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Efficacy and Impact to the Microbiome of Novel Specifically-Targeted-Antimicrobial-Peptides against *C. difficile* Infection

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Abstract

Background: *C. difficile* is a gram-positive, spore forming bacteria causing infections ranging from mild diarrhea to pseudomembranous colitis, primarily in hospitalized patients who have been exposed to antibiotic treatment. Vancomycin (VAN), Metronidazole (MTZ) and Fidaxomicin (FDX) are currently used to treat *C. difficile*-associated disease (CDAD), however, there remains a large unmet need of novel therapies for patients suffering from disease recurrence following a successful cure. Novel specifically targeted antimicrobial peptides (STAMPs) have been designed to selectively kill *C. difficile* with minimal effects on commensal bacteria and have shown rapid and selective killing of *C. difficile in vitro*. An antibiotic induced-murine model of recurrent CDI was used to investigate the efficacy and impact on the microbiome of STAMPs in comparison to current therapies.

Methods: Post antibiotic cocktail treatment, *C. difficile* susceptible C57BL/6 mice (10/group) were challenged with 4.5log$_{10}$ *C. difficile* spores (ATCC 43255). Test articles were administrated once daily on days 0-4 via a perirectal route. Efficacy and disease recurrence was evaluated by tracking changes in disease severity score, weight, and survival of mice over a 15-day period following challenge. The impact of the microbiome composition was analyzed through 16S rRNA sequencing of the fecal DNA samples collected before and after treatment.

Results: STAMP treatment resulted in significant protection against CDI with 100% survival and <5% body weight loss compared to vehicle control (50% survival and >15% body weight loss). No recurrence was noted for STAMP treated groups while VAN treatment showed initial protection followed by disease recurrence at days 8-11 characterized by significant weight loss, decreased survival and severe disease scores. Microbiome analysis indicated that STAMP treatment showed greater microbial diversity and recovery of known potentially beneficial normal flora, compared to VAN and FDX treatment.

Conclusions: STAMPs exhibited protection against CDI superior to VAN and without relapse. These results indicate that STAMPs could potentially treat CDI while preserving a greater diversity of microbiome, leading to a significantly reduced rate of recurrence.

Introduction

The current standard of care antibiotics for *C. difficile* infection (VAN and MTZ) cause broad-spectrum microbiome disruption concomitant with a high risk of disease relapse. We designed a series of STAMPs with selective bactericidal activity against *C. difficile* that can preserve the gut commensal flora. Around 350 C3CD-STAMPs were constructed based off multiple putative targeting regions found in the *C. difficile* genome in combination with different flexible linker and killing peptides. These STAMPs were screened *in vitro* for rapid and selective killing of *C. difficile*. Leading candidates were then evaluated *in vivo* using a *C. difficile* -associated diarrhea murine model.

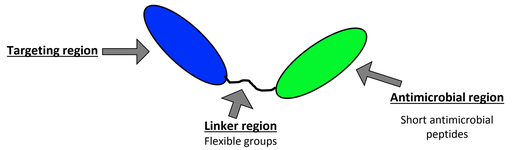

Figure 1 - STAMP Design – 65 genome peptides X Linkers X Killing Peptides

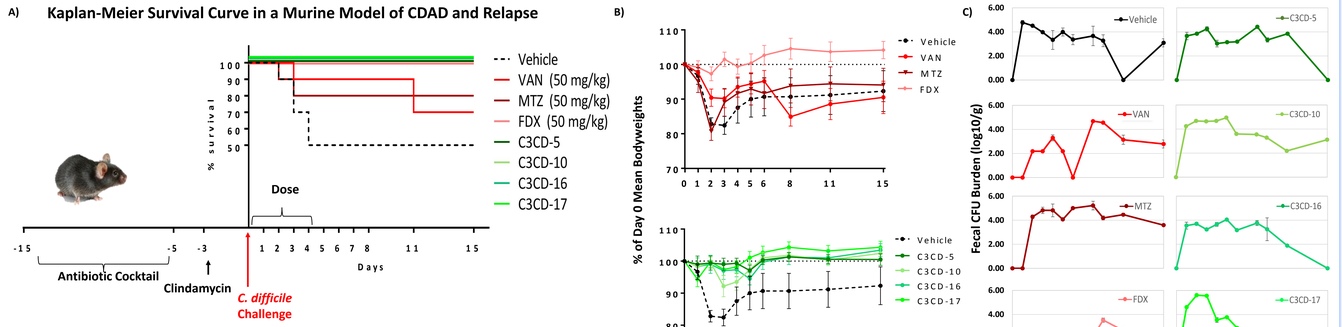

Figure 2 – C3CD-STAMPs and Standard of Care Treatment Outcome Comparison - A) Study design and Kaplan-Meier survival curves for antibiotic-induced murine CDI model; **B)** Percent bodyweight change post-treatment with standard of care antibiotics or *C. difficile*-specific C3CD-STAMPs; **C)** Fecal *C. difficile* spore burden (CFU/g) changes post challenge through Day 15.

