

Ambush of *Clostridium difficile* Spores by Ramoplanin: Activity in an *In Vitro* Model

Carl N. Kraus,^a Matthew W. Lyerly,^b Robert J. Carman^b

Nanotherapeutics, Inc., Alachua, Florida, USA^a; TechLab, Inc., Blacksburg, Virginia, USA^b

Clostridium difficile infection (CDI) is a gastrointestinal disease caused by *C. difficile*, a spore-forming bacterium that in its spore form is tolerant to standard antimicrobials. Ramoplanin is a glycolipodepsipeptide antibiotic that is active against *C. difficile* with MICs ranging from 0.25 to 0.50 $\mu\text{g/ml}$. The activity of ramoplanin against the spores of *C. difficile* has not been well characterized; such activity, however, may hold promise, since posttreatment residual intraluminal spores are likely elements of disease relapse, which can impact more than 20% of patients who are successfully treated. *C. difficile* spores were found to be stable in deionized water for 6 days. *In vitro* spore counts were consistently below the level of detection for 28 days after even brief (30-min) exposure to ramoplanin at concentrations found in feces (300 $\mu\text{g/ml}$). In contrast, suppression of spore counts was not observed for metronidazole or vancomycin at human fecal concentrations during treatment (10 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$, respectively). Removal of the *C. difficile* exosporium resulted in an increase in spore counts after exposure to 300 $\mu\text{g/ml}$ of ramoplanin. Therefore, we propose that rather than being directly sporicidal, ramoplanin adheres to the exosporium for a prolonged period, during which time it is available to attack germinating cells. This action, in conjunction with its already established bactericidal activity against vegetative *C. difficile* forms, supports further evaluation of ramoplanin for the prevention of relapse after *C. difficile* infection in patients.

Clostridium difficile infection (CDI) remains a clinically important disease, with increasing incidence and mortality rates in the United States; it now surpasses methicillin-resistant *Staphylococcus aureus* as the most common health care-associated infection (HAI) and results in an annual cost burden to the health care system of \$4.8 billion (1–3). A prolonged cure for treated patients remains elusive, as evidenced by the high rates of relapse (same ribotype within 30 days) and reinfection (different ribotype within 30 days). Recurrence rates have ranged from 15% to 40% (4–6) and are most commonly associated with the ribotype 027 epidemic strain (7). One contributing factor for high relapse rates is the presence of spores that persist within the intestinal lumen and evade elimination by standard anti-CDI treatments (8, 9). *C. difficile* spores are tolerant to standard antimicrobial therapy; hence, antimicrobial interventions targeting vegetative cells alone are unlikely to eradicate this disease reservoir (10).

Ramoplanin is a glycolipodepsipeptide antibiotic produced from *Actinoplanes*, which inhibits peptidoglycan biosynthesis by limiting lipid II availability (11); it is bactericidal to many Gram-positive aerobic and anaerobic bacteria, including *C. difficile* and vancomycin-resistant *Enterococcus* (11). Ramoplanin has known bactericidal activities against wild-type *C. difficile* and strains with reduced susceptibilities to vancomycin and has been studied in an open-label phase 2 clinical trial evaluating early clinical efficacy (12, 13). Ramoplanin was also previously reported to have activity against *C. difficile* spores in an *in vitro* gut model and an *in vivo* hamster model (14). Since residual intraluminal spores are considered essential for subsequent disease relapse, we have used *in vitro* models to define in more detail the activities of ramoplanin against *C. difficile* spores.

MATERIALS AND METHODS

Bacterial strain and spore preparation. *C. difficile* spores (ribotype 027) were prepared as previously described (15). Spore pellets were resuspended in 1/10 the original volume (50 ml) in deionized water. The spores

were counted by serial 10-fold dilution in prereduced anaerobically sterilized (PRAS) dilution blanks (Anaerobe Systems, Morgan Hill, CA) and subsequently plated on brain heart infusion (BHI) agar (anaerobic incubation for 48 h at 37°C \pm 2°C) supplemented with horse blood and sodium taurocholate, a known germinant (15).

