Ileal Dosing of a Nisin and Miconazole Combination is Efficacious in the Hamster *Clostridium difficile* Associated Disease Model

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Abstract

Objectives: *Clostridium difficile* infections (CDI) continue to be a significant cause of morbidity and mortality among patients suffering from clinical infectious diarrhoea and with the emergence of epidemic strains that are antibiotic resistant, new therapeutic approaches are needed for the treatment of CDI. The current study describes the evaluation of a potential novel therapy for CDI that involves a combination of nisin, an antibacterial food preservative, and miconazole, an antifungal. Minimum inhibitory concentration (MIC) values were determined for the combination, and the hamster *C. difficile* associated disease (HCDAD) model was used to determine in vivo efficacy of the combination.

Methods: Brucella broth, with or without 5% (w:v) sterile hamster cecal contents, was used to determine the MIC of nisin alone, miconazole alone, and the nisin/miconazole combination (1:1) against the *C. difficile* strain used in the hamster studies. A group of male Golden Syrian hamsters were ileal cannulated 10 days prior to infection with an externally ported catheter, while another group of hamsters remained non-cannulated. *C. difficile* was cultured for 48 hours to 6.4 log10 colony-forming units (CFU)/mL, and animals were orally infected with 0.5 mL of the 48-hour culture. Animals were subcutaneously dosed with clindamycin 24 hours after infection, and the nisin:miconazole (400 mg/kg:200 mg/kg) combination was administered either orally or through the ileal port every 6 hours for 5 days beginning 18 hours after clindamycin dosing. Survival was recorded for 18 days after infection and Kaplan-Meier survival plots were generated for the dose groups.

Results: The MIC value for either nisin or miconazole alone in media containing 5% cecal matter was >64 µg/mL, while the nisin:miconazole (1:1) combination in the same media resulted in an MIC value of 16 µg/mL. In media without cecal matter, the MIC values were 4 µg/mL for miconazole, 64 µg/mL for nisin, and 8 µg/mL for the nisin:miconazole combination. In the HCDAD model, all of the orally dosed animals died within 5 days. Dosing the same nisin:miconazole combination through the ileal port resulted in 63% of the animals surviving 5 days post-infection and 36% of the animals surviving to the end of the study (18 days after infection).

Conclusion: The lower MIC value of the nisin:miconazole combination in the presence of cecal matter suggested that this combination had the potential for the treatment of CDI. The in vivo efficacy for the combination was confirmed in the HCDAD model when dosed directly into the ileum every 6 hours for 5 days. The results from this study suggest that this combination could be used to treat CDI and should be further investigated.

Introduction

Since its cause was identified in 1978, *Clostridium difficile*-associated disease (CDAD) has become as a global healthcare issue due to the continued use of broad-spectrum antibiotics, like fluoroquinolones, and the associated emergence of epidemic strains in 2000 (1). Epidemic strains of *C. difficile* harbor resistance to antibiotics and have been reported to express significantly more toxin A/B than other non-epidemic strains (2). Subsequently, the percent morality associated with CDAD increased in the US by 400% from 2000 to 2007 and has been directly linked to the increased prevalence of epidemic strains among clinical isolates. In the 2013 Threat Report (3), the Centers for Disease Control and Prevention identifies *C. difficile* as an urgent threat level organism. The report stated that CDAD accounts for 14,000 deaths in the US each year, and that medical costs for the treatment of CDAD exceeds more than one billion US dollars annually. These numbers clearly indicate that CDAD is an important healthcare issue and that better treatment options are critically needed.

Orally dosed metronidazole and vancomycin have been the standard of care for the treatment of CDAD for more than two decades (4), but reduced efficacy and increased recurrence of CDAD after treatment has ended are major issues with both of these antibiotics. Alternative therapies, like oral fidaxomicin and fecal bacteriotherapy, are available, but their use can be prohibitive due to high costs or lack of regulatory oversight (5, 6). Therefore, an ideal therapy would need to be efficacious, have a low cost-of-goods, and have minimal regulatory issues.

Here, we describe the evaluation of a therapy that utilizes the combination of a known antibacterial food additive, nisin, and a marketed antifungal, miconazole, for the treatment of CDAD in hamsters. Nisin is a natural antibacterial produced by *Lactococcus lactis* and is the only lantibiotic approved food supplement used to extend the shelf life of products since the1960s (7). Miconazole is an antifungal that was developed in the 1960s for the treatment of mucosal and skin-associated fungal infections (8). It has low oral bioavailability, is rapidly metabolized by the liver, and has been shown to have antibacterial activity (9).