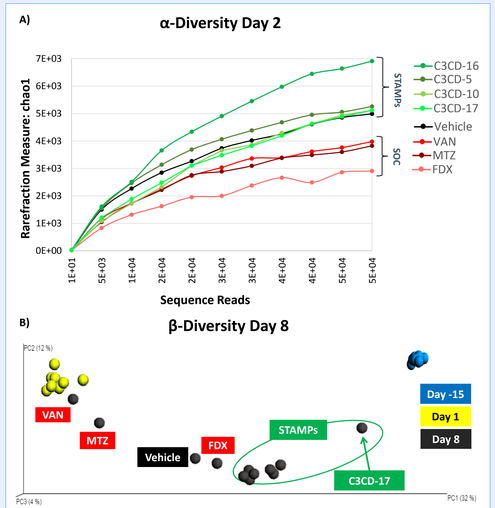

Figure 3 - Microbiome Diversity Comparison Between C3CD-STAMP-Treated Groups and Standard of Care - A) Alpha diversity (within-sample diversity) and rarefaction measured using chao1, a phylogenetic diversity metric at Day2 **B)** Beta diversity (between-sample) visualized with Principal coordinate analysis (PCoA) by Day. Coordinate axis have been sized according to principal component percentage. Day-15: prior to antibiotic-cocktail induction; Day 1: one day post-treatment; Day 8: VAN relapse

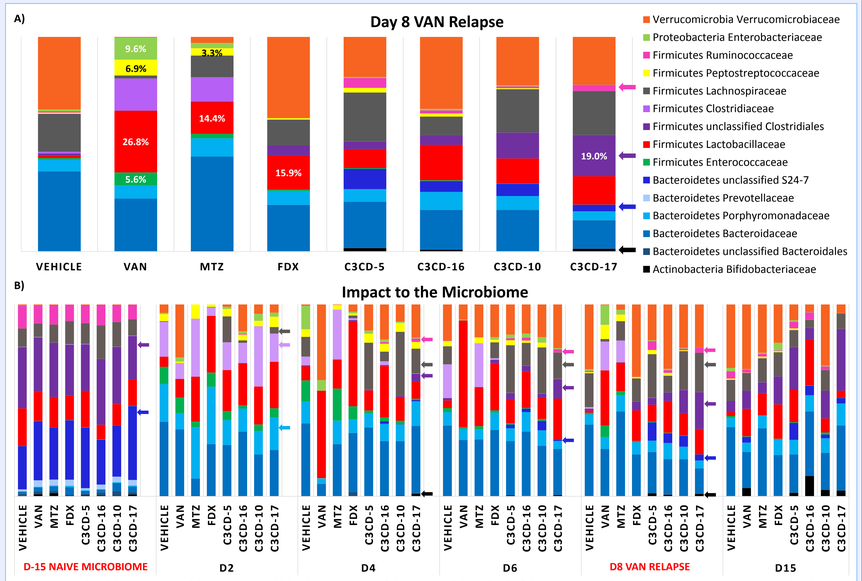

Figure 4 - Microbiome Composition Between C3CD-STAMP-treated Groups and Standard of Care - A) Microbial composition comparison at day 8, VAN relapse. **B)** Changes in the diversity of the microbiome throughout the study; antibiotic treatment leads to less diversity; C3CD-STAMP treatment was associated with greater microbial diversity.

Results

C3CD-STAMPs demonstrate superior protection from CDI without recurrence

- 100% survival and <5% weight loss with no recurrence through Day 15.
- VAN provided initial protection from infection, but VAN-treated animals relapsed at Days 8-11 (>15% more weight loss and 20% more death) (fig 2).
- C3CD-17 treated group showed steady decrease of *C. difficile* load that remained undetectable from Day 8~15.
  - ➢ FDX displayed inhibition of spore shedding during treatment period but rebounded post-treatment and remained detectable through Day 15.

C3CD-STAMPs promote faster recovery of microbiome diversity

- Ranking of overall microbial diversity during treatment period (Day 2) (fig 3a)
  - ➢ C3CD-STAMPs (highest) > Vehicle > VAN, MTZ, FDX
- The diversity of microbiome post C3CD-17 treatment at Day 8 was dissimilar to SOC, but more similar to the naive microbiome prior to model induction (fig 3b).
- A considerable increase in the population of *Enterococcaceae*, *Peptostreptococcaceae*, *Enterobacteriacea*, and *Clostrdiaceae* were found in VAN and MTZ treated groups, but not in C3CD-STAMP treated groups at Day 8 (fig 4a).
- An earlier increase in the population of healthy microbial flora, *Lachnospiraceae* *Bifidobacteriaceae*, *Ruminococcaceae*, and an undesignated *Bacteroidales* group were found in C3CD-STAMP treated groups, some as early as Day 2(fig 4ab).

Conclusion & Discussion

Our results demonstrate that C3CD-STAMPs can be utilized for treatment of primary and recurrent CDI. The rapid and specific *C. difficile* killing property of C3CD-STAMPs enable a faster recovery of the healthy gut microbiota, and a lower risk of recurrence.

- The recovery of a diverse microbiota is correlated with the efficacy in C3CD-STAMP-treated group.
- The earlier recovery of a diverse microbiota steadily reduced the *C. difficile* load and prevented relapse.
  - ➢ C3CDSTAMP treatment showed a steady decrease in fecal *C. difficile* spore load without recurrence, while SOC relapsed after initial spore inhibition.
- VAN and MTZ treatments created a less diverse microbiome and promoted bacterial populations associated with increased susceptibility to recurrent CDI.
- STAMP treatment showed earlier recovery of unclassified *Clostridiales* and *Bacteroidales* populations that resembled a naïve and healthy microbiome.
  - ➢ A better resolution of microbial profiling may further elucidate the importance of microbiome composition and diversity in resolving CDI and preventing recurrence.
- A colonic targeted oral formulation has been developed to assist in evaluating the efficacy of C3CD-STAMPs in hamster model and future human clinical trials.