Antibacterials Developed to Target a Single Organism: Mechanisms and Frequencies of Reduced Susceptibility to the Novel Anti-*Clostridium difficile* Compounds Fidaxomicin and LFF571

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Clostridium difficile is the most common cause of antibacterial-associated diarrhea. Clear clinical presentation and rapid diagnostics enable targeted therapy for *C. difficile* infection (CDI) to start quickly. CDI treatment includes metronidazole and vancomycin (VAN). Despite decades of use for CDI, no clinically meaningful resistance to either agent has emerged. Fidaxomicin (FDX), an RNA polymerase inhibitor, is also approved to treat CDI. Mutants with reduced susceptibility to FDX have been selected in vitro by single and multistep methods. Strains with elevated FDX minimum inhibitory concentrations (MICs) were also identified from FDX-treated patients in clinical trials. LFF571 is an exploratory agent that inhibits EF-Tu. In a proof-of-concept study, LFF571 was safe and effective for treating CDI. Spontaneous mutants with reduced susceptibility to LFF571 were selected in vitro in a single step, but not via serial passage. Although there are several agents in development for treatment of CDI, this review summarizes the frequencies and mechanisms of *C. difficile* mutants displaying reduced susceptibility to FDX or LFF571.

Therapy for a suspected infection is often started empirically, based on the most likely syndrome and local epidemiological data well before the causative organism is identified. Because many clinical syndromes can be caused by a number of different pathogens, singly or in combination, the initial empiric therapy of severe infection is selected to cover the most likely organisms; this often involves broad-spectrum coverage to ensure that the condition of the patient does not worsen while the precise etiology

is being determined microbiologically. However, in some circumstances, a physician can narrow the list of potential pathogens quickly and choose an initial therapy that is more organism specific. One of those indications is antibiotic-associated diarrhea, in which the leading cause is infection with *C. difficile* and rapid diagnostics can quickly identify whether it is likely to be the pathogen.

Clostridium difficile infection (CDI) can cause mild to severe intestinal disease (Cohen

Editors: Lynn L. Silver and Karen Bush

Additional Perspectives on Antibiotics and Antibiotic Resistance available at www.perspectivesinmedicine.org

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Cite this article as *Cold Spring Harb Perspect Med* 2016;6:a025445

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et al. 2010). A recently published survey of hospital inpatients from geographically diverse sites in the United States indicated that *C. difficile* was the most commonly reported nosocomial pathogen, and was the cause of 12.1% of health care–related infections in the institutions studied (Magill et al. 2014). The ability of *C. difficile* to form spores makes it difficult to remove from surfaces and allows it to spread easily and quickly within a health care setting. Over the past decade, the incidence of *C. difficile* infection has increased, and hypervirulent strains, such as B1/NAP1/027, have become more prevalent (McDonald et al. 2005). The epidemiology of CDI is also changing, as the disease is now more commonly seen outside the hospital environment and in some patients with no obvious underlying risk factors, compared with what was observed historically (Hensgens et al. 2014; Evans and Safdar 2015; Lessa et al. 2015).

Because the major risks for acquiring CDI are well defined, those, in combination with the clear clinical presentation and the availability of relatively rapid and accurate diagnostic tools for identifying toxigenic *C. difficile* (reviewed in Bagdasarian et al. 2015), allow for a presumptive diagnosis to be made quite confidently within a few hours following the onset of symptoms with appropriate therapy started quickly, and a definitive diagnosis can be made within a day or two, depending on the testing algorithm applied. The current guidelines for diagnosis and treatment of CDI (Cohen et al. 2010; Debast et al. 2014), depending on disease severity and number or risk of recurrences, include the generic antibiotics vancomycin and metronidazole, the former of which is active against Gram-positive bacteria only, and the latter having activity limited to obligate anaerobes (Baxter Healthcare Corp. Metronidazole Injection, UST RTU Product Insert, Deerfield, IL, 2009 [www.accessdata.fda.gov/drugsatfda_docs/label/2009/018657s029lbl.pdf]; and Baxter Healthcare Corp. Vancomycin Injection, USP Product Insert, Deerfield, IL, 2011 [www.accessdata.fda.gov/drugsatfda_docs/label/2012/050671s018lbl.pdf]). The antibacterial actions of these agents are owing to inhibition of functions that are essential to bacterial growth and survival, and the molecular target(s) of these

