Mechanisms of Protection by the Antitoxin Antibodies Actoxumab and Bezlotoxumab in Rodent Models of *Clostridium difficile* Infection

Amy Flattery[1]; Philip Lipari[1]; Peter Warn[2]; Pia Thommes[2]; Abdul Sattar[2]; Susanne Kramer[1]; Robert Huryk[1]; MaryAnn Powles[1]; Jon Polishook[1]; Zhiyong Yang[3]; Jeremy Ramsey[3]; Hanping Feng[3]; Lorraine Hernandez[1]; Alex Therien[1]

[1]Merck & Co., Inc., Kenilworth, NJ, USA; [2]Evotec (UK) Ltd., Manchester, UK; [3]University of Maryland Dental School, Baltimore, MD, USA

Abstract

Background and objectives: *Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacterium that colonizes and infects patients whose normal gut microbiota has been disrupted by treatment with broad-spectrum antibiotics. The symptoms of *C. difficile* infection (CDI) include mild to severe diarrhea, pseudomembranous colitis, and colonic rupture and are caused by toxins A (TcdA) and B (TcdB) released by the bacterium. A combination of monoclonal antibodies, consisting of the anti-TcdA antibody actoxumab and the anti-TcdB antibody bezlotoxumab, has previously been shown to reduce the rate of recurrent CDI in patients treated with the standard of care medications vancomycin and/or metronidazole. The antibody combination is also efficacious in a number of animal models of disease. In this study, we sought to explore the mechanisms of protection of actoxumab/bezlotoxumab in the context of *C. difficile* infection in rodents.

Methods: Actoxumab and bezlotoxumab were assessed in models of primary CDI in hamsters and of primary and recurrent CDI in mouse, in both prophylactic and therapeutic paradigms. *C. difficile* gut colonization was quantified by qPCR and by culturing on selective agar. Toxin levels were evaluated by ELISA and by cell rounding assay. Intestinal damage and inflammation were assessed by evaluating gross pathology and histopathology of intestinal tissues. Gut microbiota profiling was carried out using the PhyloChip technology.

Results: Treatment with actoxumab and bezlotoxumab decreased mortality and morbidity and attenuated the damage and inflammation that is associated with CDI in multiple rodent models. The antibodies had limited impact on *C. difficile* colonization and toxin levels in the gut, but protected the animals during the period of active infection that is normally associated with severe symptoms and, in many cases, death. While some antibody-treated animals, in particular in the hamster model, showed a degree of morbidity associated with CDI, the severity of disease was markedly reduced compared to vehicle-treated animals. Furthermore, antibody-treated animals that survived the *C. difficile* challenge eventually recovered fully from disease, showing reduced to undetectable colonization by *C. difficile* and re-establishment of a normalized gut microbiota.

Conclusion: The data are consistent with a model wherein actoxumab/bezlotoxumab protects the host from the symptoms of CDI, allowing the normal gut microbiota, the host's natural defense against *C. difficile*, to recover and prevent further colonization/infection.

Introduction

- *C. difficile* is a bacterial pathogen that colonizes and infects the intestines of patients whose gut microbiota has been disrupted following treatment with broad-spectrum antibiotics[1]
- *C. difficile* infections (CDI) are typified by severe diarrhea, pseudo-membranous colitis, and, in extreme cases, colonic rupture, sepsis, and death[2]
- The key pathogenic determinants of *C. difficile* are two exotoxins, toxin A (TcdA) and toxin B (TcdB), that target the epithelial cells that line the gut lumen[3]
- Actoxumab and bezlotoxumab are monoclonal antibodies that bind to and neutralize TcdA and TcdB, respectively, and have been shown to be protective in primary or recurrent CDI preclinically[4-7] and in recurrent CDI clinically[8]
- The mechanism(s) through which actoxumab and bezlotoxumab protect against CDI are not completely understood
- In this study, we utilize various rodent models to investigate the effects of actoxumab and bezlotoxumab on multiple aspects of CDI, including mortality, morbidity, gross and microscopic pathologic changes, inflammation, *C. difficile* burden and toxin levels, and gut microbiota

Materials and Methods

Hamster CDI models

- Animals were rendered susceptible to CDI by disrupting the gut microbiome via administration of clindamycin prior to infectious challenge with spores of *C. difficile* strain B1 (ribotype 053; ~50 spores) or strain 630 (ribotype 012; ~680 spores)
- Animals were dosed subcutaneously once a day with vehicle, or with actoxumab/bezlotoxumab (either prophylactically or therapeutically) and/or with vancomycin, at various time points (details in **Table 1** and **Figure 1**)
- Clinical signs were observed at least twice daily, and animals were euthanized either at predetermined time points or when they met predefined humane endpoints

