Sub-inhibitory concentrations of LFF571 reduce toxin production by *Clostridium difficile*

Meena Sachdeva¹ and Jennifer A. Leeds¹#

¹Novartis Institutes for BioMedical Research, Infectious Disease Area, Emeryville, CA

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Corresponding author: Jennifer Leeds

Novartis Institutes for BioMedical Research

5300 Chiron Way, Emeryville, CA 94608 USA

Phone: (510) 923-2174; Fax: (510) 923-3747

jennifer.leeds@novartis.com
Abstract

LFF571 is a novel semi-synthetic thiopeptide antibacterial that is undergoing investigation for safety and efficacy in patients with moderate *C. difficile* infections. LFF571 inhibits bacterial protein synthesis by interacting with elongation factor Tu (EF-Tu) and interrupting complex formation between EF-Tu and aminoacyl-tRNA. Given this mechanism of action, we hypothesized that concentrations of LFF571 below those necessary to inhibit bacterial growth would reduce steady state toxin levels in *C. difficile* cultures. We investigated *C. difficile* growth and toxin A/B levels in the presence of LFF571, fidaxomicin, vancomycin and metronidazole. LFF571 led to strain-dependent effects on toxin production, including decreased toxin levels after treatment with sub-inhibitory concentrations, and more rapid declines in toxin production compared to inhibition of colony formation. Fidaxomicin, which is an RNA synthesis inhibitor, conferred a similar pattern to LFF571 with respect to toxin levels versus viable cell counts. Incubation of two toxigenic *C. difficile* strains with sub-inhibitory concentrations of vancomycin, a cell wall synthesis inhibitor, increased toxin levels in the supernatant compared to untreated cultures. A similar phenomenon was observed with one metronidazole-treated strain of *C. difficile*. These studies indicate that LFF571 and fidaxomicin generally result in decreased *C. difficile* toxin levels in culture supernatants, whereas treatment of some strains with vancomycin or metronidazole had the potential to increase toxin levels. Although the relevance of these findings remains to be studied in patients, reducing toxin levels with sub growth-inhibitory concentrations of antibiotic is hypothesized to be beneficial in alleviating symptoms.
Introduction

*Clostridium difficile* infection (CDI) is a serious gastrointestinal disease. Approximately 500,000 cases occur in the United States each year, making CDI the most common hospital-acquired infection (1), (2). The ability of *C. difficile* to form spores makes it difficult to remove from surfaces and allows *C. difficile* to spread easily within a healthcare setting. Recently, the incidence of the disease has been increasing and hyper-virulent strains, such as B1/NAP1/027 have been recognized (reviewed in (3)). The epidemiology of CDI has also been changing, and the disease is now more commonly seen outside the hospital environment. The standard of care for CDI is treatment with the antibiotics metronidazole or vancomycin. Fidaxomicin was approved in 2011 for treatment of *C. difficile* associated diarrhea.

*C. difficile* are anaerobes that opportunistically colonize the gut, often after treatment with broadly-acting antibacterials. CDI is an enterotoxin-mediated disease that can be sub-clinical or have symptoms ranging from mild diarrhea to severe pseudomembranous colitis, megacolon, bowel perforation, sepsis and death (1). *C. difficile*-encoded toxins are termed A, B and CDT. There has been substantial debate about the roles of each toxin in pathogenesis, and some consider both toxins A and B to be essential virulence factors (4, 5). Toxins A and B are large, multi-domain proteins that catalyze the glucosylation of Rho-GTPases. Inactivation of the cellular enzymes leads to deregulation of cytoskeleton arrangement and cell death, followed by mucosal inflammation and diarrhea (6). Toxin CDT is an ADP-ribosylating binary toxin with an unclear contribution to disease (6).