Panel 1: MIC Values Against *C. difficile* UNT103-1

	Brucella Broth Only	Brucella Broth + 5% Cecal Contents	Fold Shift
Miconazole	4 µg/mL	>64 µg/mL	16
Nisin	64 µg/mL	>64 µg/mL	0
Miconazole:Nisin (1:1)	8 ug/mL	16 µg/mL	2
Miconazole:Nisin (1:8)	2:16 µg/mL	4:32 µg/mL	2
Vancomycin	1 µg/mL	4 µg/mL	4
Metronidazole	0.5 µg/mL	0.25 µg/mL	0

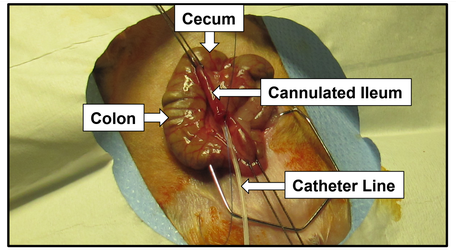
- **MIC values were determined by a modified microdilution method whereby serially diluted compounds were pre-incubated in brucella broth supplemented with 5% (v:v) sterile, naïve hamster cecal contents or non-supplemented broth for 30 minutes.**
- **Plates were inoculated with 4.1 log $_{10}$ CFU of *C. difficile* UNT103-1 and anaerobically incubated for 48 hours at 37°C.**

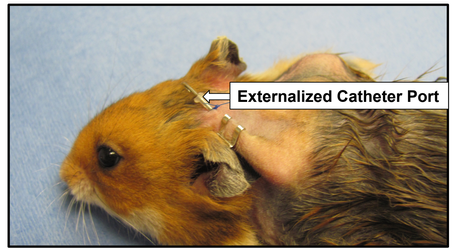
Methods and Materials

Minimum Inhibitory Concentration (MIC) Assay: A modified version of the CLSI microdilution method was used to determine MIC values. Individual and combined concentrations of nisin and miconazole were formulated and two-fold serially diluted in DMSO. The two-fold dilutions were incubated for 30 minutes in brucella broth alone or in broth that contained 5% (v:v) sterile cecal contents harvested from naïve Golden Syrian hamsters. All of the broth incubated compound dilutions were centrifuged for 10 minutes (3,700x*g*), and supernatants were transferred to 96-well plates and equilibrated to anaerobic conditions for 3 hours. Supernatants were inoculated with 4.1 $\log_{10}$ CFU/mL of UNT103-1, a non-epidemic clinical isolate of *C. difficile* (VA11; C Donskey, Cleveland VA), and microdilution plates were anaerobically incubated for 48 hours at 37°C. Vancomycin and metronidazole were included in the MIC assay as controls and were subjected to all of the conditions outlined above.

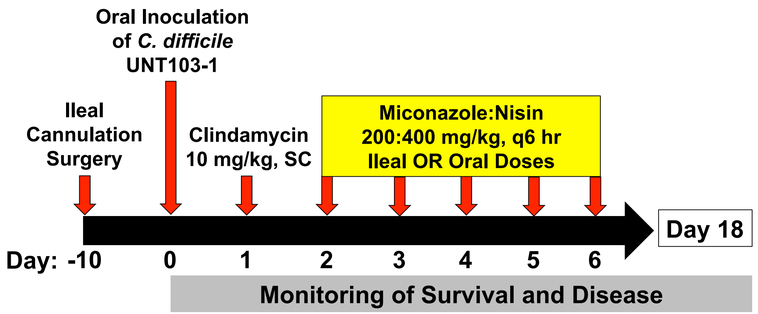
Cannulated Hamster CDAD Model: The described animal model was developed in accordance with protocol 2012/13-14 that was reviewed and approved by the IACUC at UNTHSC. Male Golden Syrian hamsters (112 – 132 grams) were obtained from Charles River Laboratories and allowed to acclimate for 5 days. On the day of surgery, each animal was anesthetized with 3 – 4% isoflurane and a midline incision was generated through the ventral peritoneum. While retracting the incision, the ileum proximal to the ileocecal junction was isolated and temporarily ligated with 2-0 silk suture. A 1 – 2 mm transverse incision was made within the ligated ileal section and the tippet of an MRE-40 catheter (Braintree Scientific) was inserted through the incision and advanced into the ileum to a length of ~1 cm towards the ileocecal junction. The catheter was anchored to the ileum with 5-0 monofilament suture, and the cannulated ileum was placed back into the peritoneal cavity. The peritoneal incision was closed and the remainder of the catheter was routed subcutaneously (SC) for external porting through the skin near the scapular region. Animals were given 2 mg/kg meloxicam as needed for pain and discomfort. Ten days after surgery, animals were orally infected with 6.0 $\log_{10}$ CFU of *C. difficile* UNT103-1, SC dosed with 10 mg/kg clindamycin 1 day after infection, and treated with a miconazole:nisin combination of 200:400 mg/kg by oral or ileal administration every 6 hours for 5 days beginning 2 days after infection. Survival and disease were monitored for 18 days after infection.

