CB-183,315 DEMONSTRATES LOW *IN VITRO* FREQUENCY OF SPONTANEOUS OR MULTISTEP RESISTANCE IN *CLOSTRIDIUM DIFFICILE*, *ENTEROCOCCUS FAECALIS* AND *E. FAECIUM*

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ABSTRACT

Background: CB-183,315 is a novel oral lipopeptide antibiotic currently in phase 3 clinical development for the treatment of *Clostridium difficile* associated diarrhea. *C. difficile* infection is the most common cause of health care-related infectious diarrhea in developed countries, accounting for up to 20% of the cases of antibiotic-associated diarrhea and nearly all cases of antibiotic-associated colitis.

Methods: We evaluated the emergence of resistance for *C. difficile* (ATCC 700057 and 3 clinical isolates from the REA groups BI, BK and K), vancomycin susceptible (VS) *E. faecalis* (ATCC 49452), vancomycin resistant (VR) *E. faecalis* (ATCC 700802), *VSE. faecium* (ATCC 6569) and *VRE. faecium* (ATCC 51559) against CB-183,315, vancomycin and rifampicin. Spontaneous resistance incidence (RI) was determined under anaerobic conditions at 8, 16 and 32X MIC. Multistep resistance under selective pressure was evaluated under anaerobic conditions by serial passage over 15 days.

Results: The RI was below the limit of detection ($<10^{-8}$ to $<10^{-9}$) for CB-183,315 and vancomycin at 16 and 32X MIC for all isolates tested. Rifampicin, used as an assay control, had RI frequencies of 10^{-7} to 10^{-8} against *C. difficile* and 10^{-6} to 10^{-7} against *E. faecalis*. In the CB-183,315 serial passage study, strains grew in 1-4 μg/mL CB-183,315 (shifted 1-4 fold compared to day 1) by day 15, with the exception of the *C. difficile* BK isolate. Against the BK isolate, the concentration permitting growth remained low (1-4 μg/mL) until day 9 and shifted up to 16-32 μg/mL by day 15. After 3 daily drug-free transfers of the BK isolate, the MICs were 2-4 μg/mL (4-8 fold shift compared to naïve). In the vancomycin serial passage, strains grew in 1-8 μg/mL vancomycin by day 15 (shifted 2-8 fold compared to day 1) for the *C. difficile*, *VSE. faecalis* and *VSE. faecium* strains. After 3 daily drug free transfers, day 15 CB-183,315 and vancomycin isolates had MICs shifted 1-8 fold compared to naïve controls for the remaining strains tested. Rifampicin was included as a positive control and exposed strains were able to grow in significantly increased concentrations of rifampicin. After 3 daily drug-free transfers, day 15 rifampicin isolates exhibited MICs shifts of >128 fold for all strains tested.

Conclusions: CB-183,315 demonstrated a low frequency of spontaneous resistance in *C. difficile* and *Enterococci*, including VRE. Similar to vancomycin, no stable resistant colonies were generated following serial passage with selective pressure for any of the strains tested. These *in vitro* data suggest the emergence of resistance to CB-183,315 against *C. difficile*, *E. faecalis* and *E. faecium* (VSE and VRE) is likely to be rare.

INTRODUCTION

- *Clostridium difficile* is a Gram-positive, spore-forming, obligate anaerobic bacillus that is the causative pathogen of *Clostridium difficile* associated diarrhea (CDAD).
- CDAD, first reported in the 1970s, is the most common cause of healthcare-related infectious diarrhea in developed countries, accounting for 20-30% of the cases of antibiotic-associated diarrhea and nearly all cases of antibiotic-associated colitis (4).
- CDAD is usually associated with prior exposure to antimicrobial agents where antibiotic treatment results in changes in the complexity and diversity of the normal fecal microbiota which allows for colonization and infection with toxigenic *C. difficile* (3).
- Toxin production by *C. difficile* leads to intestinal inflammation and damage, diarrhea and sometimes life-threatening disease, such as pseudomembranous colitis, toxic megacolon, and sepsis (2).
- High recurrence rates (20-30%) associated with current treatment options (vancomycin & metronidazole) coupled with delayed response or treatment failures and the emergence of a highly virulent strain (referred to as BI, NAP1 or 027) have increased the urgency to develop new therapeutic options (1).
- CB-183,315 (see Figure 1) is a lipopeptide antibiotic with rapid *in vitro* bactericidal activity against Gram-positive pathogens including *C. difficile*.