LFF571 (7) is a semi-synthetic thiopeptide antibiotic with potent *in vitro* activity against *C. difficile* (8, 9) and is efficacious in the treatment of *C. difficile* infection in animal models (10). LFF571 has recently been shown to be non-inferior to vancomycin in patients with moderate *C. difficile* infections (Mullane et al, submitted). LFF571 inhibits bacterial protein synthesis by binding to elongation factor Tu (EF-Tu) and preventing this translation factor from delivering an aminocyl-tRNA to the ribosome (11) (12). Because of this mechanism of action, we hypothesized that LFF571 would prevent toxin production at doses
below the growth-inhibitory concentration of the compound. In theory, this could reduce toxin-mediated diarrheal symptoms at lower concentrations and provide an advantage over other antibiotics with unrelated modes of action. Here, we show that sub-inhibitory or inhibitory concentrations of LFF571 and fidaxomicin decrease toxin levels in supernatants of *C. difficile* cultures, while sub- to inhibitory levels of vancomycin and metronidazole increase toxin levels from select strains.

**Materials and Methods**

**Antibiotics.** LFF571 and fidaxomicin (Lipiarmycin A3, prepared by fermentation of *Catellatospora* sp. Bp3323-81) were obtained from Novartis. Vancomycin and metronidazole were purchased from US Pharmacopeia (Rockville, MD).

**Organisms.** Bacterial strains used in this study were from the American Type Culture Collection (ATCC) or kindly provided by D. Low at Mount Sinai Hospital, Toronto, Canada (Table 1). All strains were routinely cultured on Brucella agar containing 5% sheep blood, 0.5 μg/ml vitamin K and 5 μg/ml hemin. Cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) with a gas mix environment of 10% hydrogen, 10% carbon dioxide and a balance of nitrogen.

**In vitro susceptibility testing, cell titering, and toxin sampling.** Minimal inhibitory concentrations (MIC) of the test agents were determined using the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (13). To select the appropriate test agent concentration ranges for toxin level and cell titer determinations, antibacterial activity was determined under the specific growth conditions used to generate the toxin assay samples. Briefly, 5-6 colonies of *C. difficile* strains from an overnight Brucella agar plate were suspended in pre-reduced tryptone yeast plus sodium thioglycolate (TY) broth supplemented with 0.5 μg/ml vitamin K, 5.0 μg/ml hemin. Duplicate suspensions of *C. difficile* (0.5 ml) were transferred to 96 deep-well plates and serial two-fold dilutions of the test agents were added to the appropriate wells. Cultures were incubated for 24 hrs at 37°C, anaerobically, unless otherwise indicated.
Once the optimal test agent concentration range was determined, experimental samples were generated from 0.5 ml cultures inoculated and incubated as described above. Viable cells were quantified by removing 110 µl and plating 10-fold serial dilutions onto Brucella agar plates, followed by incubating anaerobically at 37°C for 48 hours unless stated otherwise. The remaining culture was centrifuged for 10 min at 3500 xg and the supernatants were removed and stored at -20°C for 48-96 hours prior to assaying for toxin A/B levels.

**Toxin A/B analysis.** Total toxin A/B levels in *C. difficile* culture supernatants were measured by ELISA (Wampole, Techlab, Blacksburg, VA), according to the manufacturer’s recommendations. The assay uses microtiter plates pre-coated with polyclonal goat antibodies against toxins A and B. Briefly, 50 µl of horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse monoclonal anti-toxin A and goat polyclonal anti-toxin B) were added to each well, immediately followed by 100 µl of undiluted culture supernatant. The plates were incubated for 60 minutes at 37°C before washing five times with phosphate buffered saline (PBS). Subsequently, 100 µl of substrate (tetramethyl benzidine) were added and the samples were incubated for 10 minutes at room temperature. The reactions were stopped by the addition of 50 µl H2SO4 and signals were detected at A450 using a spectrophotometer (SPECTRAmax, Molecular Devices, Sunnyvale, CA). Purified toxin A and toxin B (tgcBIOMICS) were used as controls. The lower limits of detection in this assay were 1.25 ng/ml of purified toxin A and 6 ng/ml of purified toxin B.

