

# Mechanism of Action of and Mechanism of Reduced Susceptibility to the Novel Anti-*Clostridium difficile* Compound LFF571

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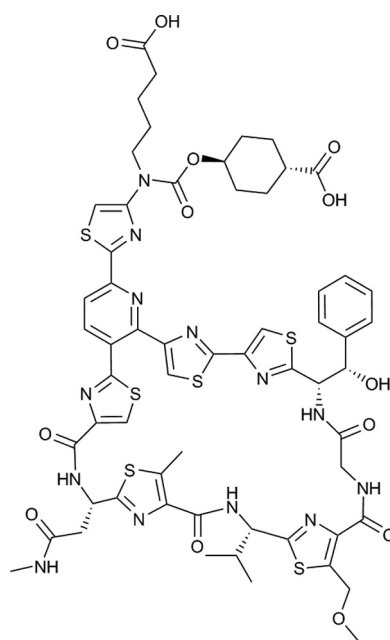
**LFF571 is a novel semisynthetic thiopeptide and potent inhibitor of Gram-positive bacteria. We report that the antibacterial activity of LFF571 against *Clostridium difficile* is due to inhibition of translation. Single-step mutants of *C. difficile* with reduced susceptibility to LFF571 were selected at frequencies of  $<4.5 \times 10^{-11}$  to  $1.2 \times 10^{-9}$ . Sequencing revealed a G260E substitution in the thiopeptide-binding pocket of elongation factor Tu. Importantly, this mutation did not confer cross-resistance to clinically used antimicrobials. These results support the development of LFF571 as a treatment for *C. difficile* infection.**

*Clostridium difficile* is a Gram-positive, anaerobic, spore-forming bacterium that is the leading cause of hospital-acquired antibiotic-associated diarrhea (22). Infection is potentially fatal and can involve complications such as severe colitis and toxic megacolon. The incidence of *C. difficile* infection (CDI) has risen dramatically over the past decade, and current estimates of 400,000 U.S. cases annually are almost quadruple the rates from the 1990s (1). This increase is associated with the emergence of hypervirulent strains, including B1/NAP1/027. CDIs are now more commonly found outside the hospital setting and in previously low-risk groups, such as children, pregnant women, and individuals with irritable bowel syndrome (1). Management of CDI involves treatment with the antibacterials metronidazole, vancomycin, and fidaxomicin. Unfortunately, recurrent disease occurs in 20 to 25% of patients receiving metronidazole and vancomycin (2, 11, 18). Less recurrence has been noted for fidaxomicin in clinical trials, although the risk was unchanged for patients infected with B1/NAP1/027 (8, 16, 17).

We have recently reported the discovery of LFF571 (14), a semisynthetic derivative of the natural metabolite GE2270 A (21). The structure of LFF571 is shown in Fig. 1. GE2270 A is a translation inhibitor that binds bacterial elongation factor Tu (EF-Tu) and blocks delivery of aminoacylated tRNA (aa-tRNA) to the ribosome (19). Like GE2270 A, LFF571 has antimicrobial activity against a range of Gram-positive bacteria, including *C. difficile* (4, 10, 14; S. Bushell, M. J. LaMarche, J. A. Leeds, and L. Whitehead, 18 June 2009, international patent application WO 2009/074605). LFF571 MIC<sub>90</sub> values obtained in two independent studies were 0.25 and 0.5 μg/ml against *C. difficile* isolates (4, 12). Here, we investigate the mechanism of LFF571 and the frequency and mechanism of reduced susceptibility to this compound. An accompanying article by Trzasko et al. examines the efficacy of LFF571 in a Golden Syrian hamster model of CDI (21a).

The MICs of LFF571 against the five *C. difficile* strains used in this study are shown in Table 1. LFF571, which was synthesized at Novartis according to published methods (Bushell et al., international patent application WO 2009/074605), demonstrated potent antibacterial activity against all *C. difficile* strains tested, with MICs of  $\leq 0.5$  μg/ml.

