Fidaxomicin reduces early toxin A and B production and sporulation in Clostridium difficile in vitro

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INTRODUCTION

Clostridium difficile infection (CDI) is a leading cause of hospital- and community-acquired antibiotic-associated diarrhoea worldwide. In the United States alone, more than three million cases of CDI are reported per year, costing an estimated 4.8 billion US dollars annually [1]. Increases in the clinical severity and mortality of CDI are largely attributed to the emergence and rapid dissemination of the epidemic BI/NAP1/027 strain [2, 3]. NAP1 strains are reported to produce more cytotoxin [toxin A (TcdA) and toxin B (TcdB)] and display increased sporulation and resistance to standard antimicrobial therapy when compared to previous strains [2, 3]. Recurrent episodes of CDI are also of major clinical concern, and up to 35% of patients will experience a second CDI following standard antimicrobial treatment [4], resulting in additional hospitalizations and healthcare-associated costs.

Until recently, the antimicrobial modalities for treating C. difficile infections remained virtually unchanged more than 20 years. Traditional treatment strategies for CDI included supportive care, the discontinued use of systemic antibiotic therapy (resolves ~23% of all CDI cases) and, for more serious cases, the oral administration of vancomycin or intravenous metronidazole [5]. In addition, the therapeutic options for recurrent or refractory CDIs remained largely undefined. More recently, fidaxomicin (Dificid), a narrow-
spectrum macrocyclic antibiotic, was approved by the US Food and Drug Administration as a suitable treatment option for patients with severe and recurrent CDIs [6]. Fidaxomicin is a RNA synthesis inhibitor that exhibits strong antimicrobial activity against vegetative C. difficile in vitro. During phase III clinical trials, oral administration of fidaxomicin successfully resolved CDI in patients with moderate to severe infections and was associated with non-inferior rates of CDI recurrence when compared to vancomycin [6, 7].

Toxigenic strains of C. difficile produce TcdA and TcdB in response to nutrient limitations during stationary-phase growth. However, our laboratory and several others previously demonstrated that some antibiotics at sub-inhibitory concentrations actually stimulate early and prolonged TcdA and TcdB production, even during exponential-phase growth, by historical and epidemic strains of C. difficile [8–10]. Recently, Babakhani et al. showed that when it was introduced to stationary-phase organisms at sub-inhibitory concentrations, fidaxomicin significantly decreased TcdA and TcdB production by C. difficile [11]. These same authors also demonstrated that sub-MIC levels of fidaxomicin reduced sporulation by C. difficile when added during stationary-phase growth [12]. Still, the initial effects of fidaxomicin on early exotoxin production and sporulation by C. difficile organisms have never been explored.

This study was designed to determine the effects of sub-inhibitory concentrations of fidaxomicin on early TcdA and TcdB gene expression and protein production by both historical and epidemic strains of C. difficile. In addition, the ability of fidaxomicin to promote sporulation prior to stationary-phase growth was also determined and compared to those of other antibiotics currently used to treat CDIs. Understanding the comprehensive effects of fidaxomicin on exotoxin production, virulence factor expression and sporulation is important when considering fidaxomicin as a potential treatment for severe and recurrent C. difficile infections.

**METHODS**

### C. difficile strains

Two strains of C. difficile were studied. American Type Culture Collection (ATCC) strain 9689 is a historical strain that was determined as toxinotype 0 by toxinotyping. ATCC 5325 is a B1/NAPI/027 clinical isolate collected in 1993 (a kind gift from Dr Stuart Johnson; Edward Hines, Jr VA Hospital, Hines, IL, USA) that was determined as a toxinotype III strain by toxinotyping. A Bactron II anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA) was utilized to maintain an anaerobic environment for C. difficile growth and manipulation.