Figure 1. Structure of CB-183,315 ((E)-3-(4-pentylphenyl)but-2-ene dapto core)

OBJECTIVE

To evaluate the emergence of spontaneous resistance and multistep resistance under selective pressure of recent clinical isolates of *C. difficile* (REA groups BI, BK and K), vancomycin susceptible and vancomycin resistant *Enterococcus faecalis* and *E. faecium* under anaerobic conditions.

MATERIALS & METHODS

Media

For Enterococci studies, Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA; 18 g/L of BD BactoAgar added to MHB) were used (Sparks, MD). For *C. difficile* studies BBL™ Brucella broth and BBL™ Brucella agar from BD Diagnostic Systems (Sparks, MD) were used. All assay media were prepared and sterilized according to manufacturer's specifications then supplemented with a final concentration of 50 mg/L Ca^{2+}. Additionally, Brucella broth and agar were supplemented with hemin (5 μg/mL) and vitamin K1 (1 μg/mL).

In order to grow up frozen cultures, prepared Brucella agar plates supplemented with 5% sheep blood, hemin, and vitamin K1 (BBHK, BD Diagnostic Systems) and tryptic soy agar fortified with 5% sheep blood (TSAB, bioMérieux, Durham, NC) were used.

Strains

The study was performed using 4 *C. difficile* isolates, 2 *E. faecium* and 2 *E. faecalis* (Table 1). All work was performed in a Bactron I Anaerobic Chamber (Sheldon Manufacturing) infused with anaerobic mixed gas (5% H_2, 10% CO_2, balance N_2; Airgas catalog #Z03NI8532002103, Salem, NH) using reduced media.

Resistance Incidence

Spontaneous resistance incidence (RI) was determined under anaerobic conditions. The *C. difficile* RI was performed in supplemented Brucella broth and agar, while the *E. faecalis* and *E. faecium* RIs were performed in supplemented MHB and MHA. Cultures suspended to ~1 McFarland (McF) and incubated at 37°C for ~4 hours were used as inocula (0.5 mL; between 3-4 McF) on selection plates (100 mm square plates) containing CB-183,315, vancomycin or rifampicin at 8X, 16X or 32X the MIC, in independent, duplicate assays. Plates were incubated at 37°C for 48 hours. Viable colony forming units per milliliter (CFU/mL) of each inoculum was determined and the RI was calculated using the colony frequency (equal to # of colonies on drug containing plates/total # of colonies plated) times the % resistant colonies. Colonies recovered on drug-containing plates were all evaluated by MIC after 3 transfers on drug-free media to confirm elevated MIC values compared to naïve controls. The starting MICs for CB-183,315, vancomycin and rifampicin against each organism tested are listed in Table 1.

Serial Passage

Multistep resistance under selective pressure was evaluated under anaerobic conditions by serial passage over 15 days. Serial passages of *C. difficile*, *E. faecalis* and *E. faecium* were performed in the presence of increasing concentrations of CB-183,315, vancomycin or rifampicin, with the exception of VREfs and VREfm which were passaged against only CB-183,315, as previously described (5). The *C. difficile* serial passage was performed in supplemented Brucella broth, while the *E. faecalis* and *E. faecium* serial passages were performed in supplemented MHB.

Cultures suspended in media to equal ~1 McF, incubated at 37°C for ~3 hours were inoculated into media containing various concentrations of drug around the MIC and grown overnight at 37°C for 20-24 hours. Each day, wells with the highest concentration of drug (μg/mL) permitting growth equal to ~1 McF were used to inoculate the next day's drug series; this process was repeated for 15 days with each drug/bacteria combination tested in duplicate. Colonies recovered from day 15 were all evaluated by gradient MIC (GMIC) using a spiral gradient endpoint method (Autoplater4000 Spiral Plater; Spiral Biotech, Norwood, MA, USA) after 3 drug-free transfers to confirm elevated GMIC values compared to naïve controls. The starting MICs for CB-183,315, vancomycin and rifampicin against each organism tested are listed in Table 1.

Table 1: Strains used in this study

Strain	Strain ID	ATCC #	Description	MIC in μg/mL			
				Media	CB-183,315	Vancomycin	Rifampicin
Clostridium difficile	QC	700057	Non-toxigenic, quality control strain	Supplemented Brucella Broth	1	1	<0.001
Clostridium difficile	BI	--	Clinical isolate REA group BI		0.5	0.5	<0.001
Clostridium difficile	BK	--	Clinical isolate REA group BK		0.5	0.5	<0.001
Clostridium difficile	K	--	Clinical isolate REA group K		0.5	2	<0.001
Enterococcus faecalis	VSEfs	49452	Vancomycin susceptible	Supplemented MHB	0.5	1	0.125
Enterococcus faecalis	VREfs	700802	Vancomycin resistant		0.25	64	0.5
Enterococcus faecium	VSEfm	6569	Vancomycin susceptible		0.5	0.5	16
Enterococcus faecium	VREfm	51559	Vancomycin resistant		1	>64	4