To monitor toxin A and B levels separately, culture supernatants were analyzed by ELISA (tgcBIOMICS, Mainz, Germany), according the manufacturer’s instructions. Briefly, 100 µl of undiluted *C. difficile* culture supernatants were transferred to microtiter plates coated with antibodies to both toxins A and B. Individual toxins were detected by immediately adding 50 µl of HRP-conjugated specific anti-toxin A or anti-toxin B antibodies. Coated plates containing specimens plus conjugate were incubated for 60 minutes at 37°C before washing three times with wash buffer. Subsequently, 100 µl of substrate (tetramethyl benzidine) were added and the samples were incubated for 20 minutes at room temperature. The reactions were stopped by the addition of 50 µl of H2SO4 and signals were detected by
spectrophotometry (SPECTRAmax), followed by subtracting background \((A_{620})\) from signal \((A_{450})\). Purified toxin A and toxin B (tcgBIOMICS) were used as controls. The lower limits of detection in this assay were 1.25 ng/ml of purified toxin A and 0.6 ng/ml of purified toxin B.

**Results**

**Characterization of antibiotic MIC profiles and toxin A/B production patterns of five \(C.\) difficile strains.** Table 1 describes the antibiotic in vitro activities and toxin expression phenotypes of five strains of \(C.\) difficile, including three clinical isolates. The antibacterial concentrations of the test agents used in this study were determined under the TY broth growth conditions used for toxin analysis, as well as by CLSI recommended agar dilution methods. MIC results obtained were comparable under both conditions.

To measure combined toxin A/B levels in supernatants from these strains, we used a commercially available ELISA kit (Wampole). Using this assay, we confirmed the previously reported toxigenic phenotypes of \(C.\) difficile strains NB95013 (14), NB95016 (D. Low, personal communication), NB95029 (D. Low, personal communication) and NB95031 (D. Low, personal communication) (Table 1). NB95009 (ATCC700057), a nontoxigenic Clinical Laboratory Standards Institute (CLSI) quality control strain (15), was confirmed to be negative for toxin A/B expression (data not shown) and was used as a negative control in all toxin detection experiments. Using the tcgBIOMICS ELISA assay, which detects toxins A and B independently, we verified that the clinical strain NB95029 expresses only toxin B (Table 1).

**Decrease in toxin levels in supernatants of \(C.\) difficile NB95016 cultures precedes bacterial growth inhibition by LFF571 or FDX.** Strain NB95016 is a clinical isolate of the B1/NAP1/027 type, which has been associated with severe disease in humans. Using the Wampole ELISA assay, high levels of toxins A and B were detectable in the absence of antibiotic treatment (Figure 1). Total toxin A/B levels decreased in cultures treated with sub-growth inhibitory concentrations of LFF571 or fidaxomicin. In contrast, toxin A/B levels declined only in the presence of growth-inhibitory concentrations of vancomycin or metronidazole (Figure 1). Similar results were observed when toxin A and toxin B were
quantified separately (data not shown). These results indicate that, for this toxigenic C. difficile strain, the toxin inhibition conferred by vancomycin or metronidazole is the result of growth inhibition, which contrasts sharply with reduction of toxin levels conferred by sub-growth inhibitory concentrations of LFF571 and FDX.

Toxin levels in supernatants of C. difficile NB95013 treated with LFF571 or FDX drop more sharply compared to reduction in colony forming units. Strain NB95013 is a clinical isolate that produces high levels of detectable toxin A/B in culture (14). Treatment of NB95013 with increasing concentrations of LFF571 or fidaxomicin led to a dose-dependent decrease in viable cells, however toxin titers dropped much more sharply (Figure 2). Treatment with vancomycin or metronidazole resulted in a loss of culture viability that largely paralleled the decrease in toxin levels in culture supernatants.

