Both Fidaxomicin and Vancomycin Inhibit Outgrowth of Clostridium difficile Spores

Charlotte A. Allen¹, Farah Babakhani², Pam Sears², Ly Nguyen² and Joseph A. Sorg¹*

¹Department of Biology, Texas A&M University, College Station, TX
²Optimer Pharmaceuticals, Inc., San Diego, CA

Running Title: Inhibition of Clostridium difficile spore outgrowth

*Corresponding Author. Mailing Address: Texas A&M University, Department of Biology, 3258 TAMU, College Station, TX 77843. Phone: (979) 845-6299. Fax: (979) 845-2891. Email: jsorg@bio.tamu.edu

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Fidaxomicin (FDX) is approved to treat Clostridium difficile associated diarrhea, and is superior to vancomycin in providing a sustained clinical response (cure without recurrence in the subsequent 25 days). Mechanism(s) behind the low recurrence rate of FDX-treated patients could be multifactorial. Here, we tested effects of FDX, its metabolite OP-1118, and vancomycin on spore germination and determined that none affected the initiation of spore germination but all inhibited outgrowth of vegetative cells from germinated spores.
**Clostridium difficile**, a Gram-positive, spore-forming, obligate anaerobe, causes intestinal infections, usually in people who have recently completed antibiotic therapies for unrelated conditions (17). Antibiotics cause alterations in the normally protective colonic microbiota, creating a niche for *C. difficile* to colonize (14, 23). To cause disease, *C. difficile* spores, which are unaffected by inciting antibiotics, must germinate to vegetative, or actively growing, bacteria in the anaerobic environment of the colon in order to produce the toxins that are responsible for the primary disease symptoms (8, 9, 12). Therefore, *C. difficile* spores, which are highly resistant to chemical disinfectants and antibiotics, are the source of infection. Although the exact mechanism and receptors involved in *C. difficile* spore germination are not clearly defined, both taurocholate and glycine have been identified as factors that synergistically stimulate germination of spores into virulent vegetative cells that secrete potent toxins (18, 22).

Vancomycin and metronidazole are commonly prescribed to treat *C. difficile* infections (CDI) (17). However, patients treated with vancomycin or metronidazole frequently relapse with *C. difficile* disease (17). Recently, fidaxomicin (FDX) was approved in the US, Europe and Canada as an alternative for the treatment of CDI. During Phase 3 clinical trials, FDX was shown to be superior to vancomycin in sustaining clinical response without recurrence up to 25 days following treatment (11, 21).

Multiple factors may lie behind the reduced rate of relapsing CDI in FDX-treated patients. Both FDX and its main metabolite OP-1118 have been shown to strongly inhibit *C. difficile* spore formation (3). FDX also has a reduced impact on the normally protective colonic
microbiota (4, 20). In this study, we evaluated whether FDX might block germination of *C. difficile* spores.

To test the effect of FDX on *C. difficile* spore germination, we purified spores from *C. difficile* strains CD196 (13) and *C. difficile* UK1 (7), as described previously (19). To provide a quantitative measure of the effects of FDX, OP-1118 and vancomycin on *C. difficile* spore germination, we analyzed the kinetics of the initiation of spore germination. By measuring the maximum rate of spore germination under different conditions, we are able to determine an apparent Km for taurocholic acid (TA), a *C. difficile* spore germinant, defined as the concentration that provides half-maximal germination rate. This strategy has been used to test the effects of different compounds on *C. difficile* spore germination (6, 10, 16, 19), *C. sordellii* spore germination (10, 15) and *Bacillus anthracis* spore germination (1). If the drugs were to affect the initiation of spore germination, a change in apparent Km should be detected.

*C. difficile* UK1 and *C. difficile* CD196 spores were suspended in BHIS (Brain Heart Infusion + 5 g/L Yeast Extract + 0.1% L-cysteine) alone or in BHIS supplemented with 2 mM TA or 5 mM TA or 10 mM TA or 20 mM TA or 50 mM TA and germination was assayed at room temperature by following changes in A$_{600}$ using a PerkinElmer Lambda 25 spectrophotometer. As expected, *C. difficile* UK1 spores (Figure 1A) and *C. difficile* CD196 spores (Figure 1B) did not germinate in BHIS medium alone; TA is required for *in vitro* *C. difficile* spore germination (18, 22). Moreover, we observed a concentration-dependent, TA-dependent increase in the rate of *C. difficile* spore germination. To analyze the effects of FDX, OP-1118 and vancomycin on *C. difficile* spore germination, we added 0.25 μg/mL FDX (2x MIC) or 2.5 μg/mL OP-1118 (2.5x MIC) or 2.5 μg/ml vancomycin (2.5x MIC) and measured the kinetics of *C. difficile* spore germination. Germination in the above conditions was followed
in triplicate samples; a representative sample is shown in Figure 1. Shown in Table 1 are the apparent Km values for TA in the presence or absence of the tested drugs, as determined from the data in Figure 1 and two additional replicates. The addition of FDX, OP-1118 or vancomycin had no effect on the apparent Km for TA, which was similar to the previously reported value (19). Moreover, none of these compounds induced germination, suggesting that these antibiotics do not affect the initiation of spore germination either positively or negatively. Similar findings on spore germination were observed with *C. difficile* strain UK14 (7) with concentrations that are supra-inhibitory for vegetative growth: 200x MIC FDX, 25x MIC OP-1118 (data not shown).