antibacterials are synthesized from more than one gene product (reviewed in Healy et al. 2000). This multigene/target feature coincides with the observation that there are no reports of single-step spontaneous mutations in *C. difficile* that confer high-level clinically relevant loss of susceptibility to either agent. Recently, a pan-European survey of *C. difficile* isolates from 2011 to 2012 showed >96% susceptibility to vancomycin and metronidazole, despite many decades of use of these agents to treat *C. difficile* infection (Freeman et al. 2015). Accordingly, there is little compelling clinical data from adequately powered and controlled studies to indicate that treatment failure for CDI is owing to antimicrobial resistance to either of these agents, and there are no breakpoints or routine susceptibility testing used to guide the choice of treatment for CDI (Bauer et al. 2009; Cohen et al. 2010; Debast et al. 2014). Unfortunately, recurrent disease occurs in ~20%–25% of patients whose symptoms resolved following treatment with VAN and MET (reviewed in Debast et al. 2014). As such, current clinical development programs monitor the impact of exploratory interventions on the rate of CDI recurrence following initial clinical cure.

In 2011, the first drug from a new class of antibacterials, fidaxomicin (FDX), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of *C. difficile*–associated diarrhea in adults ≥ 18 years of age (Optimer Pharmaceuticals, Difcid Package Insert, San Diego, CA, 2011 [www.accessdata.fda.gov/drugsatfda_docs/label/2011/201699s000lbl.pdf]). Marketing authorization followed shortly thereafter throughout the European Union, Japan, Australia, and Canada. Based on data from two phase III trials, FDX is also recommended, with moderate evidence, by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) as an alternative to vancomycin when patients are at risk of first recurrence or have had multiple recurrences (Debast et al. 2014). Infectious Disease Society of America (IDSA) has not yet updated its guidance to include FDX (Cohen et al. 2010); however, clinical practice advisory groups have published recommendations for its use (Surawicz et al. 2013). FDX inhibits the growth

of *C. difficile*, and other susceptible organisms, via inhibition of the clinically validated target bacterial RNA polymerase (Coronelli et al. 1975). The mechanism of inhibition and the predicted binding site for FDX on RNA polymerase (RNAP) is distinct from that of the rifamycins (reviewed in Srivastava et al. 2011) and there is no cross-resistance between FDX and the clinically used agents from this drug class (Goldstein et al. 2011).

There are multiple exploratory compounds with excellent in vitro potency against *C. difficile*, some of which have entered clinical development and have shown efficacy in patients with CDI (reviewed recently in Tsutsumi et al. 2014 and Ivarsson et al. 2015). Several of the antibacterials in clinical trials for treatment of CDI, including surotomycin and cadezolid, are derivatives of clinically used drugs (daptomycin and fluoroquinolones/oxazolidinones, respectively) and are inhibitors of clinically validated molecular targets (Locher et al. 2014b; Yin et al. 2015). LFF571 is an exploratory anti-*C. difficile* compound that has shown safety and efficacy in a phase II trial in humans for treatment of mild to moderate *C. difficile* infection (Mullane et al. 2015); however, LFF571 is derived from a novel scaffold that is not in clinical use and inhibits a molecular target, elongation factor Tu, that is also clinically unprecedented (LaMarche et al. 2012; Leeds et al. 2012).

LFF571 and FDX both interfere with the growth of *C. difficile* by inhibiting essential pathways that involve large, multimolecular complexes; however, because the molecular mechanisms by which they inhibit their respective targets can be interrupted by single amino acid substitutions within single-gene products, both are subject to reduction in antibacterial potency via selection for spontaneous, single-step mutants.

This review will summarize the mechanisms of action and review the literature reports of the frequencies and mechanisms of reduced susceptibility to FDX and LFF571. These agents exemplify relatively narrowly focused therapeutics in that, although both show antibacterial activity in vitro against a range of Gram-positive species, they have both been studied in humans

exclusively against *C. difficile* because of their remarkable potency against this pathogen (minimum inhibitory concentrations [MICs]₉₀ ≤ 0.5 μg/mL) (Citron et al. 2012; Goldstein et al. 2012; Hecht and Gerding 2012; Debast et al. 2013) and because their physicochemical properties result in the ability to achieve high drug concentrations in the target organ (colon) with very little systemic exposure (Sears et al. 2012; Ting et al. 2012; Bhansali et al. 2015).

