.

With the increase in antibiotic resistance and the clear impact of broad-spectrum antibiotics and CDAD, specific antibiotics may now be more appealing from a development perspective, particularly if they can be combined with rapid point-of-care diagnostics. To the best of our knowledge, only one other compound, SMT-19969 (Summit Therapeutics), shows similar selectivity toward *C. difficile*. SMT-19969 has excellent activity toward *C. difficile* in vitro, with superior selectivity compared to vancomycin and fidaxomicin.<sup>26</sup> A number of studies suggest that this selectivity results in reduced relapse rates in animal models of CDAD.<sup>27</sup> Further optimization of the *C. difficile*-specific compounds described herein may yield additional development candidates for the prophylactic and therapeutic treatment of CDAD.

## ■ MATERIALS AND METHODS

**Bacterial Strains and Growth Media for High-Throughput Screens.** All cultures were handled according to NIH Biosafety Level 2 guidelines, with culture manipulations restricted to biosafety cabinets or an anaerobic chamber. Bacterial strains for high-throughput screening and counter-screens were obtained from ATCC. ATCC catalog numbers are as follows: *Clostridium difficile*, BAA1382; *Acinetobacter baumannii*, BAA-1605; *Escherichia coli*, ATCC 25922; *Klebsiella pneumoniae*, ATCC 4352; *Pseudomonas aeruginosa*, ATCC 27853; *Enterococcus faecium*, ATCC 6569; *Staphylococcus aureus*, ATCC 43300; *Streptococcus pneumoniae*, ATCC 49619. *C. difficile* cultures were grown in Bacto brain–heart infusion (BHI) broth containing 2% Oxyrase in an anaerobic chamber. *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* were cultured in cation-adjusted Mueller–Hinton (MH2) broth, and *S. pneumoniae* was cultured in MH2 broth containing 2.5% lysed horse blood. *E. faecium* was cultured in



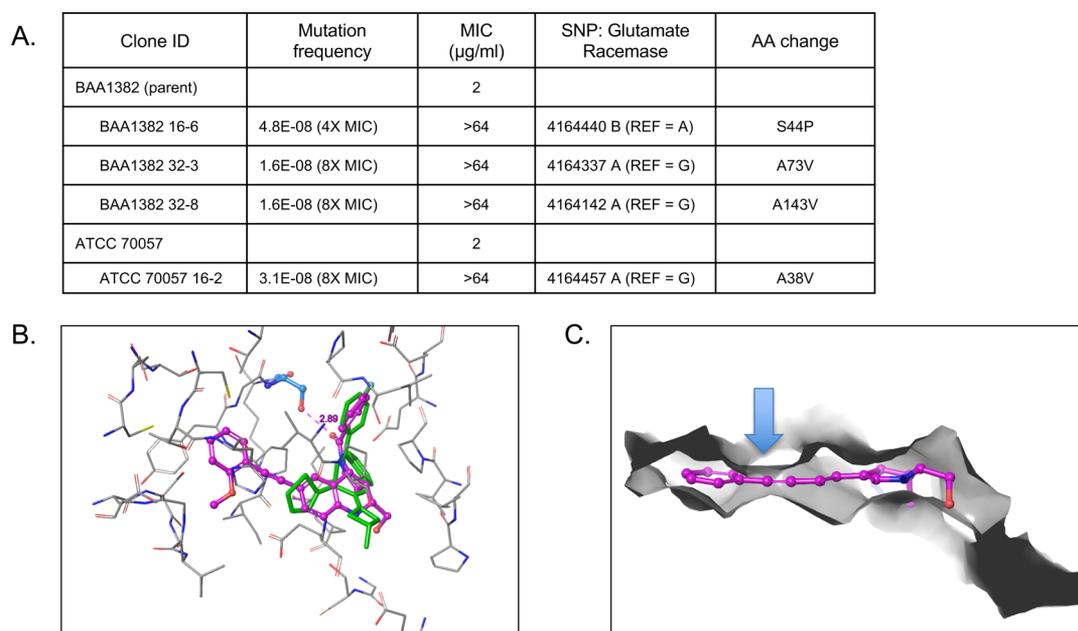
**Figure 3.** BRD3098 and BRD0761 have distinct modes of action. Time–kill kinetics studies with *C. difficile* strain BAA-1382 were performed at 4× and 8× the MICs with BRD3098 (A) and BRD0761 (B). Data were consistent with bactericidal activity for BRD0761, but not BRD3098. Macromolecular synthesis inhibition studies for cell wall biosynthesis and protein synthesis were also performed for both compounds. While BRD3098 did not show clear dose-dependent inhibition in either assays (C), BRD0761 exhibited dose-dependent reduction of [<sup>14</sup>C]N-acetylglucosamine uptake in treated cultures (D), suggesting that the compound targets cell wall biosynthesis. Inhibition of macromolecular synthesis is plotted as percent inhibition at multiples of the MIC for each compound; vancomycin and linezolid were used as positive controls at 8× their MICs in the cell wall and protein synthesis assays, respectively (denoted “8X Control”).