Antimicrobial survey. In this study, 10⁵ spores/ml were stored in deionized water anaerobically at 37°C \pm 2°C for 6 days and continuously exposed to fecal-level concentrations of metronidazole (10 $\mu\text{g/ml}$), vancomycin (500 $\mu\text{g/ml}$), or ramoplanin (300 $\mu\text{g/ml}$) (11, 16, 17). One milliliter of spores was sampled daily from day 0 (D0) to D6 and serially diluted 10-fold, as described above. Neither metronidazole nor vancomycin demonstrated spore-specific activity; hence, such activity was assessed for only ramoplanin (using the same ribotype, 027). The spores (10⁵ spores/ml) were exposed to 0, 2, 300, or 600 $\mu\text{g/ml}$ of ramoplanin for 30 min at 37°C \pm 2°C. After 30 min, excess ramoplanin was removed by six sequential centrifugation steps, each requiring 10 min at 15,000 \times g with subsequent resuspension in deionized water. After the last wash, spores were reconstituted to the original volume (50 ml) with deionized water and stored at 37°C \pm 2°C for 28 days. At each time point (days 0, 1, 7, 14, and 28), samples were centrifuged at 10,000 \times g for 2 min to separate spores from the unbound ramoplanin. The spores were reconstituted to their original sampling volume, diluted, and plated as previously described. Supernatants were held for the ramoplanin bioassay, as described below.

Received 20 November 2014 Returned for modification 23 December 2014
Accepted 30 January 2015

Accepted manuscript posted online 17 February 2015

Citation Kraus CN, Lyerly MW, Carman RJ. 2015. Ambush of *Clostridium difficile* spores by ramoplanin: activity in an *in vitro* model. *Antimicrob Agents Chemother* 59:2525–2530. doi:10.1128/AAC.04853-14.

Address correspondence to Carl N. Kraus, ckraus@nanotherapeutics.com.

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doi:10.1128/AAC.04853-14

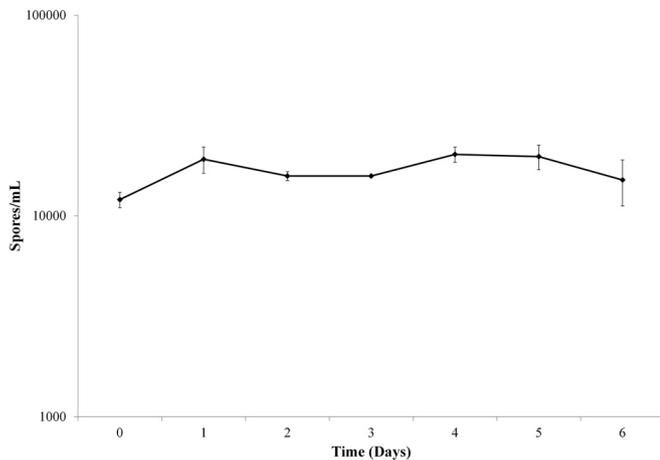


FIG 1 Spores incubated in water over the course of 6 days showed no significant variation in counts ($P = 0.24$).

Ramoplanin bioassay. The procedures used for the bioassay followed those described by Carman et al. (18). Briefly, lawns of 10^6 CFU/ml *Streptococcus salivarius* were plated on unsupplemented blood agar plates. Thirty microliters of each wash supernatant (days 0, 1, 7, 14, and 28) was pipetted aseptically onto blank sterile paper discs, which were transferred to the lawn of *S. salivarius*, in triplicate. Plates were incubated aerobically for 24 h at $37^\circ\text{C} \pm 2^\circ\text{C}$. Zones of inhibition around each disc were measured using calipers and compared to standard curves of ramoplanin for which known concentrations of ramoplanin and measured zones of inhibition had been established.

