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## Abstract

**BACKGROUND** OPT-80 is a narrow spectrum antibiotic under evaluation for the treatment of *Clostridium difficile*-associated diarrhea.

**METHODS** We tested the propensity of *C. difficile* to develop resistance to OPT-80 by the frequency of spontaneous resistance and serial passage methods. We examined the cross-resistance of OPT-80 with other antimicrobials by comparing MIC profiles of drug-resistant and drug-sensitive organisms. Finally, we studied antimicrobial synergy using the checkerboard technique.

**RESULTS** The frequency of spontaneous resistance was low: Colonies were generally not observed, at either 4x or 8x the MIC, leading to frequencies of spontaneous resistance of  $<3 \times 10^{-5}$ . Following serial passage of *C. difficile* at 0.5x MIC, the MIC remained within 1-2 dilutions of the starting MIC after 13 passages. Rifampin resistance (*RpoB* mutation), ribosomal methylation (Inducible and constitutive *Erm*), macrolide pumps (*MefA* and *MsrA*), and a variety of other resistance mechanisms did not confer resistance to OPT-80. A strain with an OPT-80 MIC of 32 [g/mL was generated after over 20 passages on steadily increasing OPT-80. This organism had very similar MICs to the wild type against rifampin, azithromycin, ampicillin, metronidazole, vancomycin, and clindamycin. The interaction of OPT-80 with other antimicrobials was studied using the checkerboard technique against *C. difficile* ATCC 43255. OPT-80 was found to be synergistic with the known RNA polymerase inhibitor rifampin. It showed neither synergy nor antagonism to ampicillin, azithromycin, telithromycin, ciprofloxacin, metronidazole, vancomycin, and Labrasol.

**CONCLUSIONS** OPT-80 has a low propensity to cause resistance development, shows no cross-resistance with any marketed antibiotic tested, and is not synergistic in *Clostridium* with most antibiotics except for rifampin.

## Introduction

*Clostridium difficile* is a significant cause of nosocomial diarrhea.(1, 2) Disruption of normal colonic flora, mainly from antibiotic use, followed by overgrowth of toxigenic strains of *C. difficile* results in illness that can range from mild watery diarrhea to severe life threatening pseudomembranous colitis. The frequency and severity of disease has been reported to be increasing with estimated annual health care costs of over \$1 billion in the United States.(3, 4) Recurrent diarrhea due to re-infection or relapse has been reported in 20% – 30% of patients receiving treatment for *C. difficile* diarrhea. The two most commonly utilized specific therapies are vancomycin and metronidazole. Although both agents are effective in treating the infection, they can lead to recurrence of disease, development of ecologically undesirable organisms (in the case of vancomycin), or frequent adverse effects (in the case of metronidazole). Both vancomycin and metronidazole are, themselves, capable of inducing *C. difficile* intestinal disease.(5, 6)

OPT-80 is a macrocyclic compound under development as a new therapeutic regimen for *C. difficile* associated diarrhea (CDAD). OPT-80 exhibits minimal activity against gram-negative bacteria, moderate activity against most gram-positive bacteria, and excellent activity toward *Clostridium* (7, 8) The narrow-spectrum profile of OPT-80 may be important in minimizing the recurrence of CDAD, as it is probably the continuing disturbance of the gastrointestinal flora caused by other CDAD treatments that leads to recolonization and overgrowth by *Clostridium*.

This study investigates the propensity of organisms to acquire resistance to OPT-80, by (i) testing the frequency of spontaneous resistance development; (ii) evaluating the increase in the MIC during serial passage; and (iii) investigating cross resistance with other antimicrobial agents. Finally, synergy studies are performed to investigate the interaction of OPT-80 with other classes of antibiotics that may be used during treatment in CDAD patients.

## Methods

**STRAINS** Laboratory strains were obtained from American Type Culture Collection (ATCC). Recent clinical isolates of *C. difficile* and resistant organisms were obtained from various sources. All strains were kept frozen at  $-70^{\circ}\text{C}$  in 10% glycerol until use.