ANTIMICROBIAL MECHANISM OF ACTION OF FDX

FDX is an 18-membered macrocyclic natural bacterial fermentation product that is identical in structure to lipiarmycin A3, tiacumicin B, and clostimicin B1 (Fig. 1) (Omura et al. 1986; Theriault et al. 1987; Bedeschi et al. 2014). For simplicity, this review will refer to this structure only as “fidaxomicin.” Published reports of the isolation of this chemical structure and the description of its mechanism of action dates back to 1975 (Coronelli et al. 1975). Macromolecular synthesis inhibition assays indicated that the mechanism of antimicrobial action of FDX was via rapid inhibition of RNA synthesis, which led to an almost simultaneous loss of protein synthesis (Coronelli et al. 1975; Sergio et al. 1975). However, the drug does not inhibit the translation machinery directly and only inhibits protein synthesis as a result of inhibition of transcription via binding to RNA polymerase (Sergio et al. 1975). FDX inhibits the very early steps in initiation of RNA synthesis, before the formation of a stable open complex, but does not inhibit elongation by RNAP (Sergio et al. 1975; Artsimovitch et al. 2012). Based on mapping of residues conferring loss of susceptibility to FDX, the drug is thought to bind portions of the β- and β'-subunits found in the RNA exit channel of RNAP and part of the switch region, including parts of switch 2 and switch 3 of the RNA polymerase (Srivastava et al. 2011; Artsimovitch et al. 2012).

Although FDX inhibits the function of RNAP from Gram-positive and Gram-negative organisms (Coronelli et al. 1975; Sergio et al. 1975; Sonenshein et al. 1977), it is active in vitro

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Fidaxomicin

LFF571

Figure 1. Chemical structures of fidaxomicin (FDX) and LFF571.

against only a limited range of Gram-positive aerobes and anaerobes, including *C. difficile* (Goldstein et al. 2012). FDX is a time-dependent inhibitor of *C. difficile* growth (Babakhani et al. 2011), is more potent in vitro against *C. difficile* than vancomycin or metronidazole (Goldstein et al. 2012), and in vitro FDX inhibits *C. difficile* toxin A/B production (Allen et al. 2013) and the outgrowth of *C. difficile* spores

(Allen et al. 2013). FDX is effective against all ribotypes of *C. difficile* tested in vitro (Goldstein et al. 2012), although the FDX MIC₉₀ is higher against B1/NAP1 strains of *C. difficile* than against other ribotypes (Goldstein et al. 2011), and clinical trials showed that FDX was less likely to prevent relapse from NAP1 strains of *C. difficile* (Louie et al. 2011; Cornely et al. 2012).

FREQUENCIES AND MECHANISMS OF REDUCED SUSCEPTIBILITY TO FDX

Point mutations conferring loss of susceptibility of *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, and *C. difficile* to FDX were typically selected in the genes encoding the β - and β' -subunits of RNAP (encoded by *rpoB* and *rpoC*, respectively) (Gualtieri et al. 2006; Kurabachew et al. 2008; Gualtieri et al. 2009; Seddon et al. 2011; Srivastava et al. 2011; Seddon and Sears 2012; Babakhani et al. 2014; Leeds et al. 2014). Shortly after the mechanism of action of FDX was first reported, Sonenshein et al. (1977) published the first report of *B. subtilis* mutants selected on superinhibitory concentrations of the compound that helped to validate RNAP as the target of the antibacterial activity. In early studies with *C. difficile*, Swanson et al. (1991) reported attempts to select mutants of a strain of *C. difficile* with reduced susceptibility to FDX; however, they were unable to do so using the reported experimental design

(the frequency of resistance was reported to be $<2.8 \times 10^{-8}$).

Seddon et al. (2011) first reported the in vitro selection of spontaneous single-step *C. difficile* mutants on superinhibitory concentrations FDX in a conference poster (Table 1). Based on the methods reported, mutant frequencies of $>1 \times 10^{-8}$ were observed. The study included two reference strains (ATCC 9689 and ATCC 700057), which were selected on four or eight times the MIC of FDX. Selected mutants had single amino acid changes in the β -subunit (Gly1074Lys, Val1143Phe, or Val1143Asp) or the β' -subunit (Asp237Tyr) of RNAP, which shifted the FDX MIC by 16- to >64 -fold above that observed for the parental strains. In a conference poster the following year, Seddon and Sears (2012) reported additional in vitro selection data for *C. difficile* (Table 1). The study included one reference strain (ATCC 9689) and one clinical isolate (ORG916) of *C. difficile*. Mutants were selected on 8–64 times the MIC of FDX at a frequency of

Table 1. Frequencies and mechanisms of spontaneous *C. difficile* mutants selected in a single step on FDX