### Determination of minimum inhibitory concentrations (MICs)

The antibiotic fidaxomicin was provided by the sponsor Optimer Pharmaceuticals (now Cubist Pharmaceuticals). The MIC of this antibiotic was determined for the C. difficile ATCC 5325 and 9689 strains by microbroth dilution assay according to the CLSI guidelines for such testing in anaerobes [13], and as we have previously described [14]. In brief, 200 µl of a stationary overnight culture was used to inoculate 20 ml of pre-reduced brain heart infusion (BHI) broth. Cultures were grown anaerobically for approximately 2 h at 37°C until a turbidity equal to the 0.5 McFarland standard [OD₆₀₀ of 0.08–0.1; ~1×10⁸ colony-forming units (c.f.u.) ml⁻¹] was achieved. Fifty µl of the C. difficile culture was then added to duplicate wells of a 96-well plate containing 50 µl of 2-fold serially diluted (2⁻⁵₀₀⁰ ng ml⁻¹) fidaxomicin. All MIC plates were prepared in the anaerobic chamber and incubated anaerobically at 37°C for 48 h. Following this, culture-containing wells were mixed by gently tapping the plate, and growth (turbidity) was assessed by a microplate reader (OD₆₅₀). MICs were defined as the lowest antibiotic concentration that inhibited measurable bacterial growth (i.e. OD₆₃₀ equal to the negative control). MICs were performed five times in triplicate.

### Growth curves and RNA isolation

C. difficile isolates were cultured anaerobically overnight in BHI, whereupon bacteria were collected by centrifugation and washed once with fresh BHI. The pellet was resuspended to the original volume (10 ml) in fresh BHI and an aliquot of washed bacteria (4–6 ml) was added to 100 ml of fresh, pre-equilibrated BHI to an OD₆₃₀ of 0.08–0.1. The culture was then added to 900 ml of fresh, pre-equilibrated BHI and grown anaerobically to an OD₆₃₀ of 0.08–0.1. Aliquots (199 mls) of this stock culture were divided among five individual sterile 500 ml flasks. Fidaxomicin was prepared in sterile water as a 200× stock solution with reference to the highest concentration required. Twofold serial dilutions were made from this stock in sterile water and 1 ml of each appropriate stock was added to 199 ml of C. difficile culture to give final concentrations of 1/4×, 1/8× and 1/16× MIC, respectively. Sterile water (1 ml) served as a negative treatment control. At times of ~3 h (stock culture split), 0 h (antibiotics added), and 6, 12, 24 and 48 h after antibiotic addition, 10 ml samples were removed from each C. difficile culture, and a small aliquot (20 µl) was used to determine viable c.f.u. The remaining organisms were collected by centrifugation (13 000 g) and used to prepare total RNA as described below for PCR analysis of toxin gene expression. Resultant supernatants were filter-sterilized and frozen at −70°C for soluble TcdA/B production by ELISA.