**MIC** MICs were determined either by agar dilution or broth microdilution procedure according to the NCCLS guidelines.(9-11)

**FREQUENCY OF SPONTANEOUS RESISTANCE (FSR)** A dense suspension of an overnight culture of *C. difficile* was spread into supplemented Brucella agar plates with antibiotics at 4x and 8x MIC concentrations. Serial dilutions of the inoculum were also plated on supplemented Brucella agar without antibiotics to determine the number of colonies inoculated. Following growth at  $35^{\circ}\text{C}$ , the frequency of spontaneous resistance was determined by dividing the number of colonies in drug plates by number of colonies inoculated.

**SERIAL PASSAGE STUDIES** Approximately  $10^8$  CFUs of ATCC strain of *C. difficile* were plated on supplemented Brucella agar containing one half the original MIC of OPT-80. Following growth, the majority of the organism was collected from the plate, suspended in Brucella broth, and  $10^8$  CFUs were plated on a fresh plate containing one half the original MIC of OPT-80. The suspension was also used to assess the current MIC. When the MIC was observed to increase, the stability of the resistant phenotype was checked after passing the variant strain on blood agar without OPT-80. A total of 18 passages were performed.

**GENERATING OPT-80-RESISTANT MUTANT STRAIN** After three passages on supplemented Brucella agar containing one half the original MIC of OPT-80, *C. difficile* ATCC 43255 was passaged an additional 25 times on steadily increasing concentrations of OPT-80 to generate resistant variants (43255-C29). At each step, the MIC was determined and the organism was re-plated at 0.5x and 1x the previous MIC. The stability of resistant phenotype was assessed on blood agar without OPT-80. The mutant strain was kept frozen at  $-70^{\circ}\text{C}$  in 10% glycerol.

**CHECKERBOARD PROCEDURE** Using the checkerboard procedure, the activity of OPT-80 vs. *C. difficile* in presence of other antimicrobial agents was investigated.(12) First, the MIC values for OPT-80 and test antibiotics vs. *C. difficile* were determined using a microbroth dilution method. Next, a 96-well plate was generated in which the concentrations of the antibiotics were varied along the two axes, from 2x the MIC down to 1/16x the MIC, with a final column or row containing each compound alone. The organism was inoculated into all wells, and after incubation, the wells containing growth were recorded.

The fractional inhibitory concentration, or FIC, was determined by finding the well with the lowest concentrations of the two compounds along the "isoeffective" diagonal (45 degree diagonal along which the compounds are at the same fraction of their MIC), and calculating the FIC as:

$$\text{FIC} = \frac{\text{MIC}_{\text{A,B}}}{\text{MIC}_{\text{A,alone}}} + \frac{\text{MIC}_{\text{B,A}}}{\text{MIC}_{\text{B,alone}}}$$

A FIC of 0.5 or lower was considered synergistic, while a FIC near 1 was additive and a FIC greater than 4 was antagonistic.

## Results

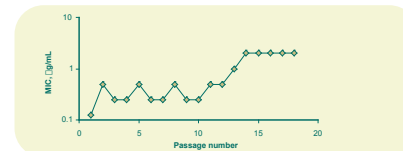
**I.** OPT-80 demonstrated a low frequency of spontaneous resistance with FSR values of  $<10^{-4}$ , similar to metronidazole and vancomycin (Table 1). This was observed with both laboratory and clinical isolates of *Clostridium difficile*.

Table 1. Frequency of Spontaneous Resistance (FSR) for metronidazole, vancomycin, and OPT-80 versus *C. difficile* isolates