Sub-inhibitory concentrations of metronidazole and vancomycin confer increased toxin levels in supernatants of C. difficile NB95031 cultures. Strain NB95031 is a clinical isolate of C. difficile. Toxins A and B were not detectable in supernatants from untreated cultures of NB95031 incubated for 24 hours (data not shown). This strain was therefore incubated in the presence of antibiotics for 72 hours prior to analysis. Viable cell counts declined with increasing concentrations of LFF571 or FDX (Figure 3). Toxin A/B levels were steady until the culture densities dropped by at least 5 logs, at which point toxin titers were near the lower limit of detection. In contrast, treatment of cultures with metronidazole or vancomycin led to an increase of toxin A/B levels at sub-inhibitory concentrations of the drugs, followed by a sharp decline at growth-inhibitory concentrations. These results suggest that these antibiotics enhance toxin production and/or release from some strains of C. difficile.

Toxin B levels in the supernatants of C. difficile strain NB95029 increase in the presence of vancomycin. Strain NB5029 is a clinical isolate that produces toxin B but not toxin A. Therefore, the tcgBIOMICS ELISA, which is more sensitive for the detection of toxin B than the Wampole assay, was used to assay this strain. In cultures treated with vancomycin, levels of toxin B in the supernatants increased before ultimately decreasing with
a decline in viable cell counts (Figure 4). This was similar to the pattern observed after vancomycin treatment of strain NB95031 (Figure 4). In contrast, toxin levels declined in cultures treated with increasing concentrations of LFF571 or FDX. Toxin levels in supernatants of cultures treated with increasing concentrations of metronidazole were steady until the antibacterial concentration was reached, after which toxin levels approached the lower limit of detection.

**Discussion**

LFF571 is a novel EF-Tu inhibitor that has been shown to be non-inferior to vancomycin in patients with moderate *C. difficile* infections and is safe and well tolerated after multiple daily doses (Mullane et al, submitted). As part of the microbiological profiling of LFF571, we investigated the effects of a range of concentrations of the compound on toxin production by *C. difficile*. A variety of *C. difficile* strains were used for analysis, including three recent clinical isolates. Two commercially available ELISA assays were used to monitor steady state *C. difficile* toxin levels in the culture supernatant. Since we could not confirm that the polyclonal antibodies used in these assays recognized all toxin A/B proteins equally, no attempts were made to quantitatively compare toxin production between strains. Instead, toxin A/B production and cell growth in the presence of increasing antibiotic concentrations were assessed for each *C. difficile* isolate independently.

Previous studies have shown that sub-inhibitory concentrations of antibiotics can have a variety of effects on bacterial species, including modulating expression of genes involved in stress, metabolism, and pathogenesis (reviewed in (16)). Sub-inhibitory concentrations of vancomycin and metronidazole, the standards of care for treatment of CDI, have previously been reported to increase toxin production in *C. difficile* cultures (17), (18). We recapitulated these results in two of four toxigenic strains treated with vancomycin and one of the strains treated with metronidazole, suggesting variability in the effects of sub-inhibitory drug concentrations between bacterial isolates. Vancomycin is an inhibitor of cell wall synthesis. Cell wall disruption may cause the release of intracellular toxins into the environment. Indeed, another cell wall biogenesis inhibitor, penicillin, has been
shown to increase *C. difficile* toxin release (18). Alternatively, vancomycin might induce gene expression or otherwise promote toxin production. Consistent with this, increased transcription of toxin A and B genes has been reported in exponentially growing *C. difficile* cultures treated with these drugs (17). The mode of increased toxin levels in supernatants after metronidazole treatment is not clear from what is known about the antibacterial mechanism of action. The sharp declines in viable cell counts in cultures treated with inhibitory versus sub-inhibitory concentrations, however, suggests a sublethal lytic effect may lead to release of toxin from some strains.