### Analysis of toxin gene expression

RNA was isolated from collected bacterial pellets using the RiboPure-Bacteria kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations. Contaminating genomic DNA was removed by two rounds of DNase treatment (DNA-free kit; Ambion), and the final RNA yield and quality was assessed by UV absorbance and agarose-gel electrophoresis, respectively. Changes in tcdA, tcdB and spo0A gene expression were assessed by real-time reverse transcriptase PCR (RT-PCR). For cDNA synthesis, 1 µg of total RNA was converted to cDNA using 10 U ml⁻¹...
M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA), 1 mM random hexamer primers (Invitrogen, Carlsbad, CA, USA) and 0.5 mM dNTPs (Invitrogen); all final concentrations. Reverse transcriptase reactions were performed at 37 °C for 2 h and terminated by heating to 95 °C for 5 min. cDNA samples were diluted 1:40 in sterile water for both C. difficile strains and used for subsequent 16S rRNA (internal control gene) PCR. For tcdA and tcdB RT-PCR, 5325 and 9689 cDNA samples were also diluted 1:40 and 1:4, respectively, in sterile water. In brief, real-time PCR was performed via a 7500 Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) by using the RT<sup>2</sup> real-time SYBR green/Rox PCR master mix (SuperArray, Frederick, MD, USA) and the following cycles: 10 min at 95 °C and then 40 cycles each at 95 °C for 15 s and 55 °C for 60 s. The primer sequences used for RT-PCR are listed in Table 1. Relative gene expression was determined using the methods we described previously [8, 14]. cDNA was generated from 1 µg of total RNA and diluted 1:40 in sterile water. C. difficile 16S rRNA served as the internal control. Real-time RT-PCR was performed on a 7500 Fast Real-time PCR system using the RT<sup>2</sup> real-time SYBR green/Rox PCR master mix and the following cycles: 10 min at 95 °C and then 40 cycles each at 95 °C for 15 s and 55 °C for 60 s. Relative gene expression was determined using the method. The sample mean Ct of 16S rRNA (internal control gene) was subtracted from the sample mean Ct of each experimental sample (ΔCt). The Delta-Ct (ΔCt) of the no-treatment control taken at 6 h was subtracted from the mean ΔCt of each experimental sample (ΔΔCt). This 2<sup>−ΔΔCt</sup> method yields the fold change in gene expression of the gene of interest normalized to the expression of the 16S rRNA internal control and relative to the no-treatment control taken at 6 h.

### Analysis of toxin production

Soluble TcdA and TcdB levels were measured (in combination) in collected culture supernatant using the Wampole Tox A/B II kit (TechLabs, Blacksburg, VA, USA) according to the manufacturer’s recommendations. TcdB purified from a NAP1 isolate (a kind gift from Dr Jimmy Ballard, University of Oklahoma Health Sciences Center; stock concentration 300 µg ml<sup>−1</sup>) was used to construct a standard curve. Samples were diluted when necessary to obtain readings within the linear range of the standard curve. All samples were tested in triplicate.

### Sporulation

To determine the number of spores produced by C. difficile strains over the growth cycle, 0.5 ml aliquots were collected from antibiotic- and control-treated cultures at 6, 12, 24 and 48 h post-treatment. Collected samples were mixed with 0.5 ml of 100 % ethanol for 1 h with rotation at room temperature to kill vegetative organisms [15]. The samples were then pelleted by centrifugation (13 000 g for 5 min) and washed twice in PBS. Following the last wash, the pellets were resuspended in 0.5 ml PBS. The spores were enumerated by serially diluting the samples in PBS and plating them onto BHI agar plates. The plates were incubated anaerobically at 37 °C for 72 h and the resultant c.f.u. ml<sup>−1</sup> were deemed to represent the relative numbers of viable spores produced.

### RESULTS

### Minimum inhibitory concentrations of historical and recent NAP1 strains

The MIC for fidaxomicin was determined for both the historical 9689 and epidemic 5325 C. difficile strains. According to the CLSI guidelines, C. difficile organisms have an expected MIC range of 0.03–0.25 µg ml<sup>−1</sup>. Both strains of C. difficile strains were sensitive to fidaxomicin. The historical 9689 strain displayed a strong sensitivity (MIC=8 ng ml<sup>−1</sup>), while the NAP1/027 5325 strain exhibited an MIC value similar to those obtained for other strains of C. difficile (MIC=250 ng ml<sup>−1</sup>) [11] (Table 2).

### Growth of historical and epidemic C. difficile strains in the presence of fidaxomicin

Similar growth dynamics were noted between the antibiotic-free cultures for the two C. difficile strains. Both strains

### Table 1. Primer sequences for amplification of C. difficile tcdA and tcdB gene sequences

<table>
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<th>Gene</th>
<th>Primer</th>
<th>Accession no.</th>
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<td>tcdA</td>
<td>tcdA-F</td>
<td>CAACACCCTAACCAGCCATA AM180355</td>
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<tr>
<td></td>
<td>tcdA-R</td>
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<tr>
<td></td>
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<td>TGATGGTGCAGAAAGAGTG AM180355</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16S-F</td>
<td>AGGGGTGAAATGGTAGATAT AM180355</td>
</tr>
<tr>
<td></td>
<td>16S-R</td>
<td>CAGCGTCAGTTACAGTCAGA AM180355</td>
</tr>
</tbody>
</table>