Exosporium processing. Spore exosporia were removed as described by Escobar-Cortes et al. (19); “intact” spores did not undergo such processing. Briefly, spores were washed four times by centrifugation (10 min at $10,000 \times g$) and resuspended in deionized water followed by final resuspension in 15 ml of phosphate-buffered saline (PBS) and sonication for 90 s. Three milliliters of 10% Sarkosyl (detergent) was added to each preparation and subsequently incubated for 15 min at room temperature with rocking. Preparations were then centrifuged for 10 min at $10,000 \times g$, and pellets were resuspended in 10 ml PBS with 0.1 ml of 1 M Tris and

10 mg of lysozyme. Spores were rocked overnight (approximately 18 h) at $37 \pm 2^\circ\text{C}$ and then sonicated for 90 s, passed through a 50% solution of sucrose using a swinging bucket rotor for 20 min at $4,000 \times g$, and resuspended in a solution containing 3 ml PBS, 200 mM EDTA, 300 ng/ml proteinase K, and 1% Sarkosyl. The spores were further rocked at room temperature for 20 min and were passed through a 50% solution of sucrose as previously described by Escobar-Cortes et al. (19). Next, the spores were washed in deionized water by two centrifugation steps for 10 min at $10,000 \times g$ and resuspended in deionized water; plating and incubation were carried out as described in the foregoing text.

For comparisons among different samples, Microsoft Excel (2010) was used for the statistical evaluation of one-way analysis of variance (ANOVA) (single factor) as well as *post hoc t* testing (two samples, assuming unequal variances).

RESULTS

Spore stability in water. Incubation in water at 37°C for 6 days resulted in spore persistence with limited variation in spore counts from D0 through D6 (Fig. 1). The mean spore counts (spores/ml) on D0 and D6 were $1.2 \times 10^4 (\pm 0.11 \times 10^4)$ and $1.5 \times 10^4 (\pm 0.39 \times 10^4)$, respectively, with no significant differences noted ($P = 0.240$). Stable spore counts permitted the assessment of continuous antimicrobial exposure in water with the expectation that no effect on spores would be identified, a finding consistent with previous observations (10).

Antimicrobial activity on spores. The effects of the assessed antimicrobials on spores were distinct, with ramoplanin clearly having a more pronounced effect than did vancomycin, metronidazole, or the water controls. Continuous exposure to vancomycin (500 $\mu\text{g}/\text{ml}$), metronidazole (10 $\mu\text{g}/\text{ml}$), and water did not significantly reduce spore counts over that at baseline. The spores exposed to ramoplanin (300 $\mu\text{g}/\text{ml}$) showed no growth after plating (Fig. 2), resulting in a significant decline (denoted by the asterisks in Fig. 2) of spore counts over time based on ANOVA at each time point after baseline (D1 to D2, $P = 0.0002$; D3 to D4, $P = 0.0001$; D5 to D6, $P < 0.0001$). *Post hoc*, two-sample *t* tests confirmed significant differences in spore counts between ramoplanin and comparators (water, metronidazole, and vancomycin)

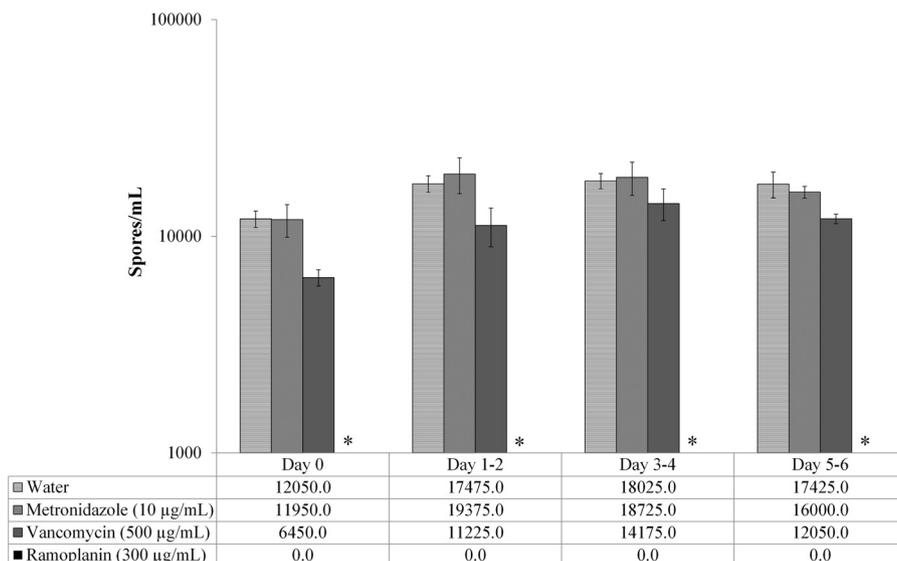


FIG 2 Continuous exposure of spores in water to metronidazole, vancomycin, or ramoplanin. Only ramoplanin-exposed spores yielded no counts ($P < 0.001$ for all time points).