C. difficile isolates	Inoculum	FSR (Vancomycin)		FSR (OPT-80)		FSR (Metronidazole)	
		4x MIC	8x MIC	4x MIC	8x MIC	4x MIC	8x MIC
ATCC 43255	$7.4 \times 10^7$	$<1.4 \times 10^4$	$<1.4 \times 10^4$	$<1.4 \times 10^4$	$<1.4 \times 10^4$	$<1.4 \times 10^4$	$<1.4 \times 10^4$
ATCC 9689	$4.3 \times 10^7$	$<2.3 \times 10^4$	$<2.3 \times 10^4$	$<2.3 \times 10^4$	$<2.3 \times 10^4$	$<2.3 \times 10^4$	$<2.3 \times 10^4$
ORGI 314	$4.9 \times 10^7$	$<2.0 \times 10^4$	$<2.0 \times 10^4$	$<2.0 \times 10^4$	$<2.0 \times 10^4$	$<2.0 \times 10^4$	$<2.0 \times 10^4$
ORGI 315	$5.3 \times 10^7$	$<1.9 \times 10^4$	$<1.9 \times 10^4$	$<1.9 \times 10^4$	$<1.9 \times 10^4$	$<1.9 \times 10^4$	$<1.9 \times 10^4$
ORGI 316	$3.7 \times 10^7$	$<2.7 \times 10^4$	$<2.7 \times 10^4$	$<2.7 \times 10^4$	$<2.7 \times 10^4$	$<2.7 \times 10^4$	$<2.7 \times 10^4$
ORGI 317	$3.9 \times 10^7$	$<2.6 \times 10^4$	$<2.6 \times 10^4$	$<2.6 \times 10^4$	$<2.6 \times 10^4$	$<2.6 \times 10^4$	$<2.6 \times 10^4$
ORGI 323	$1.03 \times 10^8$	$<9.7 \times 10^4$	$<9.7 \times 10^4$	$<9.7 \times 10^4$	$<9.7 \times 10^4$	nd	$<9.7 \times 10^4$

**II.** In the serial passage studies, OPT-80 also showed a low propensity to develop resistance (Figure 1). Thirteen passages on one half the initial MIC were required before the MIC values became significantly (more than 1-2 two-fold dilutions) elevated, and they reached a stable plateau at a relatively low value of 2 [g/mL.

Figure 1. Serial passage of *C. difficile* ATCC 43255 on blood agar plate with 0.0625 [g/mL of OPT-80 (0.5x the starting MIC value)



**III.** Cross-resistance of OPT-80 to other classes of antibiotics was performed by comparing the MIC values of *C. difficile* ATCC 43255 to that of OPT-80 resistant strain (43255-29C), generated by serial passage, and also to a macrolide resistant *C. difficile* strain (ATCC 43597). No significant elevation was observed in the MIC values for the other classes of antibiotics in the OPT-80 resistant strain as compared to the wild type strain 43255; most were identical or within a dilution of the wild type MIC (Table 2).

Table 2. Cross resistance of OPT-80 resistant *C. difficile* 43255-29C and macrolide-resistant *C. difficile* ATCC 43597 to other antibiotic classes (MIC values in [g/mL)

	C. difficile ATCC 43255 (wild type)	C. difficile ATCC 43255-29C (OPT-80-Resistant)	C. difficile ATCC 43597 (Macrolide-Resistant)
Azithromycin	16	8	>64
Telithromycin	1	0.5	>64
Ampicillin	4	8	2
Aztreonam	>64	>64	>64
Cefotaxime	64	>64	64
Ciprofloxacin	16	8	16
Vancomycin	0.5	2	0.5
Metronidazole	0.5	1	1
Rifampin	[0.125	[0.125	[0.125
OPT-80	[0.125	8	[0.125

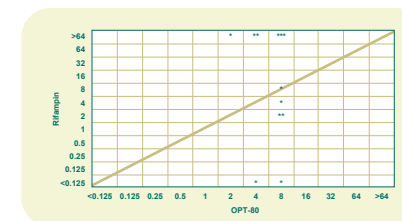
**IV.** Cross-resistance of OPT-80 with other antibiotics was also studied by comparing the OPT-80 MIC values of organisms resistant to other classes of antibiotics with the OPT-80 MIC values of sensitive versions of those species (Table 3).

Table 3. Activity of OPT-80 toward antibiotic resistant strains (MIC values in [g/mL)

	OPT-80	Rifampin	Azithromycin	Telithromycin
<i>Staphylococcus aureus</i> ATCC 29213 (Antibiotic sensitive)	4	[0.125	0.5	[0.125
<i>Staphylococcus aureus</i> ATCC BAA-44 (Rifampin resistant)	4	1	>64	>64
<i>Staphylococcus aureus</i> 96-11489 (Inducible macrolide-resistant, <i>Erm</i> )	4	[0.125	>64	[0.125
<i>Staphylococcus aureus</i> RN4220 E194 (Macrolide resistant, <i>MsrA</i> macrolide pump)	4	[0.125	64	2
<i>Staphylococcus aureus</i> ATCC 33981 ( <i>MsrA</i> , Macrolide resistant)	4	[0.125	>64	>64
<i>Streptococcus pneumoniae</i> ATCC 49619 (Antibiotic sensitive)	>32	nd	[0.125	[0.125
<i>Streptococcus pneumoniae</i> 163 (Macrolide resistant, <i>MefA</i> macrolide pump)	64	[0.125	2	[0.125
<i>Enterococcus faecium</i> ATCC 49622 (Antibiotic sensitive)	4	nd	nd	[0.125
<i>Enterococcus faecium</i> ATCC 79221 (Vancomycin resistant)	4	nd	nd	8

