A New Strategy for the Prevention of *Clostridium difficile* Infection

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(See the editorial commentary by Armstrong et al on pages 1484–6.)

**Background.** *Clostridium difficile* infection (CDI) is a leading cause of antibiotic-associated diarrhea. The infective form of *C. difficile* is the spore, but the vegetative bacterium causes the disease. Because *C. difficile* spore germination is required for symptomatic infection, antigermination approaches could lead to the prevention of CDI. We recently reported that CamSA, a bile salt analog, inhibits *C. difficile* spore germination in vitro.

**Methods.** Mice infected with massive inocula of *C. difficile* spores were treated with different concentrations of CamSA and monitored for CDI signs. *C. difficile* spore and vegetative cells were counted in feces from infected mice.

**Results.** A single 50-mg/kg dose of CamSA prevented CDI in mice without any observable toxicity. Lower CamSA doses resulted in delayed CDI onset and less severe signs of disease. Ingested *C. difficile* spores were quantitatively recovered from feces of CamSA-protected mice.

**Conclusions.** Our results support a mechanism whereby the antigermination effect of CamSA is responsible for preventing CDI signs. This approach represents a new paradigm in CDI treatment. Instead of further compromising the microbiota of CDI patients with strong antibiotics, antigermination therapy could serve as a microbiota surrogate to curtail *C. difficile* colonization of antibiotic-treated patients.

**Keywords.** CDI; *C. difficile*; spore germination; anti-germination.

*Clostridium difficile* infection (CDI) is the major identifiable cause of antibiotic-associated diarrhea in hospitals [1, 2]. In the United States alone, CDI develops in >500,000 patients, with up to 20,000 deaths per year [3]. The yearly healthcare burden has been estimated to be >$3 billion in the United States [3, 4]. A recent study reported that CDI is responsible for 25% more nosocomial infections than methicillin-resistant *Staphylococcus aureus* [5]. The rate of CDI progression to severe symptoms and death has been increasing [6–8]. The infective agent of CDI is the *C. difficile* spore, a hardy structure formed when the bacterium undergoes nutrient deprivation [9, 10]. Normally, gut microbes form a protective barrier against *C. difficile* colonization of the gastrointestinal (GI) tract, but this protective function can be weakened when undergoing antibiotic therapy [11]. Under these favorable conditions, *C. difficile* spores interact with small-molecule germinants, triggering a series of events committing the spore to germinate into toxin-producing bacteria [12–14]. Once the disease is established, a very narrow set of antibiotics can be used to treat CDI [1, 5]. The rate of failure among first-line antibiotics in the treatment of CDI can be as high as 38% [3, 15]. This is further complicated by the recent appearance of “hypervirulent” *C. difficile* strains [16, 17].

CDI patients shed dormant *C. difficile* spores in their feces [1, 3]. In contrast to oxygen-sensitive vegetative bacteria, *C. difficile* spores survive on hospital surfaces for months, allowing for CDI outbreaks and relapses [18]. Relapses of CDI happen at an alarming frequency [4, 19]. Indeed, the economic burden and mortality of CDI is due in large part to the high relapse rate [20, 21]. In many cases, once a patient
has a relapse, further relapses occur [22]. Treatment protocols are not standardized, and in some cases radical colostomy or fecal transplantation treatment have been used to combat CDI relapse [5, 23].

All current treatments for CDI focus on *C. difficile* metabolism or combating established disease [1, 4, 24]. Since *C. difficile* spore germination is required for infection, a complementary approach to curtail CDI establishment would be to prevent *C. difficile* spores from germinating in the GI tract of susceptible individuals [22, 25]. Unfortunately, there are currently no prophylactic treatments for CDI [25, 26]. A recent guideline for CDI clinical practice documented important questions that have not been addressed [1]. Preventive measures for CDI and the fate of *C. difficile* spores in the gut were among identifiable research gaps [1].

Others and we have shown that taurocholate (a bile salt) and glycine (an amino acid) bind and activate *C. difficile* spores through a complex mechanism [27, 28]. Using taurocholate and glycine analogs as chemical probes, we were able to determine structure activity relationships for germinant binding and germination activation of *C. difficile* spores in vitro [29]. Furthermore, we reported that a cholate meta-benzene sulfonic derivative (CamSA) is a strong competitive inhibitor of taurocholate-mediated *C. difficile* spore germination [29]. In the current study, we determined that CamSA can prevent CDI in mice. We present evidence that the anti-germination effect of CamSA is responsible for preventing CDI signs.

**METHODS**

**Materials**

Bile salts were purchased from Sigma-Aldrich (St. Louis, MO) or were synthesized in the Abel-Santos laboratory [29]. *Clostridium difficile* selective agar plates were purchased from BD Biosciences (Franklin, Lakes, NJ). All bacterial strains used in this study were purchased from ATCC (Manassas, VA).