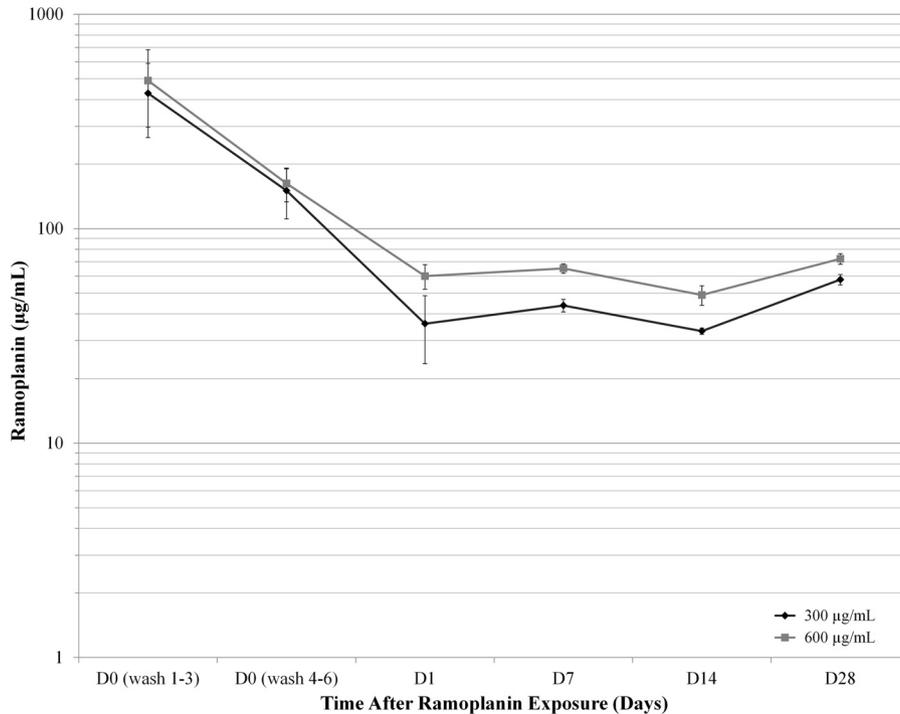


FIG 3 Unbound ramoplanin was recovered from spores exposed to two concentrations of ramoplanin for 30 min (300 and 600 µg/ml). Concentrations were stable from day 1 (D1) to D28.

at the evaluated time points (all P values, <0.02). Not only were the differences in spore counts significant between ramoplanin and each comparator, but the magnitude of the effect was striking in that ramoplanin-exposed spores, when plated on agar, exhibited no observable growth. There was no statistical evidence of spore growth suppression by vancomycin or metronidazole compared to the baseline (as assessed by ANOVA; $P = 0.18$ and 0.49 , respectively).

Persistence of ramoplanin activity. The observed effect of ramoplanin on spores was prolonged. Ramoplanin concentra-

tions were varied (0 µg/ml, 2 µg/ml, 300 µg/ml, and 600 µg/ml) to characterize a concentration-response relationship for the drug's effects. Exposure to ramoplanin was brief and limited to 30 min. Ramoplanin-exposed spores were washed six times on D0 to remove unbound drug and repelleted once at each time point. At set intervals (D0, D1, D7, D14, and D28), spore-bound and free ramoplanin concentrations were assayed (Fig. 3), and spores were counted (Fig. 4, no growth is denoted by the asterisks). From D1 through D28, the supernatant concentrations of ramoplanin remained stable, ranging from (mean \pm standard error of the mean