Since these drugs do not affect the initiation of spore germination, we tested whether later stages of germination would be affected. We assayed outgrowth by following changes in $A_{600}$ in an anaerobic environment (10% hydrogen, 5% CO$_2$, 85% nitrogen) over a 24-hour period. *C. difficile* UK1 spores (Figure 2A) or *C. difficile* CD196 spores (Figure 2B) were suspended in BHIS medium alone or BHIS medium supplemented with 2 mM TA [BHIS(TA)] or BHIS(TA) supplemented with 0.25 μg / mL FDX or 2.5 μg / mL OP-1118 or 2.5 μg / ml vancomycin. Spores suspended in BHIS medium alone did not germinate during the duration of the experiment (data not shown). Spores suspended in BHIS(TA) showed reduced absorbance initially, indicating spore germination, but showed an increase in the $A_{600}$ at approximately 2 hours, which continued for the duration of the experiment. We did not detect outgrowth (i.e., the rise in $A_{600}$ subsequent to the initial drop) when *C. difficile* UK1 spores or *C. difficile* CD196 spores were suspended in BHIS(TA) with FDX or OP-1118. However, when outgrowth was measured in the presence of vancomycin, we observed an increase followed by a decrease in $A_{600}$, suggesting that outgrowth had begun but was then inhibited by vancomycin. We
confirmed by phase-contrast microscopy that the increase in absorbance observed for \textit{C. difficile} UK1 (Figure 2A) and \textit{C. difficile} CD196 (Figure 2B) was correlated with the disappearance of phase-bright spores and appearance of phase-dark spores and vegetative cells in the suspension (Figure 2C). Spores incubated with any of the antibiotics transitioned from phase-bright to phase-dark, but no vegetative cells appeared when antibiotics were present (Figure 2C). These results suggest that FDX, OP-1118 and vancomycin inhibit outgrowth of vegetative cells from \textit{C. difficile} spores, though FDX and OP-1118 may inhibit outgrowth at an earlier stage than does vancomycin because spores germinated in the presence of FDX or OP-1118 did not show any increase in OD during germination. This result is in agreement with the different mechanism of action of the two drugs and supports previous observations that compounds which inhibit cell wall synthesis inhibit later stages of outgrowth (5). In contrast, FDX, which targets RNA polymerase and therefore inhibits production of RNA and proteins, completely inhibited outgrowth throughout the duration of the experiments (2).

In addition to inhibition of spore formation, inhibiting the conversion of \textit{C. difficile} spores to actively growing vegetative cells could be a powerful way to prevent relapsing CDI. We found that FDX, OP-1118 and vancomycin do not affect the initiation of \textit{C. difficile} spore germination, but do inhibit outgrowth of a vegetative cell from the germinating spore. This action would also prevent the synthesis of toxins and any downstream effects. Thus, FDX, OP-1118 and vancomycin inhibit some of the earliest stages in \textit{C. difficile} pathogenesis but the effects of FDX on \textit{C. difficile} spore germination, alone, cannot explain the reduced frequency of relapsing CDI. Use of a narrow spectrum antibiotic that inhibits spore formation and also prevents the outgrowth of germinated spore could be especially powerful, since it would allow most of the microbiota time to recover and reestablish colonization resistance.
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Table 1: Apparent Km values of *C. difficile* spores for taurocholate.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Strain</th>
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<tr>
<td></td>
<td><em>C. difficile</em> UK1</td>
<td><em>C. difficile</em> CD196</td>
</tr>
<tr>
<td>No drug</td>
<td>1.97 ± 0.13</td>
<td>2.16 ± 0.31</td>
</tr>
<tr>
<td>0.25 μg / ml FDX</td>
<td>1.93 ± 0.30</td>
<td>2.56 ± 1.10</td>
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<tr>
<td>2.5 μg / ml OP-1118</td>
<td>1.89 ± 0.51</td>
<td>3.49 ± 0.97</td>
</tr>
<tr>
<td>2.5 μg / ml Vancomycin</td>
<td>2.9 ± 0.49</td>
<td>2.89 ± 0.36</td>
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Values represent the average of three independent experiments and are listed in mM ± 1 standard deviation from the mean. Differences are not statistically significant (Student’s T-test).

Figure 1. Analyzing the initiation of spore germination in *C. difficile* UK1 and *C. difficile* CD196. Purified *C. difficile* UK1 spores (A) and *C. difficile* CD196 spores (B) were suspended in BHIS medium alone (●) or BHIS medium supplemented with 2 mM TA (■) or 5 mM TA (▲) or 10 mM TA (▼) or 20 mM TA (●) or 50 mM TA (○). The initiation of germination was followed at *A*<sub>600</sub> and values were normalized to T<sub>0</sub>. Experiment was performed in triplicate and quantified values of the apparent Km for TA are listed in Table 1.
Figure 2. Measuring outgrowth of *C. difficile* spores during antibiotic exposure. Purified *C. difficile* UK1 spores (A) and *C. difficile* CD196 spores (B) were suspended in BHIS medium supplemented with 2 mM TA alone (■) or with 0.25 μg / ml FDX (▲) or 2.5 μg / ml OP-1118 (▼) or 2.5 μg / ml vancomycin (♦). A$_{600}$ was followed at 5 min intervals for 60 min and then every 30 minutes for another 7 hours and finally at 24 hours. Germination and outgrowth was followed at 37°C in an anaerobic environment and normalized to $T_0$. Data points represent the average of 3 independent experiments and error bars represent 1 standard deviation from the mean and, at times, are smaller than the data point itself. (C) Samples were taken at the 150-minute time point (above) and analyzed by phase contrast microscopy. Dormant spores appear bright, germinated spores appear dark. Magnification: 1,000x. Contrast adjusted using Canvas X and applied equally to all images.