Mouse CDI models

- Animals were rendered susceptible to CDI by disrupting the gut microbiome through administration of antibiotics in drinking water for up to 10 days, followed by a single administration of clindamycin prior to infectious challenge with *C. difficile* strain UK1 (ribotype 027; 10^5 spores) or VPI10463 (ribotype 087; 0.5 – 1x10^8 vegetative cells)
- Animals were dosed with vehicle, with actoxumab/bezlotoxumab, or with vancomycin at various time points (details in **Table 1** and **Figure 1**)
- Clinical signs were observed at least twice daily, and animals were euthanized either at predetermined time points or when they met predefined humane endpoints

Ethics statement

- All animal studies adhered to the National Research Council's *Guide for the Care and Use of Laboratory Animals,* 8th ed., and were reviewed and approved by the Merck, Evotec, and University of Maryland IACUCs

Gross intestinal pathology

- At necropsy, gross pathological state of the intestines was scored on a scale of 0 to 4 or 0 to 5, with 0 indicating healthy intestine and 4/5 indicating highly inflamed and swollen intestine with clear signs of hemorrhage

Histopathology and inflammation in intestinal wall

- Sections of murine ileum were formalin-fixed, stained with hematoxylin/eosin, and scored on a scale of 0 to 15 based on inflammatory cell infiltration, mucosal hypertrophy and thickness, vascular congestion and exudates, epithelial cell and architectural disruption, and submucosal edema

C. difficile burden

- *C. difficile* spores in the intestinal content and feces were quantitated by treating samples with ethanol or denatured alcohol and plating on *C. difficile*-selective agar

Toxin levels

- Toxin concentrations in intestinal contents were quantitated using a commercial ELISA (tgcBIOMICS) and using the manufacturer's protocol

Analysis of microbiome

- The microbiome of hamster cecum content was profiled by Second Genome using the Microbiome Discovery Services G3 PhyloChip™ Assay
- Differences in microbiome composition were analyzed by principal coordinates analysis, and data are visualized along the axis that explains most of the variation across samples (PCoA1 = 44% of the variation); relative abundance of individual phyla was also compared across selected samples

References

1. Seekatz AM, Young VB. *J Clin Invest.* 2014;124:4182-4189.
2. Rupnik M, et al. *Nat Rev Microbiol.* 2009;7:526-536.
3. Carter GP, et al. *Trends Microbiol.* 2012;20:21-29.
4. Babcock GJ, et al. *Infect Immun.* 2006;74:6339-6347.
5. Yang Z, et al. *Infect Immun.* 2015;83(2):822-831.
6. Steele J, et al. *J Infect Dis.* 2013;207:323-330.
7. Zhang Z, et al. *Infect Immun.* 2015;83:405-416.
8. Lowy I, et al. *N Engl J Med.* 2010;362:197-205.

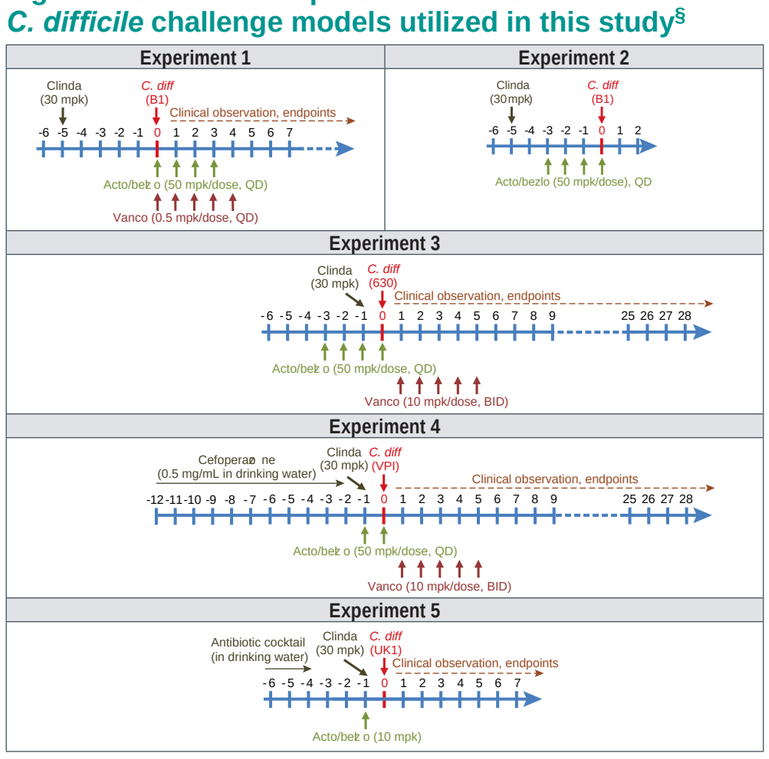
Results

Table 1. Summary of rodent CDI experiments described in this study[§]