### Table 2. Minimum inhibitory concentrations of fidaxomicin, vancomycin, metronidazole and tigecycline using the microbroth dilution method

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ATCC 9689</th>
<th>NAP1/027 5325</th>
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</thead>
<tbody>
<tr>
<td>Fidaxomicin</td>
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</tr>
<tr>
<td>Vancomycin</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.024</td>
<td>0.048</td>
</tr>
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</table>
peaked at approximately 6 h post-inoculation \(10^8\) c.f.u. ml\(^{-1}\) for the 9689 strain (Fig. 1a), \(10^9\) c.f.u. ml\(^{-1}\) for the 5325 strain (Fig. 2a), and a nearly 1 log c.f.u. ml\(^{-1}\) decrease was observed during early stationary-phase growth (6–24 h) for both organisms. From 24–48 h (stationary phase), growth by the 9689 strain recovered slightly \((-0.5\) log\) (Fig. 1a), whereas growth by the 5325 strain continued to decrease \((-0.5\) log\) (Fig. 2a).

The addition of fidaxomicin at 1/16\(\times\) and 1/8\(\times\) MIC had minimal effect on the growth of either organism (Figs 1b, c and 2b, c). However, at 1/4\(\times\) MIC, fidaxomicin reduced the viability for both the historical and epidemic strains. Specifically, a greater than 2 log reduction was observed between 6–24 h for the 9689 strain, and a 1 log decrease was observed for the 5325 strain from 6 to 10 h, whereupon both organisms recovered to have similar levels to the untreated cultures (Figs 1d and 2d).

**Fidaxomicin and tcdA and tcdB gene expression and toxin production**

Measurable tcdA and tcdB gene expression was observed at \(-12\) h and peaked at \(-24\) h in the antibiotic-free culture of the historical 9689 strain. In contrast, all sub-inhibitory concentrations of fidaxomicin significantly and dose-dependently suppressed the expression of both tcdA (solid line) and tcdB (dashed line) by the 9689 strain between 12–48 h (Fig. 1a–d). Maximal repression of tcdA and tcdB gene expression was observed in the 1/4\(\times\) MIC (2 ng ml\(^{-1}\))-treated culture, as virtually no tcdA and tcdB message was detected throughout the entire growth period (Fig. 1d). Dose-dependent reductions in toxin protein production by

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**Fig. 1.** Effects of fidaxomicin on the growth, toxin gene expression and soluble toxin production by the historical C. difficile 9689 strain. (a) Antibiotic-free cultures; (b) 1/16\(\times\) MIC (0.0005 µg ml\(^{-1}\)); (c) 1/8\(\times\) MIC (0.001 µg ml\(^{-1}\)); and (d) 1/4\(\times\) MIC (0.002 µg ml\(^{-1}\)). Fidaxomicin was added, at the final concentrations indicated, during early log-phase growth (designated time 0). Samples were collected in duplicate over 48 h for the quantification of viable C. difficile, and for the measurement of toxin gene expression by real-time RT-PCR, and TcdA/B production by ELISA. The fold-change in mRNA expression for each strain was made relative to that in its respective no-treatment control at 6 h (which was virtually zero). Filled line, tcdA mRNA; dotted line, tcdB mRNA.
the 9689 strain were also observed following exposure to all sub-MIC doses of fidaxomicin. However, substantial TcdA/B production was seen at 48 h in the 1/16 MIC (0.5 ng ml\(^{-1}\))-treated culture (Fig. 1b; peak 507 ng ml\(^{-1}\)) when compared to the untreated control culture (Fig. 1a; peak 710 ng ml\(^{-1}\)).