A strain of *Staphylococcus aureus* containing the macrolide pump *MsrA* had the same MIC value for OPT-80 as the wild type strain (ATCC 29213). Likewise, *Staphylococcus aureus* strains containing the constitutive and inducible ribosomal methylase (*Erm*) also had the same MIC values for OPT-80 as the wild type strain. The moderately rifampin-resistant *Staphylococcus aureus* ATCC BAA-44 also showed no loss of sensitivity to OPT-80. This organism is presumed to have a mutated RNA polymerase (*rpoB*) and is also resistant to other antibiotics such as [lactams, including methicillin, macrolides, aminoglycosides, clindamycin, and tetracycline. Additional clinical strains of rifampin-resistant *S. aureus* were also tested vs. OPT-80 and no cross-resistance was observed (Figure 2).

Figure 2. Correlation of sensitivity of rifampin-resistant *S. aureus*: Comparison between rifampin and OPT-80 (MIC values in [g/mL)



OPT-80 is active against *Enterococcus faecium* including the vancomycin resistant strain with a MIC of 4 [g/mL. *Streptococcus* are minimally sensitive to OPT-80, with MIC values of 16-64 [g/mL. Nevertheless, the MIC values for OPT-80 were measurable and were not increased further by the presence of the *MefA* macrolide pump.

With the exception of rifampin, all other compounds tested did not demonstrate a synergistic effect with OPT-80 activity vs. *C. difficile*. In contrast, rifampin demonstrated positive synergy with OPT-80 with a FIC value of 0.5. The effect of clindamycin on OPT-80 could not be completely delineated in the checkerboard runs. While in a couple of runs the effect appeared to be synergistic, the three other runs showed an additive interaction.

Table 4. Fractional Inhibitory Concentrations (FIC) for combinations of OPT-80 with various antimicrobials and the excipient Labrasol versus *Clostridium difficile* ATCC 43255

	Average [FIC]
Ampicillin	1.3
Azithromycin	1.3
Ciprofloxacin	1.15
Clindamycin	0.9
Labrasol	1.5
Metronidazole	0.92
Rifampin	0.5
Telithromycin	1
Vancomycin	2

The average FIC was obtained from experiments performed on separate days.

## Discussion

The frequency of spontaneous resistance is an important feature for an antimicrobial therapy, as it can allow comparison between compounds in terms of the rate at which resistance is likely to develop. OPT-80 has a low frequency of spontaneous resistance with FSR values of  $<10^{-4}$ , similar to metronidazole and vancomycin. In the serial passage studies, OPT-80 also showed a low propensity to develop resistance. Thirteen passages on one half the initial MIC were required before the MIC values became significantly (more than 1-2 two-fold dilutions) elevated, and they reached a stable plateau at a relatively low value of 2 [g/mL.

The clinical usefulness of an antimicrobial drug is determined, in part, by the rate at which organisms develop resistance to it. Organisms resistant to the other classes of antibiotics tested (macrolides, containing both ribosomal methylases and macrolide pumps, lincosamides, aminoglycosides, [lactams, and rifampin) were not found to be cross-resistant to OPT-80. An isolate of *C. difficile* ATCC 43255 with resistance induced to OPT-80 by serial passage on steadily increasing concentrations of OPT-80 was likewise as susceptible as the wild type organism toward macrolides, [lactams, quinolones, vancomycin, metronidazole, and rifampin.

A risk factor for the development of CDAD is the use of broad-spectrum antibiotics, thus patients with CDAD will be expected to either be on or have recently discontinued antibiotic use. OPT-80 was found to be synergistic with the known RNA polymerase rifampin. It showed borderline synergy with clindamycin (average FIC, or fractional inhibitory concentration, was 0.9), but showed neither synergy nor antagonism to ampicillin, azithromycin, telithromycin, ciprofloxacin, metronidazole, vancomycin, and the excipient Labrasol.

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