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Comparative Microbiological Studies of Transcription Inhibitors Fidaxomicin and the Rifamycins in *Clostridium difficile*

Farah Babakhani,* Jaime Seddon,* Pamela Sears*

Optimer Pharmaceuticals, Inc., San Diego, California, USA

Fidaxomicin (FDX) is a narrow-spectrum antibiotic for the treatment of *Clostridium difficile*-associated diarrhea. While FDX and rifamycins share the same target (RNA polymerase), FDX exhibits a unique mode of action distinct from that of rifamycins. In comparative microbiological studies with *C. difficile*, FDX interacted synergistically with rifamycins, demonstrated a lower propensity for the development of resistance to rifamycins, and exhibited no cross-resistance with rifamycins. These results highlight differences in the mechanisms of action of FDX and rifamycins.

Fidaxomicin (FDX) and rifaximin (RFX) are nonsystemic antibiotics that target RNA polymerase (RNAP) and inhibit bacterial transcription (1, 2). Unlike FDX, which is approved for treatment of *Clostridium difficile*-associated diarrhea, RFX is a broad-spectrum antibiotic indicated for treatment of travelers' diarrhea and for reducing the recurrence of overt hepatic encephalopathy (3, 4). Since RFX achieves high colonic concentrations, its use for treatment of other gastrointestinal diseases, including *C. difficile* infections (CDI), has been reported (5, 6). Most studies describe the use of RFX as a chaser following vancomycin therapy. While RFX demonstrated some success in treating recurrent CDI, rapid development of resistance has discouraged its use in patients with prior exposure to rifamycins (7–9).

In clinical trials, FDX was superior to vancomycin, the standard comparator, in sustaining clinical cure without recurrence of CDI through 25 days posttreatment (10, 11). Enhanced performance may be ascribed to the favorable attributes of FDX against *C. difficile*, as described below. Foremost, the drug and its major metabolite, OP-1118, achieve high colonic concentrations and display narrow spectra of activity (12–17). While both FDX and OP-1118 exhibit potent bactericidal activity against *C. difficile* (18) and moderate activity against some Gram-positive bacteria, *in vitro* and *in vivo* studies have indicated that they are sparing of the normal gut flora, with no activity against Gram-negative bacteria (13–16). Additionally, FDX and its major metabolite demonstrate prolonged postantibiotic effects (PAE), suppressing *C. difficile* growth for time periods of up to 10 and 3 h, respectively, which are considerably longer than those of vancomycin (19). A prolonged PAE is indicative of slow organism recovery and may confer an advantage to patients with severe CDI by potentially extending the duration of inhibitory activity between doses. Finally, both FDX and OP-1118 inhibited *in vitro* toxin production and sporulation by *C. difficile* (20, 21). *In vitro* findings are consistent with results of phase II stool analyses in which samples from FDX-treated subjects showed significantly lower spore counts and reduced incidences of toxin than samples from vancomycin-treated subjects (13).

Although FDX and rifamycins are both inhibitors of bacterial transcription, FDX acts at an earlier step in the transcription initiation pathway. While rifamycins block extension of short RNA transcripts, FDX blocks formation of the RNAP open promoter complex, the stage where template DNA has melted prior to RNA synthesis (1). This report describes results of additional comparative microbiological studies for FDX and rifamycin versus *C. difficile*, providing support for their different mechanisms of action.

Antimicrobial interactions. The microdilution checkerboard method was used to study antimicrobial synergy (22). Briefly, concentrations of FDX or OP-1118 and comparator drugs (RFX or rifampin, starting from 2× MIC for each drug) were varied along the perpendicular axes of 96-well plates, with the final column or row containing each compound alone. Plates received 10^6 CFU/ml *C. difficile* inocula, prepared by suspension of bacteria that were grown overnight on blood agar. Culture plates were incubated at 35°C under anaerobic conditions for 48 h. Fractional inhibitory concentration (FIC) indices were calculated with the equation FIC index = $(MIC_{A/B}/MIC_A) + (MIC_{B/A}/MIC_B)$, where MIC_A and MIC_B are the MICs of the drugs alone and $MIC_{A/B}$ and $MIC_{B/A}$ are the MICs of drugs A and B in the presence of the other drug, respectively. FIC indices defined antimicrobial interactions as synergistic when ≤0.5, antagonistic when >4, and indifferent when >0.5 but ≤4.