Similar to the 9689 strain, sub-inhibitory concentrations of fidaxomicin dose-dependently suppressed \(tcdA\) and \(tcdB\) gene expression by the epidemic 5325 NAP1 strain throughout the stationary phase of growth (\(\sim 12\)–48 h; Fig. 2a–d). Specifically, 50- and 10-fold decreases in \(tcdA\) and \(tcdB\) transcript, respectively, were observed in cultures containing fidaxomicin at 1/4\(\times\) MIC (62.5 ng ml\(^{-1}\)) at 24 h (Fig. 2d).

Likewise, the toxin expression levels were also lower for all other fidaxomicin treatments tested between 12–48 h when compared to the no-treatment control culture. Consistent with the findings described for toxin gene expression, 3–12 ng ml\(^{-1}\) of fidaxomicin (1/16 – 1/4\(\times\) MIC) dose-dependently decreased soluble TcdA/B levels in stationary-phase supernatants from the 5325 NAP1 strain when compared to the no-treatment control culture (Fig. 2a–d).

**Fig. 2.** Effects of fidaxomicin on the growth, toxin gene expression and soluble toxin production by the epidemic \textit{C. difficile} 5325 strain. (a) antibiotic free cultures; (b) 1/16\(\times\) MIC (0.016 µg ml\(^{-1}\)); (c) 1/8\(\times\) MIC (0.031 µg ml\(^{-1}\)); and (d) 1/4\(\times\) MIC (0.063 µg ml\(^{-1}\)). Fidaxomicin was added, at the final concentrations indicated, during early log-phase growth (designated time 0). Samples were collected in duplicate over 48 h for the quantification of viable \textit{C. difficile}, and for the measurement of toxin gene expression by real-time RT-PCR, and TcdA/B production by ELISA. the fold-change in mRNA expression for each strain was made relative to that in its respective no-treatment control at 6 h (which was virtually zero). Filled line, \(tcdA\) mRNA; dotted line, \(tcdB\) mRNA.
cultures of both strains (Fig. 3a, b). Greater sporulation was observed for the hypervirulent 5325 strain, and this was expected since NAP1 strains are commonly high spore producers [16].

Following 48 h exposure to sub-MIC fidaxomicin, dose-dependent reductions in viable spore formation were observed in both the historical 9689 and epidemic 5325 strains (Fig. 3a, b). Like tcdA and tcdB gene expression, fidaxomicin at 1/4× MIC also decreased spo0A transcription in both the 9689 and 5325 strains (data not shown), resulting in a commensurate 35 and 65 % decrease in spore production, respectively (Fig. 3a, b). While reduced at 1/16× MIC, sporulation in the 9689 (~10 %) and 5325 (~15 %) strains was at similar levels to those detected in the no-treatment control cultures. Compared to the number of spores estimated by visual inspection by light microscopic analysis (data not shown), approximately 0.1 % of spores germinated and grew as colony-forming units on BHI agar plates. These values are in agreement with the germination rates and outgrowth efficiencies reported by Lawley et al. [15].

Sub-MIC doses of vancomycin and tigecycline had similar effects on sporulation for both the 9689 and 5325 strains. As shown in Fig. 3a, b, all concentrations of tigecycline decreased sporulation in both strains considerably by 48 h. Similarly, vancomycin at 1/4× (250 ng ml⁻¹) and 1/8× (125 ng ml⁻¹) MIC reduced sporulation in both strains at 48 h (between 10–50 %). At 1/16× MIC (62.5 ng ml⁻¹), sporulation by the 5325 strain was inhibited (~15 %); however, the viable spore counts were comparable to those of the antibiotic-free control for the 9689 strain (Fig. 3a, b). In addition, all sub-inhibitory concentrations of metronidazole suppressed sporulation by the historical 9689 at 48 h (Fig. 3a). In contrast, a ~2 log increase in spore production was measured for all doses of metronidazole tested for the epidemic 5325 strain (Fig. 3b).