Study	Strain	Mutant frequency	Selecting concentration	Amino acid change	Fold change in FDX MIC
Swanson et al. 1991	ATCC 9689	$<2.8 \times 10^{-8}$	4–8 $\times$ MIC	None	None
Seddon et al. 2011	ATCC9689	$>1 \times 10^{-8}$	4–8 $\times$ MIC	β -subunit G1074K; V1143F; V1143D	32 to $>64 \times$ MIC
	ATCC 700057	$>1 \times 10^{-8}$	4–8 $\times$ MIC	β' -subunit D237Y	16 $\times$ MIC
Seddon and Sears 2012	ATCC 9689	$\leq 4 \times 10^{-10a}$	16–64 $\times$ MIC	β -subunit V1143G; V1143D; β' -subunit R89G	64–8192 $\times$ MIC
	ORG 916	$\leq 5 \times 10^{-10a}$	8–32 $\times$ MIC	β -subunit Q1074K; Q1074H; V1143-G; β' -subunit I10R; R89G	16–64 $\times$ MIC
	ATCC 9689	$\leq 1.28 \times 10^{-8}$	4–8 $\times$ MIC	β -subunit Q1074K; V1143F; β' -subunit D237Y	16 $\times$ MIC
Babakhani et al. 2014	ATCC 700057	$<1.41 \times 10^{-9}$	4–8 $\times$ MIC	None	
	ORG911	$<3.58 \times 10^{-9}$	4–8 $\times$ MIC	None	
	ORG916	$<2.71 \times 10^{-9}$	4–8 $\times$ MIC	None	
	ATCC 9689	$\leq 1.3 \times 10^{-8}$	2–8 $\times$ MIC	ND	$\geq 64 \times$ MIC
Locher et al. 2014a	NCTC 13366	$\leq 7.2 \times 10^{-7}$	4–8 $\times$ MIC	ND	16 $\times$ MIC
	A-1410	1.1×10^{-6}	8 $\times$ MIC	ND	Not reported

^aCalculated from the methods/results.

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$\leq 4 \times 10^{-10}$, based on the methods reported. The FDX MIC against the selected mutants shifted 16–8192 times the MIC reported for the parental strains. Selected mutants had single amino acid substitutions within the RNAP β -subunit (Gln1074Lys, Gln1074His, Val1143Gly, and Val1143Asp) or β' -subunit (Ile10Arg and Arg89Gly).

In a more recent publication, the same group (Babakhani et al. 2014) described the results of selecting for spontaneous single-step mutants of four strains of *C. difficile* on $4\times$ and $8\times$ MIC of FDX (Table 1). The study included two reference strains (ATCC 700057 and ATCC 9689) and two clinical isolates (ORG911 and ORG916). Rifaximin and VAN were included as comparators for the selections. Although rifaximin selected for spontaneous single-step mutants of *C. difficile* at a frequency of $\leq 1.73 \times 10^{-7}$ on $8\times$ MIC of drug, no mutants were selected on $8\times$ MIC of FDX or vancomycin (VAN) and the resistance frequency was reported to be $< 1.4 \times 10^{-9}$. When assays were conducted with $4\times$ MIC of FDX, mutants of ATCC 9689 were selected at a frequency of 1.28×10^{-8} and the MIC of FDX against these mutants shifted 16-fold. The mutants harbored amino acid substitutions in RNAP subunit β (Gln1074Lys or Val1143Phe) or in subunit β' of Asp237Tyr. The same or greater magnitude of MIC shift (16- to ≥ 64 -fold) was observed for two mutants of *C. difficile* strains ATCC 700057 and ATCC 43255 that were selected via serial passage (Babakhani et al. 2014). The investigators also describe a mutant strain of ATCC 43255 with a ≥ 64 -fold shift in FDX MIC over the parental strain, but no mutant frequencies or amino acid substitutions were reported for this strain. None of the mutants selected on FDX showed reduced susceptibility to the clinically used RNAP inhibitor rifampin (RIF), supporting the model that the binding site of FDX does not overlap with that of RIF or other RNAP inhibitors that function through sites other than the switch region of RNAP (Srivastava et al. 2011). The investigators (Babakhani et al. 2014) described in vitro synergy studies with FDX and RIF, emphasizing the nonoverlapping mechanisms of

resistance, which raises the potential to combine the two agents to suppress resistance development to either agent alone; the outcomes of such combination studies have not been reported.

Locher et al. selected for single-step spontaneous mutants of *C. difficile* on $2-8\times$ MIC of FDX, as well as by serial passage (Table 1) (Locher et al. 2014a). Mutants were selected on $8\times$ MIC of FDX at much higher frequencies ($\leq 1.1 \times 10^{-6}$) than those reported by Babakhani et al. (2014). Spontaneous mutants were selected on all four strains of *C. difficile* that were tested including ATCC 9689 and three clinical isolates. Mutants selected in a single step were then subjected to two additional sequential rounds of selection on higher concentrations of FDX. The MICs of FDX against mutants selected in a single step or in multiple steps ranged from $2 \mu\text{g/mL}$ to $> 128 \mu\text{g/mL}$ and accounted for MIC shifts of eight- to > 4000 -fold over the parental strains. The mechanisms of resistance to FDX were not reported in this publication.