Panel 2: Ileal Cannulation of Hamsters

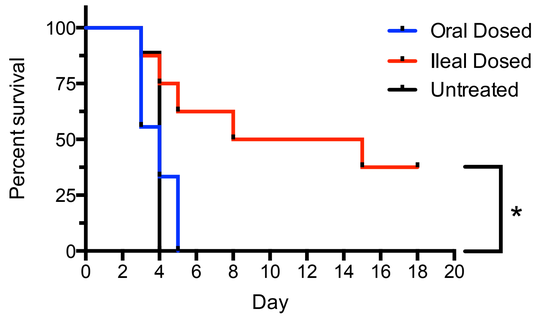




Panel 3: Experimental Timeline of Cannulated Hamster CDAD Model



Panel 4: Kaplan-Meier Survival Curves of Miconazole:Nisin Treated Hamsters



- **Oral and ileal doses of miconazole:nisin at 200:400 mg/kg were given every 6 hours for 5 days beginning 2 days after infection, and survival was monitored for 18 days post-infection with *C. difficile* UNT103-1.**
- **Statistical significance (*) for the survival curves of miconazole:nisin oral and ileal dosed groups (Log-rank *p*=0.091; Wilcoxon *p*=0.0189).**

Summary and Conclusions

- **The MIC values for the ratio combinations of miconazole and nisin (1:1, 1:8) against *C. difficile* increased 2-fold in brucella broth containing 5% hamster cecal contents when compared to MIC values generated in brucella broth alone. Similarly, the MIC value for vancomycin against *C. difficile* increased 4-fold in brucella broth containing 5% hamster cecal fluid. (Panel 1)**
- **MIC results for the miconazole and nisin combination suggested possible therapeutic efficacy for the combination in a *C. difficile* infected host. The hamster model of *C. difficile*-associated diarrhea (CDAD) was selected to evaluate the combination *in vivo*. However, nisin is known to be degraded in the small intestine (10); therefore, in order to efficiently deliver the combination into the infected cecum, the distal end of the hamster ileum was cannulated with a catheter port. (Panel 2, 3)**
- **All of the *C. difficile* infected animals (n=9) orally dosed with miconazole:nisin at 200:400 mg/kg died by the fourth day of dosing, and the untreated controls (n=9) were dead 3 days after infection. However, ileal dosing of the miconazole:nisin combination resulted in 38% (n=8) of the animals surviving 18 days after infection. Analysis of the Kaplan-Meier survival plots indicated that the results were significantly different for animals ileal dosed with miconazole:nisin as compared to those orally dosed with the combination. (Panel 4)**
- **Miconazole is an antifungal marketed for the treatment of skin- and mucosal-associated fungal infections, while nisin is an antibacterial that has been used as a food preservative since the 1960s (7-10). Survival results from the cannulated hamster CDAD model suggests that a combination of these two diverse agents could be used for clinically treating CDAD in humans and should be further investigated.**

References

1. Kelly CP and JT LaMont. Clostridium difficile — More Difficult Than Ever. N Engl J Med 2008; 359:1932-1940.
2. CDC. Antibiotic Resistance Threats in the United States, 2013. Atlanta, GA; 2013. p. 7, 36-7.
3. McDonald LC, GE Killgore, and A Thompson. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med 2005;353:2433-2441.
4. Leffler DA and JT LaMont. Treatment of Clostridium difficile-associated disease. Gastroenterology. 2009;136(6):1899.
5. Venugopal AA and S Johnson. Fidaxomicin: a novel macrocyclic antibiotic approved for treatment of Clostridium difficile infection. Clin Infect Dis. 2012 Feb;54(4):568-74.
6. Bakken JS. Fecal bacteriotherapy for recurrent Clostridium difficile infection. Anaerobe 2009; 15:285.
7. Delves-Broughton, J. (2005) Nisin as a food preservative. Food Australia, 57(12):525-527.
8. Sheehan DJ, CA Hitchcock, and CM Sibley. Current and emerging azole antifungal agents. Clin. Microbiol. Rev. 1999; 12:40–79.
9. Bossche VA, W Lauwers, G Willemsens, P Marichal, F Cornelissen and W Cools. Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: The inhibition of isoprenoid biosynthesis. Pesticide Science. 2006 APR; Volume 15, Issue 2, pages 188–198.
10. Ugurlu T, M Turkoglu, US Gurer, and BG Akarsu. Colonic delivery of compression coated nisin tablets using pectin/HPMC polymer mixture. Eur J Pharm Biopharm. 2007 Aug;67(1):202-10.