BBL BHI broth. All strains other than *C. difficile* were cultured aerobically.

**High-Throughput Screens.** Compounds (160 nL of 5 mg/mL stock solutions) were arrayed into clear, square-well 384-well plates (Aurora, EB 3030-00330) with neutral (DMSO) and positive controls (Levofloxacin, final concentration = 16 µg/mL) using an Echo 555 acoustic dispenser. For dose plates, compounds were arrayed such that the final screening concentrations ranged from 0.125 to 64 µg/mL in 2-fold dilution series. Prior to screening, 25 µL of fresh growth medium was added to each well. *C. difficile* cultures were prepared by dispensing 1 mL from a thawed cryovial into 200 mL of Bacto BHI with 2% Oxyrase. The thawed cultures were grown for 18 h in an anaerobic chamber at 37 °C; overnight cultures were diluted 1:100 in fresh medium, and 25 µL of the prepared inoculum (final concentration = 1 × 10<sup>6</sup> CFU/ml) was added to each well. The inoculated plates were briefly centrifuged to remove any bubbles and incubated for 24 h. After incubation, plates were removed from the incubator and equilibrated at room temperature for 30 min. OD<sub>600</sub> was determined in an Analyst GT (Molecular Devices). Screens and counterscreens with aerobic strains were performed similarly, except that the inoculum for each strain was prepared directly from cryovials without overnight culture, and cultures were incubated with compounds under aerobic conditions for 15–18.5 h. The cell densities of the inoculum for each strain were tested weekly to ensure that cryovial stocks maintained expected viability.

**High-Throughput Data Analysis.** Raw OD<sub>600</sub> data were imported into Assay Analyzer (Genedata AG) and normalized using the in-plate positive and negative controls. *Z'* values were calculated for each plate, and plates with *Z'* < 0.5 were repeated. Compounds inhibiting *C. difficile* growth more than 80% in the primary screen were retested at dose to determine EC<sub>90</sub> values against *C. difficile* and the comparator strains. Raw OD<sub>600</sub> values for dose testing were analyzed in the Assay Analyzer as described for high-throughput screening data, but growth inhibition curves were determined in the Condoseo analysis module (Genedata AG). The HTS data and EC<sub>90</sub> values were further analyzed using the Broad's internal SAR and stereostructure–activity (SSAR) analysis tools<sup>28</sup> and Spotfire (Tibco) to identify scaffolds with selective activity against *C. difficile*. HTS data have been deposited to PubChem (AIDs 1259249, 1224920, 1224921, and 1259246).

**Minimum Inhibitory Concentration (MIC) Determination and Selectivity Testing.** Bacterial strains for susceptibility and selectivity testing were obtained from ATCC or were clinical isolates obtained by Micromyx, LLC (Kalamazoo, MI, USA). Strain details are provided in Table 2. MIC values of representative compounds from each of the two active series against a panel of *C. difficile* isolates and other bacterial strains common to the intestinal flora were determined using a broth microdilution method as described by CLSI,<sup>29</sup> with the exception that BHI was used as the test medium in place of supplemented Brucella broth. Plates were incubated anaerobically at 35 °C for approximately 42–48 h. Following



**Figure 4.** BRD0761 targets cell wall biosynthesis. (A) Four independent resistant strains were isolated and sequenced, three from strain BAA1382 and one from strain ATCC 700057. Each isolate contained a single nucleotide polymorphism (SNP) within the *murI* gene resulting in a different amino acid change in the glutamate racemase protein. (B) In silico modeling of BRD0761 (shown as magenta-colored ball and stick) suggested that this ligand could bind to the inhibitor binding pocket of glutamate racemase in a manner similar to a pyridodiazepine amine inhibitor A1 (shown as green tube). The OH group of serine 44 from MurI (cyan color) is predicted to interact with the carbonyl O of BRD0761, which is approximately 2.89 Å away. Modeling of *C. difficile* glutamate racemase was based on the crystal structure for *Helicobacter pylori* glutamate racemase (PDB file 2W41<sup>22</sup>). (C) A cut-away of the binding site surface illustrates that the methoxy-phenyl moiety (left) extends into the glutamate substrate binding pocket via a constricted region of the enzyme (blue arrow).

incubation, microplates were removed from the anaerobic chamber and viewed from the bottom using a plate viewer. The MIC was read and recorded as the lowest concentration of drug that inhibited visible growth of the organism.