**Animals**

The Institutional Animal Care and Use Committee at the University of Nevada, Las Vegas, reviewed and approved all animal protocols used in this study. All experiments were performed according to the National Institutes of Health guidelines in the Guide for Care and Use of Laboratory Animals. Weaned female C57BL/6N mice were purchased from Harlan Laboratories (Indianapolis, IN). Animals were housed in groups of 5 mice per cage in the University of Nevada, Las Vegas, animal care facility. All water, food, and bedding were autoclaved prior to contact with animals. On arrival, mice were allowed to acclimate for 1 week prior to experimentation. Animal manipulations were performed in a biosafety level 2 laminar flow hood.

**Preparation of Purified *C. difficile* Spores for Infection**

*C. difficile* strains 630 (ATCC BAA-1382) and VPI 10463 (ATCC 43255) were plated onto brain heart infusion (BHI) agar supplemented with 1% yeast extract, 0.1% l-cysteine HCl, and 0.05% sodium taurocholate to yield single-cell clones. Individual *C. difficile* colonies were grown in BHI broth until turbid and replated to obtain bacterial lawns. Plates were incubated for 7 days at 37°C in an anaerobic environment (5% CO₂, 10% H₂, and 85% N₂). Spores were harvested by washing and gently scraping the bacterial lawns from plates with ice-cold nanopure water. The cells and spores were centrifuged at 8,000 × g for 5 minutes at 4°C. Pellets were washed 3 times with nanopure water. To separate spores from bacterial cells, bacterial pellets were centrifuged through a 20%–50% Histodenz gradient at 18,200 × g for 30 minutes at 4°C with no brake. The resulting purified spores were washed 5 times with nanopure water. To determine spore purity, selected samples were stained using the Shaeffer–Fulton staining method [30]. Spore preparations were generally >95% pure after centrifugation through the Histodenz gradient.

Before infection, purified spores were heat activated at 68°C for 30 minutes and washed another 5 times with water. Purified *C. difficile* spores were resuspended in water to obtain an optical density (OD) of 1.0 at 580 nm. A spore aliquot was serially diluted onto BHI agar supplemented with cysteine and taurocholate to enumerate colony-forming units (CFU).

**Prevention of CDI by CamSA**

Spores of *C. difficile* 630 and VPI 10463 strains were used to infect mice, as described elsewhere [31, 32]. Briefly, an antibiotic cocktail containing kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL) was prepared in autoclaved water and sterile filtered. For 3 consecutive days, mice were allowed to drink the antibiotic cocktail ad libitum. The antibiotic water was refreshed daily. After 3 days of antibiotic water, all mice received autoclaved water for the remainder of the experiment. A single dose of clindamycin (10 mg/kg) was administered by intraperitoneal injection on the fourth day (24 hours before *C. difficile* challenge). At this time, groups of 5 antibiotic-treated mice received 0 mg/kg (ie, neat dimethyl sulfoxide [DMSO]), 5 mg/kg, 25 mg/kg, or 50 mg/kg CamSA by oral gavage. The day of infection, animals received 10⁶ CFU of *C. difficile* spores by oral gavage. One hour after infection, animals received a second dose of CamSA or DMSO. A third dose of CamSA or DMSO was administered 24 hours after infection. All animals were observed twice daily for signs of CDI, such as decreased weight, hunchbacked posture, wet tail, lethargy, anal redness, and increased soiling of cages. Disease signs were scored using the following rubric: pink anogenital area (score of 1), red anogenital area (score of 2), lethargy (score of 1), diarrhea/increase in soiled bedding (score of 1),
Effect of CamSA on Mice Challenged With Vegetative C. difficile Bacteria

A freezer stock of C. difficile 630 was inoculated into BHI broth and allowed to grow overnight in an anaerobic chamber at 37°C. The overnight culture was diluted 10-fold into fresh BHI broth and incubated for 4 hours at 37°C to reach the exponential growth phase. The presence of vegetative bacterial cells and the absence of spores were verified by microscopic observation of Shafer-Fulton stained samples [30]. The bacterial culture was harvested by centrifugation at 13 700 × g for 1 minute. The resulting pellet was resuspended in 1 mL of sterile water and serially diluted onto BHI agar containing cysteine and taurocholate to determine CFU. To prevent oxygen-induced cellular death, the remaining bacterial culture was canned inside the anaerobic chamber prior to overnight storage at 4°C. Antibiotic-treated mice received 3 consecutive 50-mg/kg doses of CamSA and were challenged with 10⁸ CFU of C. difficile 630 vegetative cells. As a control, a second group of mice was challenged with C. difficile 630 spores. Animals were observed for CDI signs as described above.

