Mechanism of action of, and mechanism of reduced susceptibility to the novel anti-\textit{Clostridium difficile} compound LFF571

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ABSTRACT

LFF571 is a novel semi-synthetic thiopeptide and potent inhibitor of Gram-positive bacteria. We report that the antibacterial activity of LFF571 against \textit{Clostridium difficile} is due to inhibition of translation. Single-step mutants of \textit{C. difficile} with reduced susceptibility to LFF571 were selected at frequencies of $<4.5 \times 10^{-11} - 1.2 \times 10^{-9}$. 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 \textit{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 US cases annually are almost quadruple rates from the 1990s (1). This increase is associated with the emergence of hyper-virulent strains, including B1/NAP1/027. CDI is 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 20-25% 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 with B1/NAP1/027 (8, 16, 17).

We have recently reported the discovery of LFF571 (14), a semi-synthetic derivative of the natural metabolite GE2270 A (21). 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* (3, 4, 10, 14). LFF571 MIC$_{90}$ 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.

The minimum inhibitory concentrations (MIC) of LFF571 against the five *C. difficile* strains used in this study are show in Table 1. LFF571, which was synthesized...
at Novartis according to published methods (3), demonstrated potent antibacterial
activity against all C. difficile strains tested, with MICs ≤ 0.5 μg/ml.

To investigate LFF571 mechanism of action, we monitored translation and cell
wall biosynthesis by the incorporation of 3H-leucine (Perkin Elmer, Billerica, MA) or 3H-
N-acetyl glucosamine (American Radiolabeled Chemicals, St. Louis, MO), respectively.
Mid-exponential cultures of C. difficile NB95026 in chemically defined medium (13)
supplemented with 0.2% glucose, 0.5 μg/ml vitamin K₁ and 5 μg/ml hemin were treated
with radiolabeled precursor for 60 minutes at 37°C under anaerobic conditions (15). For
LFF571, the fifty-percent inhibitory concentration (IC₅₀) for 3H-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 was
observed, even after treatment with >30x 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 3H-UDP-GlcNAc, but not 3H-leucine. These results indicate
that the antibacterial activity of LFF571 is via inhibition of C. difficile protein synthesis.

To characterize the frequency of selecting spontaneous mutants with reduced
susceptibility to LFF571, C. difficile suspensions (10⁹-10¹⁰ colony forming units
(CFU)/ml) were plated on Brucella agar containing 0.5-1 μg/ml (1-4x MIC) antibiotic and
incubated anaerobically for 48-72 hours 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 x 10⁻¹⁰ (NB95002 selected at 0.5
and 1 μg/ml LFF571), 1.2 x 10⁻⁹ and <6.2 x 10⁻¹⁰ (NB95013 at 0.5 and 1 μg/ml,
respectively), and $3.0 \times 10^{-11}$ and $<3.0 \times 10^{-11}$ (NB95026 at 0.5 and 1 $\mu$g/ml, respectively). We were unable to select colonies of NB95031 under the conditions tested ($<4.5 \times 10^{-11}$).

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'-CTTACCTATAAGCTTATTGTGAGCA-3' (forward) and 5'-GAGGAGCATAACCCCTCTTT-3' (reverse) for *tufA*, and 5'-ATTGCATTATGAAGCAGTTT-3' (forward) and 5'-TATATGCTTTAGCGCTACTTGTCC-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 prepared at Novartis by fermentation of *Catellatospora* sp. Bp3323-81; its activity against the Clinical and Laboratory Standards Institute (CLSI) quality control strain of *C. difficile* (ATCC 700057) 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 from US Pharmacopeia. As expected, an increase in LFF571 MIC was observed for the selected mutants (>128 $\mu$g/ml versus 0.25-0.5 $\mu$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 \textit{tufB} mutation.