**Time–Kill Kinetic Studies.** Representative *C. difficile* strains were incubated with compounds at 4 $\times$  or 8 $\times$  the determined MIC under anaerobic conditions at 35 °C. Samples were removed at 0, 2, 4, 6, and 24 h for determination of viable counts as measured by colony-forming units per milliliter (CFU/mL).

**Macromolecular Synthesis Assays.** *C. difficile* ATCC 43602 cells in early exponential growth were collected by centrifugation and resuspended in 4 mL of minimal essential media (1 $\times$  M9 salts, 0.4% glucose, 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>) plus 0.1% sodium thioglycolate. One hundred microliters of cells was aliquoted to sterile screw-cap tubes, and 2.5  $\mu\text{L}$  of 40 $\times$  drug was added. Test compounds were added at 0.25-, 0.5-, 1-, 2-, 4-, and 8-fold the MIC. Cultures treated with diluent alone served as the “no drug” control for all experiments. Cultures were pre-incubated with compound for 15 min at 37 °C prior to addition of the appropriate radioactive assay component.

For inhibition of protein synthesis, reactions were initiated by the addition of [<sup>3</sup>H]leucine (1.0  $\mu\text{Ci}$ /reaction) and subsequently carried out at 37 °C for 60 min. The reactions were stopped through the addition of 25  $\mu\text{L}$  of 50% TCA + 2% casamino acids and were allowed to precipitate on ice for at least 30 min. The reactions were then filtered through glass fiber filters, followed by three washes with 5 mL of cold 5% TCA and two washes with 5 mL of ethanol. After drying, the filters were placed into scintillation vials, liquid scintillation fluid was added, and counts were determined using a Beckman

LS 3801 scintillation counter. All drug concentrations were tested in triplicate. Linezolid, at 8 $\times$  the MIC, was included as a positive control.

For inhibition of cell wall biosynthesis, reactions were initiated by the addition of [<sup>14</sup>C]N-acetylglucosamine (0.4 Ci/reaction) and then incubated at 37 °C for 60 min. The reactions were stopped through the addition of 8% SDS (100  $\mu\text{L}$ /tube) and boiled at 95 °C for 30 min. SDS-treated cells were collected on a 0.45  $\mu\text{m}$  HA filter and washed three times with 5 mL of 0.1% SDS. Filters were then rinsed two times with 5 mL of dH<sub>2</sub>O, allowed to dry, and then counted via liquid scintillation counting using a Beckman LS 3801. Vancomycin at 8 $\times$  the MIC was included as a positive control.

**In Vivo Efficacy (Murine CDAD Model).** In vivo efficacy testing was adapted from that published by Chen et al.<sup>17</sup> and performed at TransPharm Preclinical Solutions (Jackson, MI, USA). Each control and test article group included a total of 10 mice. C57BL/6 mice were exposed to a cocktail of antibiotics in their drinking water for a period of 9 days, starting on day –14. The antibiotic cocktail consisted of 10% glucose, kanamycin (0.5 mg/mL), gentamicin (0.044 mg/mL), colistin (1062.5 U/ml), metronidazole (0.269 mg/mL), ciprofloxacin (0.156 mg/mL), ampicillin (0.1 mg/mL), and vancomycin (0.056 mg/mL). The consumption of the antibiotic cocktail was monitored daily. Normal drinking water was returned on day –5. On day –3, mice received a single dose of clindamycin (10 mg/kg) in a volume of 0.5 mL by oral gavage. On day 0, each mouse was challenged with *C. difficile* spores (ATCC 43255) at approximately 4.5 log<sub>10</sub> spores per mouse in a dose volume of 0.5 mL by oral gavage. Two hours following challenge, mice were administered test article in 15% vitamin E–TPGS via oral gavage (po) in a volume of 0.5 mL. Test treatments continued

twice daily (bid) through day 4. Vancomycin was administered once daily at 50 mg/kg as a control. To monitor shedding of *C. difficile*, feces were collected daily from the cages for CFU determination. Fecal pellets were suspended in 50% EtOH and PBS and vortexed, maintained at room temperature for 1 h, vortexed, and serially diluted in PBS. The resulting suspensions were plated on TCCFA agar and grown in an anaerobic atmosphere at 37 °C overnight to enumerate viable CFU. Mice were observed at least twice daily during the study period for signs of morbidity and mortality. Clinical scores were assigned as follows: 0, normal; 1, lethargic; 2, lethargic + hunched; 3, lethargic + hunched + wet tail/abdomen; 4, lethargic + hunched + web tail/abdomen + hypothermic. Mice that lost  $\geq 25\%$  body weight were humanely euthanized. Kaplan–Meier survival plots were prepared for the resulting data using GraphPad Prism v.5.0. Survival curve comparisons were made between the negative control and all other treatments using the Logrank Test in GraphPad Prism. All in vivo protocols were performed according to standard NIH guidelines and were approved by the TransPharm IACUC (protocol no. TP-21-13) and the MIT Committee for Animal Care (CAC, protocol no. 0212-022-15).