Experiment #	1	2	3	4	5[5]
Animal	**Hamster**			**Mouse**	
Dosing	Therapeutic	Prophylactic	Prophylactic	Prophylactic	Prophylactic
Strain	B1	B1	630	VPI 10463	UK1
Treatment arms	• Vehicle • Vancomycin (subefficacious) • Acto/bezlo + vancomycin (subefficacious)	• Vehicle • Acto/bezlo	• Vehicle • Vancomycin • Acto/bezlo • Acto/bezlo + vancomycin	• Vehicle • Acto/bezlo • Vancomycin	• Vehicle • Acto/bezlo
Endpoints	• Survival • Gross pathology (scale 0-4) • *C. difficile* burden • Toxin levels • Gut biota	• Survival • Gross pathology (scale 0-4) • *C. difficile* burden • Toxin levels • Gut biota	• Survival • Gross pathology (scale 0-5) • *C. difficile* burden • Toxin levels	• Survival • Gross pathology (scale 0-5) • *C. difficile* burden • Toxin levels	• Survival • Gross pathology (visual) • Histopathology • Inflammation

[§]See **Figure 1** for dosing paradigms.

Figure 1. Schematic representations of timelines for *C. difficile* challenge models utilized in this study[§]



[§]See **Table 1** for details.

Figure 2. Actoxumab/bezlotoxumab improves survival in hamster and mouse CDI models

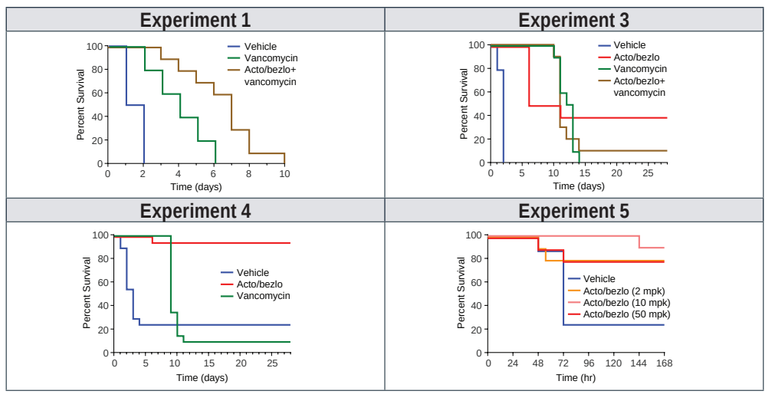
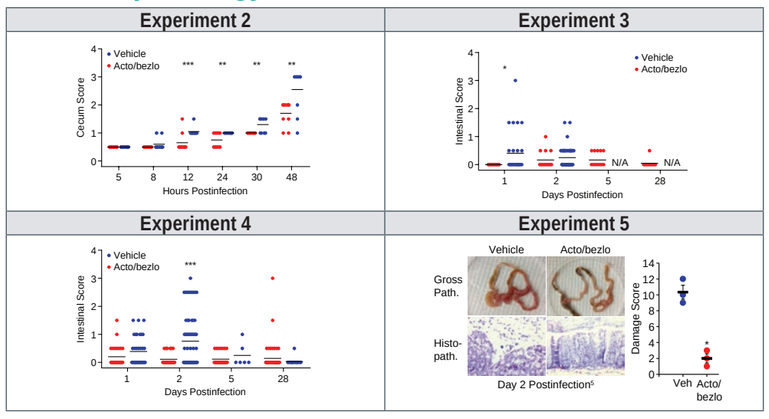


Figure 3. Actoxumab/bezlotoxumab protects from intestinal pathology in hamster and mouse models of CDI



*P<0.05; **P<0.01; ***P<0.001.

Figure 4. Actoxumab/bezlotoxumab has no impact on intestinal *C. difficile* burden, and surviving animals eventually clear the infection