To investigate LFF571's mechanism of action, we monitored translation and cell wall biosynthesis by the incorporation of [<sup>3</sup>H]leucine (Perkin Elmer, Billerica, MA) or *N*-[<sup>3</sup>H]acetylglucosamine (American Radiolabeled Chemicals, St. Louis, MO), re-



**FIG 1** Chemical structure of LFF571. LFF571 is a semisynthetic thiopeptide derived from the natural product GE2270 A (21).

spectively. Mid-exponential-phase cultures of *C. difficile* NB95026 in chemically defined medium (13) supplemented with 0.2% glucose, 0.5 μg/ml vitamin K<sub>1</sub>, and 5 μg/ml hemin were treated with radiolabeled precursor for 60 min at 37°C under anaerobic conditions (15). For LFF571, the 50% inhibitory concentration (IC<sub>50</sub>) for [<sup>3</sup>H]leucine incorporation (0.06 μg/ml) was similar to the antibacterial concentration under the same testing conditions (0.03 μg/ml) (Table 2). In contrast, no inhibition of cell wall synthesis

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**TABLE 1** MICs of LFF571 against *C. difficile* strains used in the study<sup>a</sup>

Strain	Description	LFF571 MIC (µg/ml)
NB95002	Clinical isolate <sup>b</sup>	0.125
NB95013	ATCC 43255	0.5
NB95026	Clinical isolate (MOH838) <sup>c</sup>	0.5
NB95031	Clinical isolate (MOH082, REA type AA) <sup>c</sup>	0.5
NB95047	Clinical isolate (MOH108, REA type J) <sup>c</sup>	0.25

<sup>a</sup> MICs were determined by agar dilution methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5). REA, restriction endonuclease analysis.

<sup>b</sup> Novartis collection.

<sup>c</sup> Kindly provided by Donald Low at Mount Sinai Hospital, Toronto, Canada.

was observed, even after treatment with >30× the antibacterial concentration. Similar results were seen for translational inhibitor tetracycline (Sigma-Aldrich, St. Louis, MO), while the peptidoglycan synthesis inhibitor vancomycin (US Pharmacopeia, Rockville, MD) (20) blocked incorporation of [<sup>3</sup>H]UDP-GlcNAc, but not [<sup>3</sup>H]leucine. These results indicate that the antibacterial activity of LFF571 is via inhibition of *C. difficile* protein synthesis.

To characterize the frequency of selection of spontaneous mutants with reduced susceptibility to LFF571, *C. difficile* suspensions (10<sup>9</sup> to 10<sup>10</sup> CFU/ml) were plated on brucella agar containing 0.5 to 1 µg/ml (1 to 4× MIC) antibiotic and incubated anaerobically for 48 to 72 h at 37°C. Resistance frequency was defined as the number of colonies selected divided by total CFU plated. Reduced susceptibility to LFF571 was observed at the following frequencies: 1.7 × 10<sup>-10</sup> (NB95002 selected at 0.5 and 1 µg/ml LFF571), 1.2 × 10<sup>-9</sup> and <6.2 × 10<sup>-10</sup> (NB95013 at 0.5 and 1 µg/ml, respectively), and 3.0 × 10<sup>-11</sup> and <3.0 × 10<sup>-11</sup> (NB95026 at 0.5 and 1 µg/ml, respectively). We were unable to select colonies of NB95031 under the conditions tested (<4.5 × 10<sup>-11</sup>).

To understand the genetic basis for reduced susceptibility to LFF571, mutants were analyzed for changes in EF-Tu. *C. difficile* possesses two identical copies of the gene encoding EF-Tu, *tufA* and *tufB* (Table 3), which were amplified using primers to non-identical flanking sequences: 5'-CTTACCATAAGCTTATTGTGAGCA-3' (forward) and 5'-GAGGAGCATAACCCCTCTTT-3' (reverse) for *tufA* and 5'-ATTCGATCACTATGAGCAAGTTC-3' (forward) and 5'-TATATGCTTTAGCGCTACTTGTC-3' (reverse) for *tufB*. All mutants exhibited *tufB* mutation G782A, resulting in amino acid substitution G260E; NB95013-JAL0759 harbored the G782A change in both *tufA* and *tufB*.

We investigated whether reduced susceptibility to LFF571 resulted in cross-resistance to other antibiotics. Fidaxomicin was

**TABLE 2** Antibacterial and macromolecular inhibitory concentrations of LFF571

Test agent	IC <sub>50</sub> (µg/ml ± SD) <sup>a</sup>		Antibacterial concn (µg/ml) <sup>b</sup>
	[ <sup>3</sup> H]leucine	[ <sup>3</sup> H]NAG <sup>c</sup>	
LFF571	0.06 ± 0.004 (n = 5)	>1 (n = 5)	0.03
Tetracycline	10.2 ± 4.3 (n = 5)	>32 (n = 5)	8
Vancomycin	>32 (n = 5)	2.6 ± 1.9 (n = 6)	4

<sup>a</sup> Values represent means ± standard deviation (SD).

<sup>b</sup> Antibacterial concentrations were determined using the same medium and inoculum with which the macromolecular synthesis experiments were performed. Strain NB95026 was used for all assays.