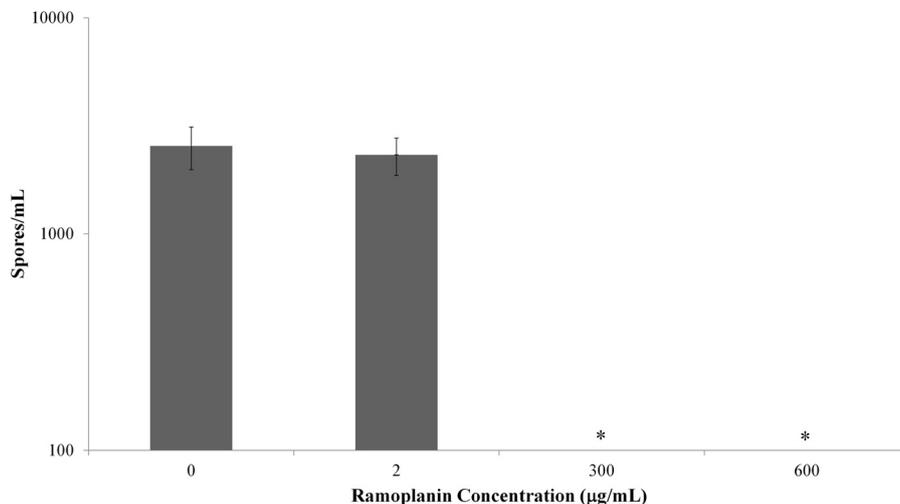


FIG 4 Summary spore counts for all time points at day 28 after 30 min of ramoplanin exposure. Higher concentrations (300 and 600 µg/ml) resulted in no counts for the duration of the incubations in water.

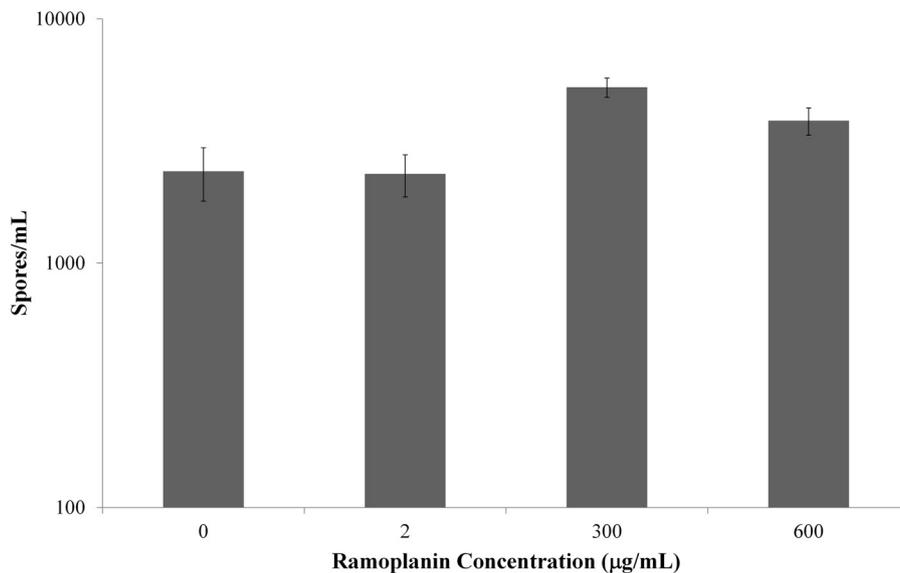


FIG 5 Diluting ramoplanin-exposed samples 10-fold resulted in growth not measurable at previous dilution.

[SEM]) 33.2 ± 1.1 to 57.8 ± 3.2 µg/ml for the spores exposed to 300 µg/ml and from 49.0 ± 5.1 to 72.3 ± 4.1 for the those exposed to 600 µg/ml (Fig. 3). There were no significant differences in supernatant ramoplanin concentrations among the evaluated time points for spores exposed to 300 µg/ml ($P = 0.116$) or to 600 µg/ml ($P = 0.065$).