Statistical Analysis

Mice survival (n = 5) was analyzed by Kaplan-Meier survival analysis. Statistical comparison to untreated infected controls was calculated using the log-rank test. Signs of severity were analyzed as box-and-whisker plots. Data were expressed as mean ± SD in box-and-whisker plots. Analysis of nonparametric severity data was performed by 1-way analysis of variance, with statistical significance set at a P value of < .01. SDs represent at least 3 independent measurements, unless otherwise stated. Recovered CFU represent mean values from a pool of 5 animals. The unpaired Student t test was used to determine the significance of differences between mean values.

Results

CamSA Dose

A single 50 mg/kg dose of CamSA was administered by oral gavage to antibiotic-treated mice 24 hours (T-24) before challenge with 10⁸ CFU of C. difficile 630 spores. A second group of antibiotic-treated mice received a single 50-mg/kg dose of CamSA at 10 minutes (T0) following spore challenge. Animals were observed for CDI signs as described above.

Enumeration of C. difficile Vegetative Cells and Spores

Cages from infected animals were changed, and feces were collected at different time points. Feces were weighted and homogenized in autoclaved water. An aliquot of the fecal suspension was heated to 70°C for 15 minutes. Heated and unheated feces and GI tract contents were diluted as necessary in water and plated on C. difficile selective agar. Plates were incubated anaerobically for 48 hours, and colonies were counted to enumerate CFU. CFU obtained from unheated samples represent the sum of C. difficile vegetative cells and spores. CFU obtained from heated samples represent the number of C. difficile spores only. The presence of C. difficile colonies was verified by PRO disk.
content was watery and purulent. Mice treated with 5 mg/kg CamSA developed moderate-to-severe CDI, although signs of onset were delayed by approximately 24 hours (Figures 1 and 2B). Animals with moderate CDI signs eventually recovered. Mice treated with 25 mg/kg CamSA developed mild CDI signs with delayed signs onset (Figure 2C). All animals recovered before reaching the clinical end point. In contrast, all animals treated with 50 mg/kg CamSA showed no sign of CDI (Figures 1 and 2D). The intestines of these animals looked healthy and were undistinguishable from those of noninfected animals. All surviving CamSA-treated animals were treated with a second course of antibiotics, but no relapse signs were observed even after 14 days. Similar results were observed for CamSA-treated mice challenged with spores from *C. difficile* strain VPI 10463 (data not shown).

**Figure 2.** Severity of *Clostridium difficile* infection (CDI) signs decreases with increasing doses of CamSA. Animals challenged with *C. difficile* spores were treated with 3 doses of 0 mg/kg (A), 5 mg/kg (B), 25 mg/kg (C), or 50 mg/kg (D) CamSA. The severity of CDI signs was scored using the Rubicon scale discussed in Methods. Analysis of data was performed by 1-way analysis of variance, and a *P* value of < .01 was considered statistically significant.

**Figure 3.** CamSA partially protects mice from *Clostridium difficile* infection (CDI) when the dose was received before infection. Kaplan-Meier survival plot for *C. difficile*-infected mice treated with single doses of CamSA. Groups of 5 mice were treated with dimethyl sulfoxide (DMSO; ○), a single 50-mg/kg dose of CamSA at the time of *C. difficile* spore challenge (T0; □), or a single 50-mg/kg dose of CamSA 24 hours prior to *C. difficile* spore challenge (T24; Δ). *P* = .0495, by the log-rank test, compared with untreated infected animals.

**Refining the CamSA Dose**

CamSA completely protected animals from CDI with a single 50 mg/kg dose when administered at T0 (Figures 3 and 4A). These mice remained free of CDI signs. When the single 50
mg/kg CamSA dose was administered at T-24 animals developed moderate-to-severe CDI with delayed illness onset (Figure 3). Disease severity was more variable in T-24 mice (Figure 4). All animals remained healthy 24 hours after infection. Two animals displayed severe signs 48 hours following spore challenge, whereas 3 presented only mild signs. Of these, 2 mice began to recover 72 hours following infection, and 1 mouse worsened to reach the clinical end point. Animals that did not reach the clinical end point eventually recovered.

**CDI From Vegetative Cells and Spores**

Murine models can develop CDI signs when infected with vegetative *C. difficile* cells [31]. While 3 doses of 50 mg/kg of CamSA prevented CDI in spore-challenged mice, the same dose regime was unable to prevent CDI in vegetative cell-challenged mice. Three animals became moribund 48–72 hours after challenge (Figure 5). Two other animals developed moderate CDI signs and eventually recovered.