**Selection of Resistant Mutants.** *C. difficile* mutants resistant to BRD0761 were selected by agar dilution assay on BHI agar plates with compound at 4 $\times$  or 8 $\times$  the MIC (2 and 4  $\mu\text{g}/\text{mL}$  against strains 700057 and BAA-1382, respectively). Spontaneous mutants were streaked onto *C. difficile* selection agar (CDSA; Becton Dickinson) in a Bactron II Anaerobic Chamber to presumptively confirm their identity as *C. difficile*. Each mutant that grew on CDSA was then subcultured in an anaerobic chamber onto Supplemented Brucella Agar plates (SBA; Remel) and was incubated in BD GasPak Anaerobe containers with sachets for 48 h. Frozen stocks were made from the isolated colonies and were also used directly for broth microdilution susceptibility testing (MIC, as described above) and Crystal ID testing to confirm *C. difficile* identity. Isolated bacterial colonies of the mutants and parent isolates from SBA plates were suspended at a density equivalent to a no. 4 McFarland standard in BD test medium provided with the BD Crystal ANR Kits (Becton Dickinson). Crystal identification cartridges were then incubated at 35 °C for 4 h and read using the BBL Crystal Autoreader. *B. fragilis* ATCC 25285 was tested in parallel for purposes of quality control.

BRD3098 resistant mutant generation was attempted via serial passage. *C. difficile* 700057 and BAA-1382 were incubated in BHI medium for 15 generations. At each passage, bacteria were incubated with 0.002–2  $\mu\text{g}/\text{mL}$  BRD098 anaerobically at 35 °C for approximately 42–48 h. Following incubation, microplates were viewed from the bottom using a plate viewer to determine the MIC. After the MIC was recorded, a 10  $\mu\text{L}$  sample was removed from the well containing the highest drug concentration that permitted growth and plated onto drug-free supplemented Brucella Agar. The plates were incubated for 24–48 h in the Bactron II anaerobic chamber. These plates were then used to prepare a standardized cell suspension for inoculation of prepared microplates for the next serial transfer as described above. This process was repeated to complete a total of 15 serial transfers of each culture. Following completion of the 15 serial transfers, all isolates were tested for susceptibility in BHI broth simultaneously along with the original parent strain using freezer stocks that had been prepared during passage throughout the study.

### Whole Genome Sequencing and Mutation Analysis.

Genomic DNA was isolated from mutant strains using the DNeasy Blood and Tissue kit (Qiagen). Isolated DNA was quantitated using a NanoDrop 1000, and Illumina fragment libraries were generated as previously described<sup>30</sup> with the following modifications. For each sample, 100 ng of genomic DNA was sheared to 200 bp in size using a Covaris LE220 instrument (Woburn, MA, USA) with the following parameters: temperature, 7–9 °C; duty cycle, 20%; intensity, 5; cycles per burst, 200; time, 90 s; shearing tubes, Crimp Cap microTUBES with AFA fibers (Covaris). DNA fragments were end repaired, 3' adenylated, ligated with indexed Illumina sequencing adapter, and PCR enriched, as previously described.<sup>31</sup> The resulting Illumina fragment sequencing libraries were normalized and size selected to contain inserts of 180 bp  $\pm 3\%$  in length using a Pippin Prep system (Sage Science, Beverly, MA, USA) following the manufacturer's recommendations. Libraries were 101bp paired end sequenced using the HiSeq 2000 instrument (Illumina, San Diego, CA, USA).

For all sequenced strains, reads were mapped onto the finished reference genome *C. difficile* 630 (Genbank accession no. GCA\_000009205.1) using BWA mem version 0.5.9.<sup>32</sup> Variants were identified using Pilon version 1.8.<sup>33</sup> Pilon identifies single-nucleotide polymorphisms, as well as longer insertion and deletion events. Variants shared between a mutant strain and its parent strain were removed from the analysis; only variants differing between the mutant strain and the parent strain were analyzed. All sequences have been deposited at NCBI, accession no. PRJNA285473.