RESULTS

Table 2: Resistance Incidence of *C. difficile* BI, BK, K and QC strains against CB-183,315, Vancomycin and Rifampicin

Strain ID	Compound	Concentration Tested (X MIC)	SAMPLE A Resistance Incidence	SAMPLE B Resistance Incidence
C. diff. BI	315	8	4.4E-09	<5.4E-09
C. diff. BI	315	16 & 32	<4.4E-09	<5.4E-09
C. diff. BI	VAN	8, 16 & 32	<4.4E-09	<5.4E-09
C. diff. BI	RIF	8	<4.4E-09	3.2E-08
C. diff. BI	RIF	16	1.3E-08	5.4E-09
C. diff. BK	315	8	<4.2E-08	<6.0E-08
C. diff. BK	315	16	<4.2E-08	6.0E-08
C. diff. BK	315	32	<4.2E-08	<6.0E-08
C. diff. BK	VAN	8, 16 & 32	<4.2E-08	<6.0E-08
C. diff. BK	RIF	8	<4.2E-08	1.2E-07
C. diff. BK	RIF	16	4.2E-08	<6.0E-08
C. diff. K	315	8, 16 & 32	<5.8E-09	<4.9E-09
C. diff. K	VAN	8, 16 & 32	<5.8E-09	<4.9E-09
C. diff. K	RIF	8	<5.8E-09	9.9E-09
C. diff. K	RIF	16	1.7E-08	9.9E-09
C. diff. QC	315	8, 16 & 32	<6.5E-09	<5.5E-09
C. diff. QC	VAN	8, 16 & 32	<6.5E-09	<5.5E-09
C. diff. QC	RIF	8	1.3E-08	1.1E-08
C. diff. QC	RIF	16	2.6E-08	5.5E-09

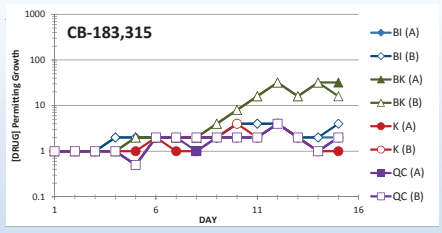
- Only 2/18 (11%) CB-183,315 isolates had MICs ≥1 μg/mL (MIC range = <0.06-2 μg/mL)
- Vancomycin isolates remained unchanged compared to naïve controls (MICs = 0.5 μg/mL)
- 14/15 (93%) rifampicin isolates had MICs 30-1000-fold higher than naïve controls
- No cross resistance was seen to daptomycin, CB-183,315, vancomycin, ampicillin, metronidazole and rifampicin

Table 3: Resistance Incidence of vancomycin susceptible and resistant *E. faecalis* and *E. faecium* against CB-183,315, Vancomycin and Rifampicin

Strain ID	Compound	Concentration Tested (X MIC)	SAMPLE A Resistance Incidence	SAMPLE B Resistance Incidence
VSEfs	315	8, 16 & 32	<1.33E-09	<2.34E-09
VSEfs	VAN	8, 16 & 32	<1.33E-09	<2.34E-09
VSEfs	RIF	8 & 16	>1.3E-06	>2.3E-07
VSEfm	315	8, 16 & 32	<2.9E-09	<1.97E-09
VSEfm	VAN	8, 16 & 32	<2.9E-09	<1.97E-09
VREfs	315	8	3.2E-09	<9.09E-10
VREfs	315	16	<1.6E-09	9.1E-10
VREfs	315	32	<1.6E-09	<9.09E-10
VREfs	RIF	8	>8.0E-07	>1.6E-07
VREfs	RIF	16	1.4E-08	7.8E-08
VREfm	315	8, 16 & 32	<3.39E-09	<4.04E-09

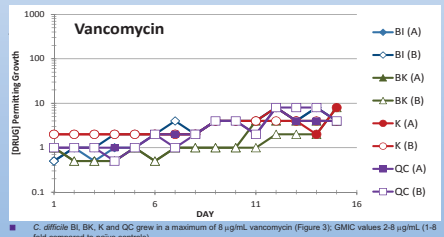
- 3/5 (60%) CB-183,315 had MICs of 1 μg/mL (MIC range = 0.5-1 μg/mL)
- 100% rifampicin isolates tested had MICs 32 to >64-fold higher than naïve controls
- No cross resistance was observed against daptomycin, CB-183,315, vancomycin, ampicillin, moxifloxacin and rifampicin