FDX demonstrated synergy with rifamycins (Table 1, FIC indices of 0.25), but not with its metabolite, OP-1118, or with vancomycin (FIC indices of 1). Similarly, OP-1118 showed synergy with rifamycins (tested in four separate experiments) but not with vancomycin or its parent compound, FDX (data not shown). The combined activity of FDX or OP-1118 with rifamycins on *C. difficile* exceeded the sum of the activities of the drugs alone, consistent with FDX and its metabolite inhibiting a different (earlier) step in the transcription initiation pathway compared to rifamycins.

Frequency of spontaneous single-step resistance development. The frequencies of spontaneous single-step mutations for FDX, RFX, and vancomycin were determined in 4 strains of

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Address correspondence to Farah Babakhani, fbabakhani@optimerpharma.com.
* Present address: Farah Babakhani, Jaime Seddon, and Pamela Sears, Cubist Pharmaceuticals, San Diego, California, USA.
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TABLE 1 Fractional inhibitory concentrations for combinations of fidaxomicin with antimicrobials

Antimicrobial agent[a]	FIC	Interpretation
Rifampin	0.25	Synergy
Rifaximin	0.29	Synergy
Vancomycin	1	Indifference
OP-1118	1	Indifference

[a] Experiments were performed 2 times, 6 times, 3 times, and 5 times with rifampin, rifaximin, vancomycin, and OP-1118, respectively.

C. difficile, including ATCC strains 9689 and 700057 plus two clinical isolates. Briefly, a dense suspension of bacteria ($\sim 10^9$ CFU/ml), which was prepared by dilution of bacteria grown overnight on blood agar, was inoculated onto brucella agar with 4× and 8× the MIC of drug. Following anaerobic incubation for 48 h, the frequency of spontaneous single-step resistance development (FSR) (mutation frequency) was calculated by dividing the number of colonies on antibiotic plates by the number of colonies inoculated.

RFX selected for the highest number of spontaneous resistant mutants with mutation frequencies ranging from 3.58×10^{-9} to 1.73×10^{-7} at 8× MIC of drug (Table 2). RFX MIC values for resistant colonies ranged from 0.5 to >1 μg/ml (an increase of ≥32-fold). Though sequencing was not performed, RFX-resistant strains with high MICs have been reported following *C. difficile* exposure to rifamycins, the result of single-nucleotide substitutions in hot spot regions (*rpoB* amino acids 136 to 550) (9, 23, 24).

In contrast, neither FDX nor vancomycin produced resistant mutants at 8× MIC (FSR $<1.4 \times 10^{-9}$ for ATCC strains and $<2.72 \times 10^{-9}$ and $<3.58 \times 10^{-9}$ for the clinical strains). *C. difficile* clones with elevated FDX MIC values emerged sporadically from the ATCC strain 9689 only at 4× MIC (Table 2). These clones demonstrated stable reduced susceptibility, with FDX MIC values of 2 or 4 μg/ml, and carried mutations in either the *rpoB* (Gln1074Lys or Val1143Phe) or *rpoC* (Asp237Tyr) genes, which lie outside areas targeted by rifamycins.

The fitness cost of such mutations was not investigated in this study; however, Kuehne et al. demonstrated that a laboratory-generated *C. difficile* strain, with reduced susceptibility to FDX (obtained through directed mutagenesis in RNA polymerase at Val1143Asp), had impaired fitness and exhibited delayed growth (25). In support of the above-mentioned laboratory findings, as of this publication and 2 years of surveillance data, only one clinical isolate (*rpoB* Val1143Gly) with reduced susceptibility to FDX (MIC 16 μg/ml) has been identified (26).

Cross-resistance. During phase 3 clinical trials, rifaximin-resistant *C. difficile* strains were observed in approximately 8% of pretreatment strains; however, none demonstrated cross-resistance to FDX (27). To further examine the lack of cross-resistance between the two drugs, susceptibilities of laboratory-generated clones with reduced susceptibility to FDX were compared with those of the wild-type (i.e., antibiotic-sensitive) strains using Clinical and Laboratory Standards Institute (CLSI) broth microdilution methods (28, 29).

FDX showed no cross-resistance with rifamycins. *C. difficile* strains with reduced susceptibility to FDX (generated from FSR studies or serial passages on brucella agar under FDX selection, i.e., 3× on agar with one-half the original MIC of FDX followed by an additional 25× with steadily increasing concentrations of FDX) were sensitive to rifamycins (Table 3). Likewise, RFX-resistant *C. difficile* strains, generated via FSR, displayed no cross-resistance to FDX, with FDX MIC values equivalent to those of the wild-type strains. Additionally, the multidrug-resistant strain, *Staphylococcus aureus* ATCC BAA-44 (resistant to β-lactams, macrolides, aminoglycosides, clindamycin, tetracycline, and rifampin), and antibiotic-sensitive *S. aureus* ATCC 25921 were similarly sensitive to FDX, indicative of a lack of cross-resistance to other classes of antibiotics, including the rifamycins. Similar observations have been observed during FDX clinical trials; the rifaximin MIC_{90} for 716 clinical *C. difficile* isolates was reported to be >256 μg/ml, but none of the strains showed elevated FDX MICs (26).

