Inhibitory effect of REP3123 on toxin and spore formation in Clostridium difficile, and in vivo efficacy in a hamster gastrointestinal infection model

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Objectives: REP3123 is a fully synthetic methionyl-tRNA synthetase inhibitor in pre-clinical development as a novel agent to treat Clostridium difficile infection (CDI). This novel agent was investigated for its ability to block the production of toxins and spores, and was tested for efficacy in vivo in a hamster model.

Methods: Clostridial toxin levels were determined qualitatively using monoclonal antibodies and by cytotoxicity assays. Spores were detected by staining and by quantitative dilution plating after ethanol treatment. Efficacy of REP3123 was tested in a clindamycin-induced C. difficile hamster gastrointestinal (GI) infection model.

Results: REP3123 at concentrations as low as 1 mg/L inhibited de novo toxin production in high cell density, stationary phase cultures of C. difficile. Among comparator agents currently used for CDI therapy, vancomycin required much higher levels of 20 mg/L, and metronidazole had no effect on toxin levels. REP3123 caused a >10-fold reduction of the sporulation rate in vitro. Vancomycin and, in particular, metronidazole appeared to promote the formation of spores. REP3123, at concentrations as low as 0.5 mg/kg, demonstrated efficacy in the hamster model of CDI and was superior to vancomycin in the overall survival of the animals at the end of the study (33 days).

Conclusions: REP3123 inhibited growth of C. difficile, affected the production of toxins and spores and demonstrated superior efficacy compared with vancomycin in the hamster GI infection model. This agent may be a promising candidate for CDI treatment; in particular, the inhibition of toxin production and spore formation may reduce the severity and spread of the disease, respectively.

Keywords: C. difficile infection, sporulation, hamster efficacy model

Introduction

C. difficile infection (CDI) is an inflammatory condition of the large bowel characterized by diarrhoea and can range in severity from mild to fulminant. CDI is associated with the appearance of distinct raised plaques and neutrophil accumulation in the lumen of the intestinal lining.1,2 Use of antibiotics such as clindamycin, cephalosporins, aminopenicillins, trimethoprim/sulfa-methoxazole and, more recently, fluoroquinolones, is a strong predisposing factor for CDI due to the disruption of the normal gut flora that otherwise suppresses C. difficile.3–5 The inflammation of the intestinal lining is caused by toxins TcdA and TcdB expressed by some of the strains of C. difficile.6,7 TcdA and TcdB are glucosyltransferases that target small host GTPases in the Ras superfamily and are encoded on the 19.6 kb pathogenicity locus (PaLoc), which also contains the regulatory factors TcdC and TcdR (formerly TcdD), and TcdE, a putative holin.8 Toxinogenic strains of C. difficile can be further classified into toxigenotypes according to sequence variability within the PaLoc.9 It is clear that both toxins contribute to CDI. Antibodies that specifically recognize and neutralize TcdA protect the host from pseudomembranous colitis.10,11 TcdB is an inflammatory enterotoxin, and it has been shown that TcdA− TcdB+ strains are capable of causing disease.12,13
addition, some *C. difficile* strains produce a binary toxin encoded by the *cdaA* and *cdaB* genes, which possesses adenosine diphosphate (ADP)-ribosyltransferase activity,\(^{14}\) but its role in pathogenesis is unclear.

Ingestion of spores is the main route of colonization of the human gut by *C. difficile*.\(^{15}\) Spores are extremely resistant to disinfectants and can persist in the environment for more than 12 months with little loss of viability or pathogenicity.\(^{16}\) Spores are also implicated in the 20% to 25% of CDI cases that relapse after treatment.\(^{17}\) It is hypothesized that the spores can remain in the crypts of the intestine and, once antibiotic treatment is completed, cause renewed disease.

Recent reports from several countries indicate that the incidence of CDI is on the rise worldwide. Most CDI cases occur in elderly patients in a hospital setting, but hospitalization increases the risk of colonization for healthy adults also.\(^ {18}\) In the USA, CDI hospitalizations and CDI-related case-fatality rates doubled between 2000 and 2005.\(^ {19}\) A number of recent outbreaks have been reported, in which CDI cases were primarily clonal in nature.\(^ {20,21}\) A strain type classified as BI/NAP1/027 was responsible for more than half of the cases, and hallmarks of this epidemic ‘outbreak’ strain are high morbidity and mortality, higher resistance to antibiotics (e.g. fluoroquinolones), the presence of a *tdcC* variant gene and toxin hyper-production.\(^ {20,22–24}\)

Vancomycin and metronidazole are used to treat CDI patients, but treatment failure and relapse occur frequently with either agent.\(^ {25}\) Both metronidazole and vancomycin have been shown to promote persistent overgrowth of vancomycin-resistant enterococci during the treatment of CDI.\(^ {26}\) Overall, options for the treatment of CDI are currently very limited, and there is a medical need for the development of new and improved agents. REP3123 is a novel, fully synthetic agent that targets methionyl-tRNA synthetase of Gram-positive bacteria, including *C. difficile*, but has very limited activity against beneficial organisms of the normal gut flora.\(^ {27,28}\) This study was performed to investigate the effects of a protein synthesis inhibitor such as REP3123 on toxin production and sporulation in vitro, thus potentially reducing the virulence and limiting the spread of *C. difficile*, and to test the in vivo efficacy of REP3123 in a hamster model.