Leeds et al. subjected *C. difficile* strain ATCC 43255 and three clinical isolates of *C. difficile* to 10 passages on a range of concentrations of FDX (Leeds et al. 2014). The genomes of selected *C. difficile* mutants with reduced susceptibility to the selecting agents and their isogenic parental strains were fully sequenced to determine the single-nucleotide polymorphisms (SNPs) that were observed in the mutant background compared with the susceptible parent. When passaged on FDX, a 16-fold decrease in susceptibility was observed for clinical strain NB95013, and a 64-fold decrease in susceptibility was observed for clinical strain NB95026. There was a twofold change in FDX susceptibility for clinical strain NB95031 and a fourfold shift in FDX susceptibility for clinical strain NB95047. The NB95026 mutant showing a 64-fold decrease in susceptibility to FDX harbored a Gln1073Arg substitution in RNAP subunit β . Previous work by Seddon et al. (2011) had identified lysine and histidine substitutions at the same residue (numbered residue 1074 in Seddon et al. 2011 and Seddon and Sears 2012). The NB95013 mutant showing a 15-fold decreased

susceptibility to FDX harbored a deletion in CD22120 (*marR* homolog), resulting in a frameshift after amino acid 117 of a homolog of the MarR family of transcriptional regulators. This finding represented the first report of a mechanism outside RNAP that may alter susceptibility to FDX. Although MarR is associated with multidrug resistance, the investigators did not observe reduced susceptibility to antibiotics other than FDX in that study.

Although notable, the high variability observed for frequencies of selecting mutants of *C. difficile* with reduced susceptibility to FDX (Table 1) may be owing to variations in methodology combined with too few replicates in any one study; therefore, it is not clear if the differences are biologically meaningful.

***C. difficile* WITH REDUCED SUSCEPTIBILITY TO FDX ISOLATED FROM PATIENTS TREATED WITH FDX IN PHASE III CLINICAL TRIALS (STUDIES OPT-80-003 AND -004)**

Goldstein et al. reported that the FDX MIC for all baseline isolates from the modified intent-to-treat (mITT) and per protocol populations across both phase III studies was 0.25 µg/mL (Goldstein et al. 2011). Although there were no instances of FDX MIC >0.5 µg/mL among the baseline *C. difficile* isolates from the 26 patients in the per protocol population who were clinical failures following treatment with FDX, a single strain of *C. difficile* with reduced susceptibility to FDX was isolated from a FDX-treated, clinically cured patient who suffered a recurrence. The strain, which harbored a Val1143Gly substitution in the RNAP β-subunit was isolated at the time of recurrence and the FDX MIC was 16 µg/mL (Goldstein et al. 2011).

Eyre et al. reported whole genome sequences of the 28 pairs of isolates available at baseline and recurrence in FDX-treated patients from the ITT population in the pooled phase III studies (~41% of the total intent-to-treat (ITT) population receiving FDX who suffered recurrences of CDI during the study) (Eyre et al. 2014). In this analysis, isolates from two ITT patients who suffered recurrences were reported

to have mutations in the target gene *rpoB*. One of those isolates is the same one reported previously by Goldstein et al. (2011) against which FDX had a MIC of 16 µg/mL. In addition, a second strain was isolated from a patient with recurrence that showed a 10-fold reduction in susceptibility to FDX (FDX MIC = 0.015 µg/mL at baseline and 0.125 µg/mL at follow-up). The strain harbored a Val1143Leu substitution in RNAP β-subunit. Eyre et al. (2014) indicated that because neither mutation was observed in isolates from any other patient, it suggests that the mutants are uncommon. However, sequencing data from only 28 baseline and matched-patient postbaseline isolates from the FDX arm of the combined phase III trials, which included a total of 572 subjects who received FDX, have been reported, so more surveillance and posttreatment isolates would need to be tested to understand the true prevalence of such mutants. Eyre et al. (2014) also reported other nonsynonymous SNPs, including one in a hypothetical protein and one in a putative PTS system, which were identified in strains from patients treated with FDX who experienced a recurrence of disease following clinical cure at end of treatment. The extent to which any of these substitutions, whether in the target of FDX or elsewhere, contribute to recurrence of disease is unknown. Regular postmarketing surveillance data will be required to inform whether the use of FDX to treat *C. difficile* infection results in the selection for additional mutants with reduced susceptibility in patients or in the environment.