At each time point for which the ramoplanin drug concentrations were assessed, spore pellets free of unbound ramoplanin were plated for spore counts to determine if the spore-related activity would persist past the short-term time points initially evaluated (D0 to D6 of the antimicrobial survey). Specifically, the intent was to determine whether a brief 30-min ramoplanin exposure that was followed by multiple washes can inhibit spore growth over the course of 28 days. Those spores exposed to low concentrations of ramoplanin (2 µg/ml) demonstrated no inhibition relative to the controls (0 µg/ml ramoplanin) over the course of 28 days (Fig. 4), with mean count \pm SEM values of $2.3 \times 10^4 \pm 4.6 \times 10^2$ and $2.5 \times 10^4 \pm 5.71 \times 10^2$, respectively. Those spores exposed to higher concentrations of ramoplanin (300 µg/ml and 600 µg/ml) had no growth observed when plated undiluted. The difference in spore counts after ramoplanin exposure compared to deionized water controls was significant ($P < 0.0001$). However, diluting these samples 10-fold resulted in spore counts similar to those for the spores exposed to 0 and 2 µg/ml (Fig. 5). At an additional dilution (10 \times), spore counts of samples exposed to higher concentrations of ramoplanin (300 µg/ml and 600 µg/ml) did not demonstrate an effect that was similar to those with the lower dilution spore counts relative to those of the water controls ($P = 0.19$ and 0.53 , respectively, paired two-sample t -tests for means).

Adherence of ramoplanin to *C. difficile* exosporium. In order to determine whether the exosporium was essential to the observed growth-inhibiting effects of ramoplanin, the exosporia were processed as previously described (19), resulting in spores with “stripped” exosporia, and then compared to unprocessed spores (with “intact” exosporia) under three separate conditions (the ramoplanin concentration was 300 µg/ml in each condition):

(i) ramoplanin exposure with no subsequent spore processing (i.e., “intact”), (ii) ramoplanin exposure with subsequent spore processing (i.e., “stripped”), and (iii) water exposure with subsequent spore processing (Fig. 6). Ramoplanin-exposed spores that had not been processed yielded no growth when plated; in contrast, when ramoplanin-exposed spores were processed (i.e., their exosporia had been removed), the spore counts were similar to that for control water-exposed spores. After ramoplanin exposure, the difference in spore counts between samples that were not processed (with presumed intact exosporia) and samples that were processed (without presumed exosporia) was significant ($P < 0.0001$). No difference in spore count was noted when comparing processed spores that had been exposed to ramoplanin to spores exposed only to water ($P = 0.389$).

DISCUSSION

C. difficile infection, as a leading cause of hospital-acquired infection and as a particular source of morbidity and mortality among the elderly, is a cause for concern to clinicians across multiple specialties. Unlike most acute illnesses from infections, for which treatment results in a prolonged cure, *C. difficile* infections carry with them a high risk of disease recurrence that can affect approximately 25% of successfully treated patients. This disease recurrence is, at least in part, a consequence of *C. difficile* spores that persist in the gut after the acute infection has been treated. These forms of *C. difficile* remain tolerant to standard antimicrobials and, if present in sufficient quantity, can reemerge as a threat to the host not long after hospital discharge.

Ramoplanin has demonstrable *in vitro* activities against many pathogens of clinical concern, such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *C. difficile*. While its previously described mechanism of action, the inhibition of cell wall synthesis through peptidoglycan binding, remains distinct from those of other agents that affect the cell wall, such as β -lactams and cell wall-active glycopeptides (e.g., vancomycin), the effect of ramoplanin on the spore, the antimicrobial-tolerant form of *C. difficile*, has not been well characterized. Chilton et al. spec-