Lack of detectable cross-resistance provides additional microbiological evidence to support the distinct mechanisms by which FDX and rifamycins inhibit *C. difficile* transcription. While both FDX and RFX bind to the bacterial RNAP, each drug

TABLE 2 Spontaneous mutation frequencies of fidaxomicin, rifaximin, and vancomycin versus *C. difficile* strains

C. difficile strain	Drug[a]	MIC (μg/ml)	FSR at[b]: 4× MIC	FSR at[b]: 8× MIC
ATCC 9689 (ORG74)	FDX	0.125	1.28×10^{-8} to $<1.41 \times 10^{-9}$	$<1.41 \times 10^{-9}$
	VAN	0.5	$<1.41 \times 10^{-9}$	$<1.41 \times 10^{-9}$
	RFX	0.03	1.92×10^{-8} to 1.59×10^{-7}	2.24×10^{-8} to 1.73×10^{-7}
ATCC 700057 (ORG830)	FDX	0.25	$<1.41 \times 10^{-9}$	$<1.41 \times 10^{-9}$
	VAN	1	$<1.41 \times 10^{-9}$	$<1.41 \times 10^{-9}$
	RFX	0.008	6.67×10^{-9} to 2.07×10^{-8}	4.13×10^{-9} to 1.23×10^{-8}
Clinical strain (ORG911)	FDX	0.5	$<3.58 \times 10^{-9}$	$<3.58 \times 10^{-9}$
	VAN	0.5	$<3.58 \times 10^{-9}$	$<3.58 \times 10^{-9}$
	RFX	0.031	1.43×10^{-8}	3.58×10^{-9}
Clinical strain (ORG916)	FDX	0.25	$<2.71 \times 10^{-9}$	$<2.71 \times 10^{-9}$
	VAN	1	$<2.71 \times 10^{-9}$	$<2.71 \times 10^{-9}$
	RFX	0.016	2.17×10^{-8}	1.90×10^{-8}

[a] FDX, fidaxomicin; RFX, rifaximin; VAN, vancomycin.
[b] FSR experiments were performed 3 times with the ATCC strains and once with the clinical strains.

TABLE 3 Activity of fidaxomicin and rifampin toward antibiotic-resistant strains

Microorganism (resistance characteristic)	MIC (μg/ml)	
	Fidaxomicin	Rifamycin[a]
C. difficile ATCC 700057 (antibiotic sensitive)	0.25	0.016
C. difficile 700057-8A (ORG 1632) (rifaximin-elevated MIC)	0.125	0.5
C. difficile 700057-6 (ORG 1629) (fidaxomicin-elevated MIC)	4	0.016
C. difficile ATCC 9689 (antibiotic sensitive)	0.125	0.016
C. difficile 9689-4B (ORG1626) (rifaximin resistant)	0.125	>1
C. difficile 9689-7A (ORG 1669) (fidaxomicin-elevated MIC)	2	0.016
C. difficile ATCC 43255 (antibiotic sensitive)	≤0.125	≤0.125
C. difficile 43255-29C (fidaxomicin-elevated MIC)	8	≤0.125
S. aureus ATCC 29213 (antibiotic sensitive)	4	≤0.125
S. aureus ATCC BAA-44 (rifampin resistant)	4	1

[a] All strains were tested versus rifaximin, with the exception of *C. difficile* ATCC 43255, its derivative (43255-29C), and *S. aureus* strains, which were tested versus rifampin.

has been shown to interact with separate regions on the enzyme, inhibiting transcription at different steps. In contrast to RFX, which inhibits extension of nascent RNA, FDX acts earlier in the transcription cycle by preventing complete melting promoter DNA, thereby blocking the formation of productive promoter complexes (1).

In summary, differences in rates of resistance development, lack of cross-resistance, and synergistic interactions support the distinct modes of action of FDX and RFX. Such differences in mechanisms of action may explain the narrow spectrum of activity and differential inhibition of sporulation by FDX versus RFX reported previously.

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Since Optimer has been acquired by Cubist Pharmaceuticals, all three authors are currently employed by Cubist Pharmaceuticals, San Diego, CA.

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