ANTIMICROBIAL MECHANISM OF ACTION OF LFF571

LFF571 is a semisynthetic derivative of the natural product GE2770A (Fig. 1) (LaMarche et al. 2012). Scientists at Le Petit Research Center identified GE2270A, a thiopeptide-based secondary metabolite isolated from the fermentation broth of *Planobispora rosea*, in a screen for *Staphylococcus aureus* growth inhibitors that were antagonized by the addition of exogenous elongation factor Tu (EF-Tu) into the assay system (Selva et al. 1991, 1997; Landini et al. 1996);

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Figure 2. EF-Tu functional cycle with point of LFF571 inhibition indicated. GDP, Guanosine diphosphate; GTP, guanosine triphosphate.

the structure assignment for GE2270A was corrected in subsequent publications (Tavecchia et al. 1994, 1995). As a human therapeutic candidate, LFF571 has a novel mechanism of action, in that, like the natural product precursor GE2270A, it binds to and inhibits the function of bacterial EF-Tu (Landini et al. 1996; Leeds et al. 2012).

EF-Tu is one of three proteins (along with EF-Ts and EF-G) that are required, after formation of the 70S initiation complex of the ribosome, to catalyze the subsequent steps in peptide chain elongation (Fig. 2) (reviewed in Voorhees and Ramakrishnan 2013). EF-Tu is the chaperone that delivers aminoacylated tRNAs to the A site of the ribosome in the form of the ternary complex EF-Tu • guanosine triphosphate (GTP) • aa-tRNA (Fahnestock et al. 1972; Stark et al. 1997). Subsequent GTP hydrolysis causes

the release of EF-Tu in complex with guanosine diphosphate (GDP). EF-Tu is then reactivated by the action of EF-Ts, which catalyzes the GDP/GTP nucleotide exchange and renders EF-Tu competent for carrying out the next round of tRNA delivery (Miller and Weissbach 1970).

Because the binding site overlaps with that of tRNA, LFF571 inhibits protein synthesis by interfering with the ability for EF-Tu to deliver aminoacylated tRNA to the ribosome (Deng et al. 2011; Leeds et al. 2012). There are several other natural products that are known to inhibit the function of EF-Tu by the same or other mechanisms including kirromycin, pulvomycin, thiomuracin, amithiamycin, and efrotomycin (Parmeggiani and Nissen 2006; Morris et al. 2009). Synthetic small molecule inhibitors of EF-Tu have also been disclosed in publications (Deibel et al. 2004; Jayasekera et al. 2005).

To date, no EF-Tu inhibitors have been registered for clinical use in humans, although a different analog of GE2270A with an unreported structure (BI-K-0376 or “VIC-acne”) was investigated as a topical agent for the treatment of acne (reviewed in Butler 2005).

LFF571, like FDX, is active in vitro against its molecular target (EF-Tu) from Gram-positive and Gram-negative organisms (Deng et al. 2011); however, the antibacterial spectrum of LFF571 is limited to Gram-positive aerobes and anaerobes (Citron et al. 2012). The K_d of LFF571 binding to *C. difficile* EF-Tu • GDP is ~ 130 nM (Deng et al. 2011). LFF571 is effective against all ribotypes of *C. difficile* tested ($MIC_{90} = 0.25$ $\mu\text{g/mL}$) and is more potent in vitro against *C. difficile* than vancomycin and metronidazole (Citron et al. 2012; Debast et al. 2013). LFF571 was shown to be efficacious and safe in a proof-of-concept study in humans with mild to moderate CDI (Mullane et al. 2015), and to have low oral bioavailability and high fecal concentrations in infected patients (Bhansali et al. 2015). Anticipated by the novel mechanism of antibacterial activity, LFF571 is potent against bacteria with reduced susceptibility or resistance to marketed antibiotics (Citron et al. 2012). Supporting its use as a therapeutic for CDI, LFF571 reduces *C. difficile* toxin A/B levels in culture supernatants even at sub-MIC concentrations (Sachdeva and Leeds 2015).