Because LFF571 inhibits translation, we hypothesized this antibiotic might decrease, rather than increase, toxin levels in *C. difficile* cultures. We observed that LFF571 led to decreases in toxin production at or below the growth-inhibitory concentrations of the compound in all four of the strains tested. While this is the first study to test the effects of LFF571 on toxin synthesis, other protein translation inhibitors, including REP3123 (19), RBx-11760 (20), and cadezolid (21), have previously been shown to reduce *C. difficile* toxin A/B levels *in vitro*. Interestingly, the protein synthesis inhibitor linezolid has been reported to increase toxin A and B transcription (17). In contrast, the transcription inhibitor fidaxomicin and its metabolite, OPT-1118, have been shown to inhibit toxin gene transcription and toxin production from strains B1/NAP1/027 and UK1 (22, 23). In our experiments, sub-growth inhibitory to inhibitory concentrations of fidaxomicin reduced toxin levels in a manner similar to LFF571.

In summary, *in vitro* treatment with the novel semi-synthetic thiopeptide LFF571 led to a reduction in toxin A/B production from various *C. difficile* strains. This differentiates the microbiological profile of LFF571 from that of metronidazole and vancomycin. The relevance of *in vitro* toxin inhibition to clinical infection, however, remains to be determined.

**Acknowledgments**

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References


linezolid on toxin gene transcription and production in *Clostridium difficile*. J Med Microbiol **57**:776-783.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Source and description</th>
<th>Toxin phenotypes</th>
<th>MIC µg/mL</th>
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<tr>
<td>NB95009</td>
<td>ATCC 700057 (VPI 11186)\textsuperscript{a}</td>
<td>A-/B-</td>
<td>0.5</td>
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<td>NB95013</td>
<td>ATCC 43255 (VPI 10463)</td>
<td>A+/B+</td>
<td>0.25</td>
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<td>A+/B+/CDT+</td>
<td>0.5</td>
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<tr>
<td>NB95029</td>
<td>Clinical isolate\textsuperscript{b} (MOH118, ribotype AA)</td>
<td>A-/B+</td>
<td>0.5</td>
</tr>
<tr>
<td>NB95031</td>
<td>Clinical isolate\textsuperscript{b} (MOH082, ribotype AA)</td>
<td>A+/B+</td>
<td>0.5</td>
</tr>
</tbody>
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\textsuperscript{a}CLSI MIC quality control strain \textsuperscript{b}kindly provided by D. Low, Mt. Sinai Hospital, Toronto, Canada
Figure Legends

Figure 1. Toxin production and cell viability after antibiotic treatment of strain NB95016. Strain NB95016 was grown for 24 hours in the presence of the indicated antibiotics. Remaining viable cells (log_{10} CFU/ml, lines) and toxin A/B production (A_{450}, bars) were measured. FDX, fidaxomicin; VAN, vancomycin; MET, metronidazole. Data are mean and SEM of three independent experiments, each with duplicate antibiotic treatments.

Figure 2. Toxin production and cell viability after antibiotic treatment of strain NB95013. Strain NB95013 was grown for 48 hours in the presence of the indicated antibiotics. Remaining viable cells (log_{10} CFU/ml, lines) and toxin A/B production (A_{450}, bars) were measured. FDX, fidaxomicin; VAN, vancomycin; MET, metronidazole. Data are mean and SEM of duplicate antibiotic treatments.

Figure 3. NB95031 demonstrates increased toxin levels at sub-inhibitory concentrations of metronidazole and vancomycin. Strain NB95031 was grown for 72 hours in the presence of the indicated antibiotics. Remaining viable cells (log_{10} CFU/ml, lines) and toxin A/B production (OD_{450}, bars) were measured. FDX, fidaxomicin; VAN, vancomycin; MET, metronidazole. Data are mean and SEM of duplicate antibiotic treatments.

Figure 4. NB95029 demonstrates increased toxin B levels at sub-inhibitory concentrations of vancomycin. Strain NB95029 was grown for 24 hours in the presence of the indicated antibiotics. Remaining viable cells (log_{10} CFU/ml, lines) and toxin B production (OD_{450}-620, bars) were measured. FDX, fidaxomicin; VAN, vancomycin; MET, metronidazole. Data are mean and SEM of duplicate antibiotic treatments; toxin B values for metronidazole-treated samples represent a single experiment.