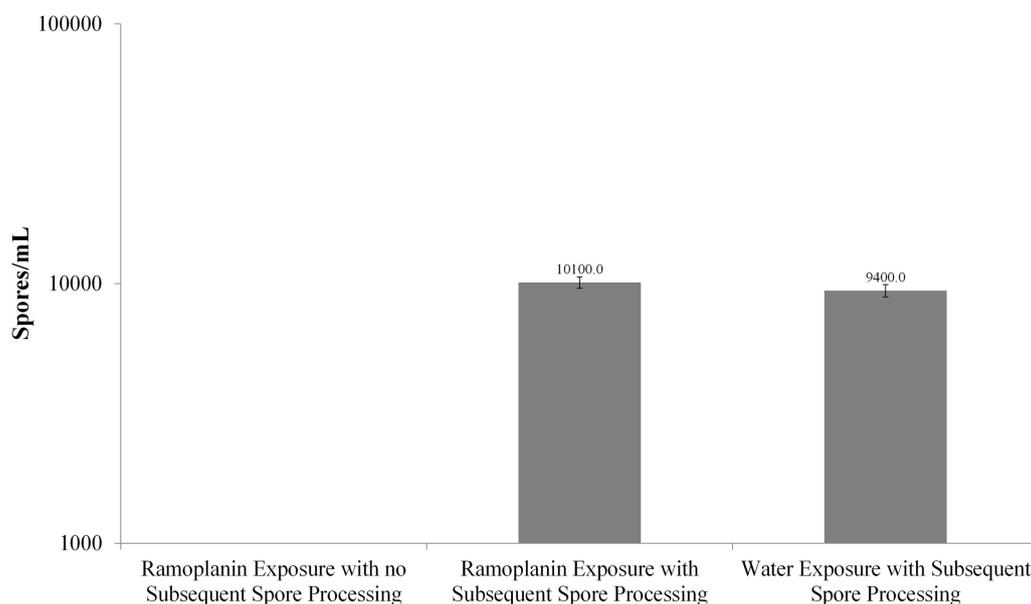


FIG 6 Removal of exosporia after exposure to ramoplanin resulted in counts equivalent to those after water exposure; unprocessed spores, similarly exposed, exhibited no measurable counts.

ulated that certain antimicrobial agents, such as nisin and oritavancin, adhere to the exosporium of *C. difficile* because of electrostatic charges resulting from cross-linkages on the spore surface (20). It is possible that such exosporial adherence might persist and permit sufficient concentrations of antimicrobial activity to kill any vegetating organisms. This would explain the observations reported here, where ramoplanin was adherent to the exosporial surface and prevented emergence of viable *C. difficile* organisms.

We have presented evidence supporting the concept that antimicrobial spore binding may play a role in the activity of ramoplanin against *C. difficile*. Based on the prolonged suppression of spore counts and the presence of assayable ramoplanin in the supernatant samples, ramoplanin appears to bind to the *C. difficile* exosporium for an extended period of time. We postulate that ramoplanin remains adherent to the exosporium and achieves a concentration equilibrium with the supernatant over the course of the 28-day incubation. Because ramoplanin is a relatively large molecule with a molecular mass of 2,554 Da (21), it is unlikely to penetrate the exosporium. Instead, it remains adherent to the exosporium and only acts as an effective antimicrobial as it ambushes the germinating cell. Supporting this mechanism further is the observation that removal of the exosporium after ramoplanin exposure, prior to plating, resulted in spore counts no different from those of control water-exposed spores with intact exosporia (Fig. 6). More challenging to explain are increasing spore counts when the same samples are diluted 10-fold (Fig. 5). We suggest that, with a 10-fold dilution, the local ramoplanin concentration on the agar plate falls below the MIC, thereby permitting spore growth.

Firm conclusions cannot yet be made from these data since observed counts can be confounded by cell turnover. This seems unlikely since initial assessment with a glutamate dehydrogenase assay did not support a high turnover rate (data not shown). Future experiments should, however, include an alternative evaluation of spore germination, such as phase contrast (22). Furthermore, although “fecal concentrations” were used for this *in vitro*

evaluation, these studies were carried out without the presence of fecal material, a limitation which will need to be addressed in follow-up studies.

Treatment of recurrent *C. difficile* infection includes the use of pulsed vancomycin (23), which is, in part, an attempt to eliminate the intraluminal spores that are germinating at different times. Emerging data indicate that sporulation rates may vary based on ribotype, with a heightened rate of sporulation for *C. difficile* ribotype 027 (24), which is associated with the higher disease recurrence rate for this particular ribotype (25). Enhancing the therapeutic options to address such disease recurrence is clearly an unmet need that is currently being evaluated with strategies such as fecal microbiota transplantation (FMT) and *C. difficile* vaccines (26–30). Should the activity observed in these *in vitro* experiments mirror activity in the human gut, then the use of ramoplanin as an agent for secondary prophylaxis may be a therapeutic option for clinicians.

Given the prior clinical experience with ramoplanin that includes a phase II study in CDI (13), the need to identify strategies for relapse reduction in patients with CDI, and the evidence reported here that supports an ambush type of activity against CDI spore forms, definitive determination of the clinical efficacy of ramoplanin in the secondary prophylaxis of CDI is appropriate and timely.

ACKNOWLEDGMENTS

We thank R. G. McAllister for manuscript review and editing.

This study was funded by Nanotherapeutics, Inc.

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