FREQUENCIES AND MECHANISMS OF REDUCED SUSCEPTIBILITY TO LFF571

LFF571 is a semisynthetic derivative of the natural EF-Tu inhibitor GE2270A. The GE2270A-producing strain of *P. rosea* has a naturally occurring, functional EF-Tu variant (EF-Tu1) that is not susceptible to inhibition by GE2270A (Mohrle et al. 1997). Zuurmond et al. (2000) systematically introduced individual amino acid substitutions that are found in the native *P. rosea* EF-Tu1 into the GE2270A-sensitive EF-Tu protein from *E. coli*. They found that only two individual substitutions could account for the naturally occurring resistance to GE2270A, namely, Gly257Ser or Gly275Ala (*E. coli* num-

bering), both of which are found in GE2270A-binding region of EF-Tu domain II. The locations of the substitutions in the protein are supported by the cocrystal data showing that GE2270A binds to domain II of bacterial EF-Tu, making contact with residues 215–230, 256–264, and 273–277 (Heffron and Jurnak 2000).

EF-Tu is one of the most abundant proteins in the cell, representing up to 10% of the total protein content (Ishihama et al. 2008). There is approximately one EF-Tu molecule for every tRNA and this ratio is constant under different growth conditions (Furano 1975). In many organisms, including *C. difficile*, there are two copies of the genes encoding EF-Tu (*tufA* and *tufB*) (Jaskunas et al. 1975; Sela et al. 1989; Ke et al. 2000; Leeds et al. 2012). One altered copy of EF-Tu is sufficient for reduced susceptibility to GE2270A or LFF571 (Zuurmond et al. 2000; Leeds et al. 2012), unlike the mechanism of resistance to kirromycin, an inhibitor of the GTPase activity of EF-Tu, against which reduced susceptibility is only achieved when both copies of *tuf* are mutated (reviewed in Parmeggiani and Nissen 2006). Nevertheless, despite the high copy number of the target protein and the dominance of resistance, single-step spontaneous mutants of *C. difficile* with reduced susceptibility to LFF571 are selected in vitro at low frequencies ($\leq 1.2 \times 10^{-9}$) (Table 2) (Leeds et al. 2012). Mutant selections were attempted with four strains of *C. difficile* (three clinical strains and one high-toxin expressing-type strain ATCC 43255); reduced susceptibility to LFF571 was observed at the following frequencies: 1.7×10^{-10} (NB95002 selected at 0.5 and 1 $\mu\text{g/mL}$ LFF571), 1.2×10^{-9} and $< 6.2 \times 10^{-10}$ (NB95013 at 0.5 and 1 $\mu\text{g/mL}$, respectively), and 3.0×10^{-11} and $< 3.0 \times 10^{-11}$ (NB95026 at 0.5 and 1 $\mu\text{g/mL}$, respectively). No mutants of NB95031 were selected on LFF571 under the conditions tested ($< 4.5 \times 10^{-11}$).

All *C. difficile* mutants selected in vitro on LFF571 showed *tufB* mutation G782A, resulting in amino acid substitution G260E; NB95013-JAL0759 harbored the G782A change in both *tufA* and *tufB*, which are identical in sequence in this pathogen (Leeds et al. 2012). Residue

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Table 2. Frequencies and genotypes of spontaneous *C. difficile* mutants selected in a single step on LFF571

Strain	Selecting concentration	Mutant frequency	Mutant selected on LFF571	Amino acid change	Fold change in LFF571 MIC
NB95002	4 × MIC	1.7×10^{-10}	NB95002-JAL0777	<i>tufB</i> : G260E	> 256 × MIC
NB95013	1 × MIC	1.2×10^{-9}	NB95013-JAL0758	<i>tufB</i> : G260E	> 512 × MIC
			NB95013-JAL0759	<i>tufA</i> : G260E <i>tufB</i> : G260E	> 512 × MIC
NB95026	2 × MIC	$< 6.2 \times 10^{-10}$	None	None	
	1 × MIC	3.0×10^{-11}	NB95026-JAL0792	<i>tufB</i> : G260E	> 512 × MIC
	2 × MIC	$< 3.0 \times 10^{-11}$	None	None	
NB95031	1 × MIC	$< 4.5 \times 10^{-11}$	None	None	
	2 × MIC	$< 4.5 \times 10^{-11}$	None	None	