In this study we characterized the mechanisms of action and loss of susceptibility to LFF571. We hypothesized that the mechanism of LFF571 against \textit{C. difficile} would parallel that of related semi-synthetic monoacidic derivatives of GE2270 A against \textit{S. aureus} (15). Indeed, LFF571 specifically blocked \textit{C. difficile} protein synthesis. Furthermore, selection on inhibitory concentrations of LFF571 resulted in a substitution at the \textit{C. difficile} residue analogous to G257 in \textit{E. coli} EF-Tu. Substitutions at \textit{E. coli} G257 render the protein resistant to the inhibitory activity of GE2270 A when measured in an \textit{in vitro} translation assay (23). Along with our previous observation that LFF571 interacts directly with \textit{E. coli} EF-Tu \textit{in vitro} (9), these data support the hypothesis that LFF571 inhibits \textit{C. difficile} translation by binding EF-Tu. The frequency of selecting single-step mutants with reduced susceptibility to LFF571 was low (≤1.2 \times 10^{-9}); for comparison, Critchley et al. (7) reported frequencies of \textit{C. difficile} with reduced susceptibility to the \textit{metRS} inhibitor REP3123 of ≤1 \times 10^{-8}. Our observation that only one amino acid substitution is selected in \textit{C. difficile} \textit{in vitro} likely explains the lower frequency of resistant colonies obtained for LFF571 compared to agents that select multiple, independent substitutions, including REP3123 (7). Overall, the excellent potency of LFF571, low frequency of susceptibility loss \textit{in vitro}, and no cross-resistance to standard of care antibiotics support the development of LFF571 for treatment of CDI in humans.
We acknowledge Jade Bojkovic and Stacey TiamFook technical assistance and Catherine Jones for editing the manuscript.

REFERENCES


FIGURE LEGENDS

Figure 1. Chemical structure of LFF571. LFF571 is a semi-synthetic thiopeptide derived from the natural product GE2270 A (21).

TABLES

Table 1. Minimum inhibitory concentrations of LFF571 against *C. difficile* strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>LFF571 MIC (µg/ml)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB95002</td>
<td>Clinical isolate⁶</td>
<td>0.125</td>
</tr>
<tr>
<td>NB95013</td>
<td>ATCC 43255</td>
<td>0.5</td>
</tr>
<tr>
<td>NB95026</td>
<td>Clinical isolate (MOH838)³</td>
<td>0.5</td>
</tr>
<tr>
<td>NB95031</td>
<td>Clinical isolate (MOH082, REA type AA)⁵</td>
<td>0.5</td>
</tr>
<tr>
<td>NB95047</td>
<td>Clinical isolate (MOH108, REA type J)⁶</td>
<td>0.25</td>
</tr>
</tbody>
</table>

¹Minimum inhibitory concentrations (MIC) were determined by agar dilution methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5).
²Novartis collection
³Kindly provided by Dr. Donald Low at Mount Sinai Hospital, Toronto, Canada
⁴Kindly provided by Dr. Donald Low at Mount Sinai Hospital, Toronto, Canada
Table 2. Antibacterial and macromolecular inhibitory concentrations of LFF571

<table>
<thead>
<tr>
<th>Test agent</th>
<th>IC₅₀ (µg/ml ± SD)</th>
<th>Antibacterial concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>³H Leucine</td>
<td>³H NAG</td>
</tr>
<tr>
<td>LFF571</td>
<td>0.06 ± 0.004 (n=5)</td>
<td>&gt;1 (n=5)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10.2 ± 4.3 (n=5)</td>
<td>&gt;32 (n=5)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>&gt;32 (n=5)</td>
<td>2.6 ± 1.9 (n=6)</td>
</tr>
</tbody>
</table>

*Values represent mean and standard deviation (SD). 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.

Table 3. Genetic changes in *C. difficile* upon selection with LFF571

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleotide/ Amino acid change</th>
<th>Minimal inhibitory concentration (µg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tuftA</td>
<td>tuftB</td>
</tr>
<tr>
<td>NB95009</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>NB95002</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>NB95002-JAL0777</td>
<td>g782a/G260E</td>
<td>&gt;128</td>
</tr>
<tr>
<td>NB95002-JAL0783</td>
<td>g782a/G260E</td>
<td>&gt;128</td>
</tr>
<tr>
<td>NB95013</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>NB95013-JAL0758</td>
<td>g782a/G260E</td>
<td>&gt;128</td>
</tr>
<tr>
<td>NB95013-JAL0759</td>
<td>g782a/G260E</td>
<td>&gt;128</td>
</tr>
<tr>
<td>NB95026</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>NB95026-JAL0792</td>
<td>g782a/G260E</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

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

*Minimum inhibitory concentrations (MIC) were determined by agar dilution methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines (5).

*b abbreviations: VAN = vancomycin, MET = metronidazole, FDX = fidaxomicin, TET = tetracycline, CLI = clindamycin, ERY = erythromycin, MOX = moxifloxacin, LNZ = linezolid. NC, no change.