### Materials and methods

**Microorganisms, media and chemicals**

*C. difficile* strains ATCC 43596 and ATCC 43255 (VPI 10463) were from the American Type Culture Collection (Manassas, VA, USA); strains MB898 and MB903 were clinical isolates from Eurofins (formerly Focus Bio-Inova, Herndon, VA, USA). RMA 18383 and RMA 18386 are BI/NAP1/027 strains obtained from R. M. Alden Research Laboratory (Santa Monica, CA, USA) and represent epidemic, moxifloxacin-resistant *tdcC* variant strains.

*C. difficile* was maintained anaerobically on modified cycloserine ceftauxin fructose agar (CCFA) agar from Remel (Lenexa, KS, USA). Brucella agar (BD Biosciences, San Jose, CA, USA) was supplemented with vitamin K/haemin (Remel) and 5% laked sheep blood (Hardy, Santa Maria, CA, USA), and antibacterial agents were added to the molten agar to the desired final concentrations. TV (3% tryptose/2% yeast extract) from BD Biosciences was used as broth medium for *C. difficile*. All media were pre-reduced over-night prior to inoculation. REP3123 was synthesized at Replidyne, Inc.; other agents used in this study included vancomycin, metronidazole, clindamycin phosphate and amoxicillin (Sigma)/clavulanic acid (USP, Rockville, MD, USA).

**Detection of toxins**

Since toxin production typically starts in the late-exponential growth phase when the cell density in a *C. difficile* culture reaches a plateau,\(^ {29}\) the effect of antibacterial agents on toxin production was determined in stationary phase, high cell density cultures. In brief, an overnight culture of *C. difficile* in TY broth was diluted 10-fold with fresh broth, grown for 4–6 h to late exponential growth phase and harvested by centrifugation (10 min, 4000 rpm). The cells were washed with broth and resuspended in fresh broth to 10–20× the density of a 0.5 McFarland standard, corresponding to \(10^{8}–10^{9}\) cfu/mL. The exact viable cell counts in the inoculum were determined by plating 100 μL of appropriate dilutions \((1/10^4, 1/10^5\) and \(1/10^6\)) on CCFA, followed by the enumeration of colonies the following day. Portions (1 mL) of the culture were dispensed into individual 17×100 mm tubes, antibacterial agents were added to achieve the desired final drug concentrations and the cultures were incubated anaerobically at 35–37°C. Samples were removed at desired time-points, and cell-free supernatants were obtained by centrifugation (10 min, 4000 rpm) and frozen at −20°C until use. Toxins were detected using the 96-well enzyme-linked immuno-flow assay (ELIFA) system (Thermo Fisher, Rockford, IL, USA; formerly Pierce) for the vaccine-assisted transfer of native protein antigens onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Also, samples were mixed with 50 μL of 2× denaturing (SDS) loading buffer, boiled for 3 min, electrophoretically separated on NuPage Bis–Tris gels (4% to 12%, 1 mm×17 well) and blotted onto Immobilon-P membranes (Millipore, Billerica, MA, USA), including standards of purified toxin A or toxin B (VWR International, USA). The ELIFA and western blots were blocked for 1 h with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and then incubated for 2 h with anti-toxin A or anti-toxin B monoclonal antibodies (Novus Biologicals, Centennial, CO, USA) diluted 1/500 and 1/250, respectively, in blocking buffer. The blots were washed twice for 5 min with TBST, followed by incubation for 1 h with goat anti-mouse IgG (H+L) alkaline phosphatase-conjugate (Bio-Rad) diluted 1/2000 in blocking buffer. The blots were washed three times for 5 min with 20 mL TBST and once with TBS for 2 min, and developed with NBT/BCIP (Thermo Fisher; formerly Pierce). In addition to the immunological methods, *C. difficile* toxin activity was monitored by the ‘gold standard’ tissue culture assay. IMR-90 human fibroblasts (ATCC CCL-186) were grown for 7 days in 5% CO\(_2\) in Eagle’s minimum essential medium (EMEM) from CellGro (Manassas, VA, USA) containing 10% fetal bovine serum and penicillin/streptomycin until the culture was confluent. The growth medium was aspired, and the cells were rinsed with phosphate-buffered saline (PBS), trypsinized, resuspended in fresh EMEM and used to seed 24-well plates, which were incubated overnight to allow attachment. The growth medium was then replaced with 1 mL of *C. difficile* supernatants that had been filter sterilized (0.2 micron) and were 10-fold serially diluted in EMEM over a range of 1:10 to 1:1000000. The plates were incubated for 18–24 h, and the percentage of rounded cells to normal cells was determined through microscopic examination.