**Molecular Modeling of Interaction with BRD0761 Glutamate Racemase.** Molecular modeling studies were done using software developed by Schrodinger Inc.<sup>34</sup> The 2W4I PDB file (chains A and B) was used as starting crystal structure of *H. pylori* glutamate racemase.<sup>22</sup> A BLAST<sup>35</sup> sequence comparison of the *C. difficile* and *H. pylori* glutamate racemase sequences provided an alignment to use for modeling. Four amino acid mutations within 5 Å of the inhibitor binding site were identified as necessary changes to produce the *C. difficile*-like inhibitor binding pocket. Amino acid mutations and side-chain scanning were done via Prime.<sup>36</sup> Models of BRD0761 bound to the protein were produced using Glide<sup>37</sup> and refined with Prime MMGBSA simulations, which allowed both the ligand and protein to relax via energy minimization.

**Compound Synthesis.** The synthesis of library members from series 1 (SNAr 8-ortho) has been previously published,<sup>38</sup> the same route was utilized in the resynthesis of active compounds. Compounds were characterized through LRMS and <sup>1</sup>H NMR experiments to give data consistent with the expected products. The synthesis of library scaffolds from series 2 and initial library synthetic steps have been previously published.<sup>18</sup> The active library members were prepared through a Sonogashira coupling in the final capping step, which has not been previously described. The following protocol was used for the Sonogashira coupling prior to cleaving the final compounds from the solid-phase Lanterns.

To each flask containing Lanterns was added dimethylformamide (DMF) (0.800 mL/Lantern) followed by the desired alkyne (20 equiv), CuI (3 equiv), diisopropylethylamine (30 equiv), and Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (1 equiv). The resulting mixture was degassed with a stream of N<sub>2</sub> before shaking at 60 °C overnight. After 24 h, the reaction mixture was removed and the Lanterns were washed with the following solvents for 30 min intervals:

dichloromethane (DCM), DMF, NaCN solution (0.1 M) in 1:1 tetrahydrofuran (THF)/H<sub>2</sub>O (30 min), DMF, 3:1 THF/H<sub>2</sub>O, 3:1 THF/isopropyl alcohol (IPA) THF, DCM. The Lanterns were then dried on a lyophilizer overnight.

Characterization data for BRD0761: <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.64 (s, 1H), 7.58–7.52 (m, 2H), 7.43 (dd, 1H, J = 1.5, 7.7 Hz), 7.26–7.21 (m, 1H), 7.08 (t, 2H, J = 8.6 Hz), 6.92–6.83 (m, 2H), 6.49 (d, 1H, J = 8.3 Hz), 5.89 (d, 1H, J = 7.2 Hz), 4.46 (s, 1H), 3.86 (s, 3H), 3.82–3.75 (m, 1H), 3.74–3.66 (m, 1H), 3.63–3.52 (m, 2H), 3.28 (dd, 1H, J = 8.5, 9.5 Hz), 2.44–2.34 (m, 1H), 2.23–2.17 (m, 1H), 2.14–1.98 (m, 1H), 1.91–1.81 (m, 1H). LRMS calcd for C<sub>28</sub>H<sub>26</sub>FN<sub>2</sub>O<sub>3</sub>, [M + H]<sup>+</sup> 457.19, found 457.44.

BRD3098: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.50 (brs, 1H), 8.14 (d, 1H, J = 7.9 Hz), 8.00 (brs, 1H), 7.46 (d, 2H, J = 7.7 Hz), 7.36 (dd, 2H, J = 7.7, 8.0 Hz), 7.29–7.26 (m, 4H), 7.12 (dd, 1H, J = 7.2, 7.2 Hz), 7.00–6.94 (m, 5H), 6.89–6.86 (m, 2H), 4.91 (brs, 1H), 4.75 (brs, 1H), 4.53 (brs, 1H), 3.65–3.45 (m, 4H), 3.32 (brs, 1H), 3.04–2.97 (m, 2H), 2.76–2.73 (brm, 1H), 2.19–2.09 (m, 4H), 1.14 (d, 3H, J = 6.7 Hz), 0.89 (d, 3H, J = 6.0 Hz). LRMS calcd for C<sub>36</sub>H<sub>41</sub>N<sub>4</sub>O<sub>5</sub>, [M + H]<sup>+</sup> 609.73, found 609.34.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.6b00206.

ADME/PK data for key compounds BRD3098, WXL1631, and BRD0761 (Supplementary Table 1), methods associated with Table 1, and characterization data of medicinal chemistry analogues (Supplementary Table 2) (PDF)

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### Notes

The authors declare no competing financial interest.

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