**DISCUSSION**

Fidaxomicin (Dificid, Dificlir), a macrocyclic antibiotic secreted by the actinomycete Dactylosporangium aurantiacum, is a narrow-spectrum antibacterial that interferes with RNA transcription by binding to the DNA template–RNA polymerase complex. It is minimally absorbed when administered orally and displays potent bactericidal activity against *C. difficile* while disrupting the normal bowel flora minimally [17]. Following its approval, fidaxomicin has achieved clinical success in treating CDIs, and in some cases was considered to be superior to vancomycin in reducing recurrent cases of disease [18].

It is widely accepted that the pathogenesis of *C. difficile* infection is commonly initiated by the disruption of the normal gut microbiota by various antibiotics, thus providing a niche for the germination of spores from toxigenic strains of *C. difficile*. However, a newer perception of CDI pathogenesis has emerged, suggesting that antibiotics themselves cause *C. difficile* organisms to become more virulent. Specifically, we and others have demonstrated that several strains of *C. difficile*, when exposed to sub-inhibitory doses of antibiotics that either promote CDI (i.e. amoxicillin, clindamycin and cephalosporins) or treat the infection (i.e. metronidazole and vancomycin), up-regulated toxin production by these organisms, despite having different mechanisms of action [8–10].

In our study, all sub-MIC fidaxomicin-containing cultures reduced toxin gene expression and soluble toxin production during both exponential and early stationary-phase growth for both the historical 9689 and epidemic 5325 strains. While still below the levels of the antibiotic-free control,
moderate amounts of tcdA/tcdB message and soluble exotoxin protein production were detected by both the historical and epidemic strains of C. difficile when exposed to extremely low levels of fidaxomicin (1/16× MIC: 0.5 and 15.6 ng mL⁻¹, respectively). These observations agree with those made by Babakhani et al. who looked at the effects of sub-inhibitory concentrations of fidaxomicin on TcdA and TcdB production in late stationary-phase organisms (up to 14 days) [11]. In vivo, fidaxomicin is poorly absorbed into the bloodstream following oral administration and collects mainly in the gastrointestinal tract, where concentrations in the stool are well above the MICs determined for C. difficile [19]. We hypothesize that fidaxomicin’s ability to block mRNA synthesis is largely responsible for reducing the production of TcdA and TcdB message and protein.

The transmission and recurrence of C. difficile infection is facilitated by environmentally resistant spores. Our study showed that even at sub-inhibitory levels, fidaxomicin reduced sporulation at 48 h in both the historical 9689 and epidemic 5325 strains. These findings also paralleled observations made by Babakhani et al., who demonstrated that when added to late stationary-phase organisms, sub-MIC fidaxomicin reduced spore formation in multiple isolates of C. difficile [12]. These data suggest that the decrease in sporulation is attributable to the inhibition of mRNA transcript production by fidaxomicin and may explain, at least in part, the extremely low recurrence rate of CDI in patients receiving fidaxomicin therapy [7, 20]. While similar decreases in sporulation were observed for both strains following exposure to tigecycline and vancomycin, only metronidazole stimulated sporulation (~2 log increase) by the epidemic 5325 strain at 48 h. These data support the observations made by us and others that the effects of antibiotics on C. difficile sporulation can vary, depending on the antibiotic and the strain of C. difficile being tested.

In conclusion, several classes of antibiotics can trigger the onset of CDI. Our study demonstrated that minimal concentrations of fidaxomicin immediately suppressed toxin gene expression and production, and reduced sporulation by both historical and epidemic strains of C. difficile, even when at sub-inhibitory concentrations. The data collected in this study suggest that fidaxomicin’s clinical success in treating CDI may be explained by its ability to reduce toxin production and sporulation by C. difficile promptly.

References
20. Morrow T. Fewer recurrent infections of C. difficile seen with fidaxomicin. This new class of antibiotic—the macrocyclic–has a greater sustained response against re-infection than vancomycin. Manag Care 2011;20:49–50.