G260 in *C. difficile* EF-Tu is synonymous with G257 in *E. coli* EF-Tu, one of the two residues in which amino acid substitutions resulted in GE2270A-resistant EF-Tu. The proposed mechanism of resistance to LFF571 is that, rather than displacing the inhibitor, the G260E gain-of-function substitution restores the interactions between aa-tRNA and EF-Tu • GTP • LFF571. Several lines of evidence support the model that LFF571 and tRNA bind simultaneously to the mutant EF-Tu • GTP complex. First, LFF571 retains weak antibacterial activity against the EF-Tu G260E mutants (MIC 128–256 µg/mL) (Leeds et al. 2012). Second, Zuurmond et al. (2000) reported that the affinity of the natural product inhibitor GE2270A for wild-type *E. coli* EF-Tu • GTP is the same as for the G260S mutant, and in the presence of the GE2270A, the affinity of aa-tRNA for the G260S mutant increases 100× over its affinity for wild-type EF-Tu • GTP/GE2270A. Finally, the affinity of GE2270A for *E. coli* EF-Tu • GDP is dramatically decreased by G260S, so on GTP hydrolysis at the ribosome, the inhibitor more quickly dissociates, leaving the aa-tRNA in the A site. Therefore, the mechanism of resistance to this thiopeptide class, in general, may not be a result of decreased affinity of the inhibitor to EF-Tu • GTP; rather, the mutant restores the ability of the GTP-bound enzyme to bind the substrate aa-tRNA and the mutant confers a more rapid dissociation of the inhibitor from the mutant EF-Tu/ribosomal complex once the aa-tRNA has engaged the A site (Zuurmond et al. 2000). This proposed mechanism of resis-

tance to LFF571 is a model based on the behavior of the natural analog GE2270A and would require structural and affinity studies with LFF571 to be validated.

CONCLUDING REMARKS

FDX and LFF571 are novel antibacterial agents with clinically unprecedented mechanisms of target inhibition that exemplify compounds with a clinical focus directed at a single organism—*C. difficile*. Both compounds select for reduced susceptibility in *C. difficile* in vitro, and mutants with reduced susceptibility to FDX have been reported to be isolated at the time of recurrence from two patients who received FDX as a treatment for CDI. Because *C. difficile* is a spore-forming species, selection for mutants in patients could lead to dissemination of mutant spores, which, over time, could shift the susceptibility of the compounds for *C. difficile* strains in the general population. Routine susceptibility testing is not used to guide the choice of appropriate therapy for CDI. Therefore, it would fall to regular surveillance studies or epidemiological data from outbreaks to inform trends in the loss of susceptibility to such agents. Whether such a shift in susceptibility would have an impact on the ability to treat *C. difficile* with such agents is unknown, especially given that the concentrations of these agents in the colon, at the site of antibacterial action, is typically well above the MIC for the mutants (Louie et al. 2011; Bhansali et al. 2015).

Although not approved for use, other drugs, such as rifaximin and fusidic acid, against which high-frequency single-step resistance can be selected in *C. difficile*, are occasionally used to treat CDI. Attempts to understand the impact of mutants with reduced susceptibility to these agents on therapeutic outcome have been reported (Noren et al. 2006; Mattila et al. 2013). For example, a study comparing fusidic acid with metronidazole for the treatment of CDI showed that although all isolates from patients randomized to fusidic acid were susceptible at baseline, 55% (11/20) of posttreatment isolates were resistant to fusidic acid (median MIC >256 µg/mL) (Noren et al. 2006). Clinical failure and relapse, among those treated with fusidic acid, occurred in 12% and 34% of the patients with fusidic acid-resistant strains posttreatment versus 6% and 17% of the patients with fusidic acid-sensitive strains posttreatment. Persistence of *C. difficile* in stool culture at follow-up predicted failure/relapse regardless of treatment assignment. One or two nonsynonymous substitutions in EF-G (the target of fusidic acid) were responsible for the shift in susceptibility in *C. difficile* (Noren et al. 2007). Fusidic acid fecal concentrations have not been directly reported; however, ~2% of an oral dose is excreted unchanged (Reeves 1987).

Recently, Adams et al. reported that the serial passage of *C. difficile* in subinhibitory concentrations of the phase III CDI compound surotomycin resulted in mutants with an eight- to 16-fold shift in susceptibility to both surotomycin and daptomycin, the clinically used natural product antibiotic from which surotomycin is derived (Adams et al. 2015). In addition, surotomycin also selected for mutants of *Enterococcus faecium* and *E. faecalis* that are cross-resistant to daptomycin (Mascio et al. 2014). Therefore, even with antibiotics for which the mechanism of inhibition involves a complex membrane target, rather than a single-gene product, one can select for loss of susceptibility that is stable and imparts cross-resistance of other pathogens to the drugs used clinically to treat infections with those organisms.

Perhaps with the increasing focus on new agents for CDI, especially in the era of target-

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based drug discovery, the paradigm for guiding appropriate treatment will shift, and will encompass some form of susceptibility testing to insure adequate therapy. Or perhaps the high drug exposures will obviate the need. The data coming from the many trials in CDI, as well as the surveillance studies for newly registered drugs, will help to inform this decision.

ACKNOWLEDGMENTS

I am grateful to Johanne Blais for helpful suggestions and assistance with the presentation of data.

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