**Recovery of *C. difficile* Cells and Spores From Feces**

Untreated mice that developed CDI signs (Figure 2A) excreted mostly vegetative cells and few spores (Figure 6). Mice treated with 5 mg/kg CamSA also excreted mostly vegetative cells and developed CDI similar to untreated animals. Interestingly, the maximum amount of shed bacteria was delayed, compared with that for untreated animals (Figure 6). This delay...
correlated with the delay in signs onset (Figure 2B). With a dose of 25 mg/kg CamSA, the proportion of excreted C. difficile spores increased (Figure 6), and disease signs were milder (Figure 2C). These mice displayed maximum shedding 72 hours following infection, corresponding with disease onset. The feces of animals treated with 50 mg/kg CamSA contained almost exclusively spores (Figure 6), and these mice did not develop CDI (Figure 2D). In these animals, C. difficile spore excretion continued for 96 hours, peaking 72 hours following challenge. In fact, by 120 hours after infection, the sum of excreted C. difficile spores over time was quantitatively identical to the number of spores given by gavage.

**DISCUSSION**

Since the first step in the establishment of CDI is the germination of C. difficile spores in the microflora-depleted gut of hospitalized patients, antigermination compounds could be used in combination therapies to supplement antibiotic treatments in immunocompromised patients [25]. Once the antibiotic regimen is completed, reestablishment of the normal gut flora will prevent C. difficile spore germination, and antigermination therapy can also be discontinued.

CamSA was able to prophylactically prevent murine CDI caused by 2 different C. difficile strains and ameliorate CDI signs in a dose-dependent manner. Furthermore, a single dose of CamSA given at the time of infection was sufficient to prevent CDI signs without any signs of relapse. No signs of inflammation or damage to the GI tract were seen in CamSA-protected mice. Furthermore, CamSA-protected animals did not display signs of acute toxicity.

It is noteworthy that CamSA gave complete protection from CDI against unnaturally massive C. difficile spore inocula: a 75-kg human must ingest hundreds of grams of infected feces to reach the same number of C. difficile spores. And yet, even in these extreme conditions, CamSA was able to completely prevent CDI. The large inocula allowed synchronization of CDI onset, thus reducing individual variability, and allowed us to probe the limits of CamSA protective action.

When CamSA was given at suboptimal concentrations, CDI onset was delayed, and signs were less severe than in untreated mice. Similar results were obtained when an optimal CamSA dose was administered 24 hours prior to infection. In both cases, we attributed late CDI onset and reduced symptom severity to the partial inhibition of C. difficile spore germination by lowered intestinal CamSA concentrations. These data suggest that CDI severity is linked to the number of C. difficile spores able to germinate in the GI tract of mice. Indeed, the ratio of C. difficile vegetative cells to spores in feces correlated with CDI symptom severity. Thus, CamSA treatment can prevent CDI and ameliorate signs in a concentration-dependent manner.

Because CDI onset roughly coincides with antibiotic treatment, one can determine a priori when a patient will be at risk for CDI. In a clinical setting, CamSA treatment can be started before the first antibiotic dose. Multiple doses of CamSA can be administered as needed. After antibiotic treatment, the CamSA dosage can be continued until the intestinal microbiota has recovered.

Vegetative C. difficile cells are not believed to be involved in human CDI transmission but can infect mice [4, 31]. CamSA was able to prevent infections from dormant C. difficile spores but not from actively dividing C. difficile cells. Although not clinically relevant, infection of mice with vegetative C. difficile cells bypasses spore germination requirements and suggests that CamSA prevents CDI by inhibiting C. difficile spore germination and not by targeting metabolic processes in the vegetative bacterium.

Since CamSA blocks C. difficile spore germination in vivo, we were able to follow the fate of ingested C. difficile spores without interference from germination and/or resporulation. CamSA-treated mice excreted C. difficile vegetative cells and/or spores in a dose-dependent manner. Ingested C. difficile spores were quantitatively recovered from feces of mice treated with 50 mg/kg CamSA, further supporting the role of CamSA antigermination activity in CDI prevention.

In conclusion, we provide multiple lines of evidence to support that CamSA prevents CDI by inhibiting C. difficile spore germination in vivo, thus allowing ingested spores to be shed before they can establish infection. To our knowledge, CamSA is the first reported C. difficile spore antigerminant that also protects mice from CDI. This approach represents a new paradigm in CDI management. Instead of further compromising the microbiota of CDI patients with strong antibiotics, antigermination therapy could serve as a microbiota surrogate to curtail C. difficile colonization of antibiotic-treated patients.

**Notes**

**Acknowledgments.** E. A. S. conceived the use of antigerminants as CDI prophylaxis and supervised the project. A. H. and M. P. established the mouse CDI model. A. H. synthesized CamSA and determined its effect on the mouse CDI model. M. P. determined CFU in feces and intestines. E. A. S. and A. H. designed experiments, analyzed data, and wrote the manuscript.

**Financial support.** This work was supported by the National Science Foundation (grant number CHE 0957400).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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