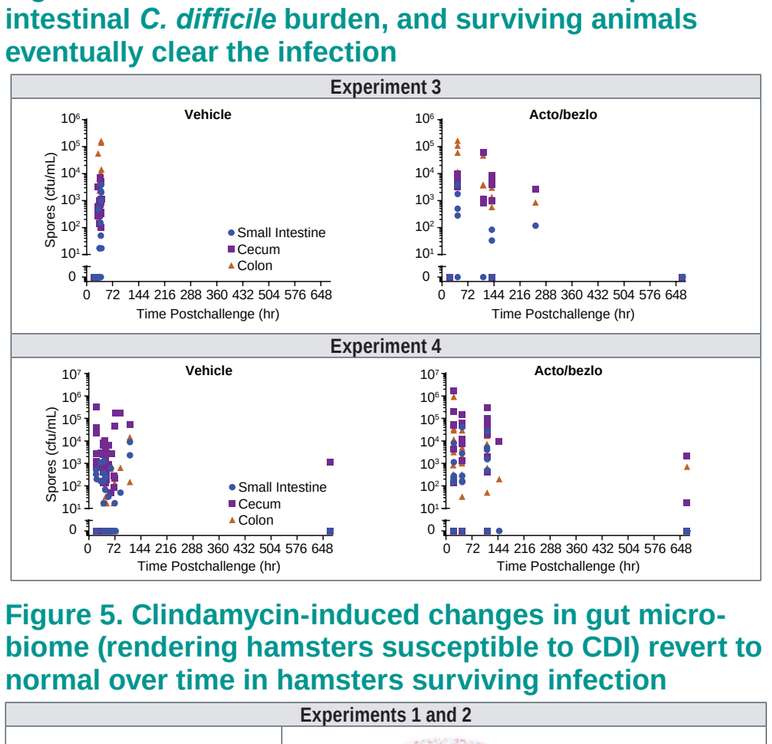
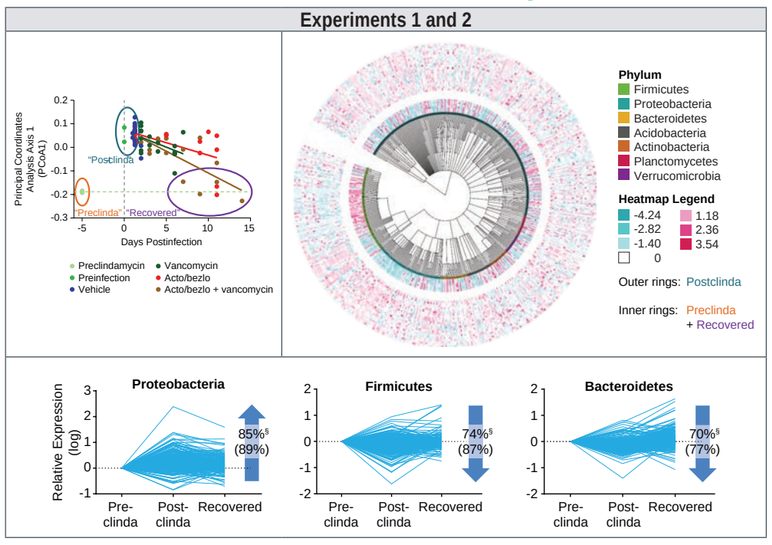
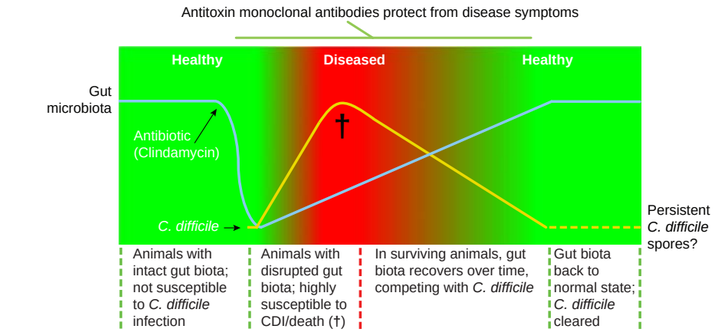


Figure 5. Clindamycin-induced changes in gut microbiome (rendering hamsters susceptible to CDI) revert to normal over time in hamsters surviving infection



§XX% = percent of organisms changing in direction of arrow in "post-clinda" group; (XX%) = percent of organisms changing back toward "pre-clinda " in "recovered" group.

Figure 6. Actoxumab/bezlotoxumab does not affect the course of infection, but protects the host from symptoms during disease window



Conclusions

- Treatment with antibiotics such as clindamycin disrupts the gut microbiota and renders mice and hamsters susceptible to infection with *Clostridium difficile*
  - Infected animals have a high intestinal *C. difficile* burden, severe intestinal pathology, and high mortality
- Actoxumab and bezlotoxumab in combination are protective in various iterations of the hamster and mouse models of CDI
  - Decreased mortality
  - Decreased morbidity (includes diarrhea, weight loss, abdominal distension, lethargy, hunched posture)
  - Decreased gross and microscopic intestinal pathology
  - Decreased inflammation
- Actoxumab/bezlotoxumab has no impact on *C. difficile* colonization
- Hamsters that survive the acute phase of CDI gradually clear the infection (*C. difficile* burden decreases), correlating with a gradual recovery of the normal gut microbiota
- The data are consistent with a model wherein actoxumab/bezlotoxumab protects the host against the symptoms of CDI during the window of time when the gut microbiota has not sufficiently recovered to prevent infection by *C. difficile*