<sup>c</sup> NAG, N-acetylglucosamine.

**TABLE 3** Genetic changes in *C. difficile* upon selection with LFF571<sup>a</sup>

Strain	Nucleotide/amino acid change		MIC (µg/ml) <sup>b</sup>										
	<i>tufA</i>	<i>tufB</i>	LFF571	VAN	MET	FDX	TET	CLI	ERY	MOX	LNZ		
NB95009	NC <sup>c</sup>	NC	0.25	1	0.5	0.03	64	2	1	2	2		
NB95002	NC	NC	0.5	0.5	1	0.125	32	4	1	1	2		
NB95002-JAL0777	NC	G782A/G260E	>128	0.5	1	0.03	64	4	0.5	0.5	2		
NB95002-JAL0783	NC	G782A/G260E	>128	0.5	1	0.03	32	4	0.5	0.5	2		
NB95013	NC	NC	0.25	0.5	0.5	0.03	0.06	4	1	0.5	2		
NB95013-JAL0758	NC	G782A/G260E	>128	0.5	0.5	0.03	0.06	4	1	0.5	2		
NB95013-JAL0759	G782A/G260E	G782A/G260E	>128	1	0.5	0.03	0.06	4	1	0.5	2		
NB95026	NC	NC	0.25	0.5	0.5	0.06	0.06	2	1	32	2		
NB95026-JAL0792	NC	G782A/G260E	>128	0.5	0.5	0.06	0.125	2	1	32	2		

<sup>a</sup> All strains were selected on 0.5 µg/ml LFF571, except NB95002-JAL0783, which was selected on 1 µg/ml.

<sup>b</sup> MICs were determined by agar dilution methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5). VAN, vancomycin; MET, metronidazole; FDX, fidaxomicin; TET, tetracycline; CLI, clindamycin; ERY, erythromycin; MOX, moxifloxacin; LNZ, linezolid.

<sup>c</sup> NC, no change.

prepared at Novartis by fermentation of *Catellatospora* sp. strain Bp3323-81; its activity against the Clinical and Laboratory Standards Institute (CLSI) quality control strain of *C. difficile* (ATCC 70057) was within the acceptable range (6). Linezolid (from Pfizer as Zyvox) and moxifloxacin (from Bayer as Avelox) were extracted and purified at Novartis. Tetracycline, ampicillin, clindamycin, and erythromycin were purchased from Sigma-Aldrich; vancomycin and metronidazole were obtained from US Pharmacopeia. As expected, an increase in the LFF571 MIC was observed for the selected mutants ( $>128 \mu\text{g/ml}$  versus 0.25 to 0.5  $\mu\text{g/ml}$  for parental strains). Strains with reduced susceptibility to LFF571 continued to be sensitive to structurally and mechanistically unrelated antibiotics, including fidaxomicin, vancomycin, and metronidazole (Table 3). Sensitivity to the protein synthesis inhibitor tetracycline was also unchanged within the acceptable 2-fold range of the assay, and the decreased susceptibility of NB95026 to moxifloxacin was not affected by the *tufB* mutation.

In this study, we characterized the mechanisms of action and loss of susceptibility to LFF571. We hypothesized that the mechanism of action of LFF571 against *C. difficile* would parallel that of related semisynthetic monoacidic derivatives of GE2270 A against *S. aureus* (15). Indeed, LFF571 specifically blocked *C. difficile* protein synthesis. Furthermore, selection on inhibitory concentrations of LFF571 resulted in a substitution at the *C. difficile* residue analogous to G257 in *E. coli* EF-Tu. Substitutions at *E. coli* G257 render the protein resistant to the inhibitory activity of GE2270 A when measured in an *in vitro* translation assay (23). Along with our previous observation that LFF571 interacts directly with *E. coli* EF-Tu *in vitro* (9), these data support the hypothesis that LFF571 inhibits *C. difficile* translation by binding EF-Tu. The frequency of selection of single-step mutants with reduced susceptibility to LFF571 was low ( $\leq 1.2 \times 10^{-9}$ ); for comparison, Critchley et al. (7) reported frequencies of *C. difficile* with reduced susceptibility to the *metRS* inhibitor REP3123 of  $\leq 1 \times 10^{-8}$ . Our observation that only one amino acid substitution is selected in *C. difficile in vitro* likely explains the lower frequency of resistant colonies obtained for LFF571 compared to those of agents that select multiple, independent substitutions, including REP3123 (7). Overall, the excellent potency of LFF571, low frequency of susceptibility loss *in vitro*, and absence of cross-resistance to standard-of-care antibiotics support the development of LFF571 for treatment of CDI in humans.

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