Figure 2. Serial passage of *C. difficile* BI, BK, K and QC strains against CB-183,315



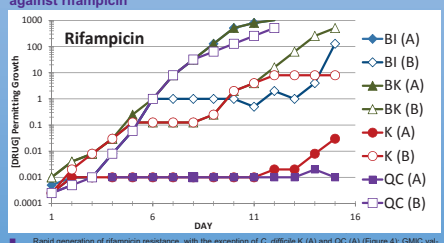
- *C. difficile* BI, K and QC grew in a maximum of 4 μg/mL CB-183,315 (Figure 2); GMIC values 0.5-1 μg/mL (1-2 fold compared to naïve controls)
- *C. difficile* BK grew in 16-32 μg/mL CB-183,315 (Figure 2); GMIC values 2-4 μg/mL (8-16 fold compared to naïve controls)

Figure 3. Serial passage of *C. difficile* BI, BK, K and QC strains against vancomycin



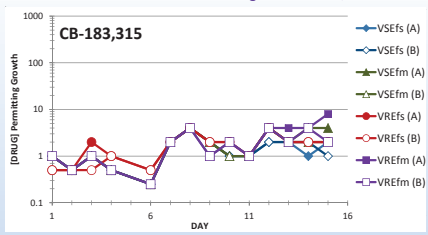
- *C. difficile* BI, BK, K and QC grew in a maximum of 8 μg/mL vancomycin (Figure 3); GMIC values 2-8 μg/mL (1-8 fold compared to naïve controls)

Figure 4. Serial passage of *C. difficile* BI, BK, K and QC strains against rifampicin



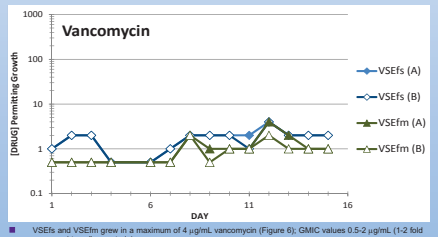
- Rapid generation of rifampicin resistance, with the exception of *C. difficile* K (A) and QC (A) (Figure 4); GMIC values confirmed resistance

Figure 5. Serial passage of vancomycin susceptible and resistant *E. faecalis* and *E. faecium* against CB-183,315



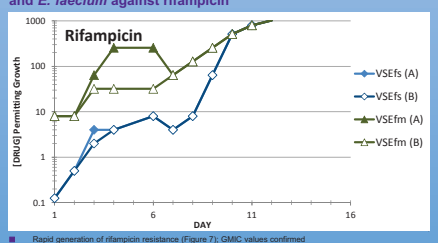
- VSEfs, VSEfm, VREfs and VREfm grew in a maximum of 8 μg/mL CB-183,315 (Figure 5); GMIC values 0.5-2 μg/mL (1-2 fold compared to naïve controls for all cases except VSEfm is 4-8 fold compared to naïve controls)

Figure 6. Serial passage of vancomycin susceptible *E. faecalis* and *E. faecium* against vancomycin



- VSEfs and VSEfm grew in a maximum of 4 μg/mL vancomycin (Figure 6); GMIC values 0.5-2 μg/mL (1-2 fold compared to naïve controls)

Figure 7. Serial passage of vancomycin susceptible *E. faecalis* and *E. faecium* against rifampicin



- Rapid generation of rifampicin resistance (Figure 7); GMIC values confirmed resistance

CONCLUSIONS

- The rate of spontaneous resistance to CB-183,315 was either low or below the limit of detection in *C. difficile* and the *Enterococci*
- Under selective pressure with CB-183,315, *C. difficile* BI, K and QC remained relatively unchanged over the 15 days of serial passages, while *C. difficile* BK remained unchanged until ~ day 9. Strain BK cells grew in 16-32 μg/mL at the end of day 15 (GMIC values 2-4 μg/mL; 8-16 fold increase compared to naïve control)
- Under selective pressure with CB-183,315,VSEfs, VSEfm, VREfs and VREfm remained relatively unchanged over the 15 days of serial passages growing in a maximum of 8 μg/mL CB-183,315 (GMIC values 2-4 μg/mL; 1-8 fold increase compared to naïve control)
- CB-183,315 drug levels in Phase 2 fecal samples were > 1000 μg/g (Cubist; data on file) which is significantly higher than the highest MIC (4 μg/mL) in any of the follow up isolates
- These *in vitro* data suggest the emergence of resistance to CB-183,315 against *C. difficile*, *E. faecalis* and *E. faecium* (VSE and VRE) is likely to be rare.

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