**Detection of spores and determination of sporulation rates**

For sporulation assays, 10 μL of a cell suspension that was prepared from fresh overnight plates and matched the turbidity of a 0.5 McFarland standard was plated on Brucella agar containing test
were determined at the time of inoculation (which the antibacterial agent was added to a final concentration of culture was split into individual 1 mL cultures in capped tubes to broth and grown for 4–6 h to the late exponential growth phase. The culture of effects of antibacterial agents on sporulation in broth, an overnight ant green spheres and vegetative cells as red rods. To examine the culture of C. difficile in TY medium was diluted 10-fold with fresh broth and grown for 4–6 h to the late exponential growth phase. The culture was split into individual 1 mL cultures in capped tubes to which the antibacterial agent was added to a final concentration of 10 mg/L. The total number of viable cells and the number of spores were determined at the time of inoculation (t=0), at 4 and at 7 days.

Hamster model of C. difficile gastrointestinal (GI) infection

In vivo efficacy of REP3123 was evaluated in a hamster intestinal infection treatment model at Ricerca Biosciences, LLC (Concord, OH, USA), in accordance with regulations outlined in the USDA Animal Welfare Act and following a protocol approved by Ricerca’s Institutional Animal Care and Use Committee. Male Golden Syrian hamsters were purchased from Charles River Laboratories (Kingston, NY, USA) and were ~6 weeks of age, with weights ranging from 89 to 109 g at the start of the study. The animals were housed individually in filtered polycarbonate shoe-box style cages equipped with water bottles, and Harlan Teklab Global Diet 2016 was available ad libitum via food hoppers. The hamsters were pre-treated with a single subcutaneous dose of clindamycin phosphate (50 mg/kg), formulated in PBS, at Day −1. After 24 h (Day 0), each hamster was inoculated via oral gavage with 0.5 mL of a suspension of C. difficile ATCC 43596 (3.2×10⁷ cfu/mL), which is a clindamycin-resistant, toxigenic reference strain. To prepare this inoculum, C. difficile was grown in fluid thioglycollate medium for 28 h at 37°C, and the cells were harvested by centrifugation, rinsed twice with sterile PBS, resuspended in sterile PBS and the exact cell density was determined using the dilution plate count method. Spores accounted for ~1% of the total viable cells in the inoculum. Oral dosing of REP3123 di-HCl salt, formulated in 5% dextrose, and of vancomycin, formulated in USP Purified Water, was commenced the following day (Day 1), which was ~20 h post-inoculation. Treatments were administered twice a day for 5 consecutive days at specified doses (5 and 0.5 mg/kg), with eight hamsters per group. Controls included an uninfected group and an infected but untreated group. The hamsters were observed daily to record clinical signs (duration, time of onset, time of recovery or death), and animals in a lethargic, clearly moribund state were euthanized. A necropsy was performed on animals that were either found dead or were euthanized at the end of the study (33 days).

Results

Inhibition of toxin production by REP3123

REP3123, vancomycin and metronidazole were evaluated for the ability to inhibit toxin production in high cell density (>10⁹ cfu/mL) cultures of C. difficile using immunological methods and cytotoxicity assays. C. difficile cells from the late-exponential growth phase were washed to remove any pre-formed toxins and re-suspended at high cell density (>10⁹ cfu/mL) in fresh broth containing the antibacterial agents, and culture supernatant samples were collected over a period of 5–6 days. Toxin production was inhibited in epidemic BI/NAP1/027 strain RMA 18386 where neither toxin A nor toxin B was detected in the supernatants of cultures treated with 4 mg/L REP3123 (Figure 1a). In contrast, vancomycin and metronidazole did not reduce toxin production when compared with drug-free control cultures, even though the drug concentrations were above the MICs of these agents. Similar results were obtained using the historic toxin hyper-producing strain ATCC 43255 (VPI 10463). REP3123 at the lowest concentration tested (1 mg/L) blocked de novo toxin production in this strain, while a much higher concentration of vancomycin (20 mg/L) was required to achieve some reduction of toxin levels, and metronidazole had essentially no effect under these conditions (Figure 1b). The protein synthesis inhibitor clindamycin was also capable of blocking toxin production, albeit at somewhat higher concentrations (4 mg/L). Inhibition of cell wall synthesis by amoxicillin/clavulanate, on the other hand, had no negative influence on toxin accumulation.

Clostridial toxins were also detected by semi-quantitative cytotoxicity assays that are based on the typical morphological changes (rounding) of human IMR-90 fibroblasts in the presence of toxins. Late-exponential cells of a clinical isolate, C. difficile MB898, were exposed to 1 mg/L of drugs for 5 days. REP3123, but not vancomycin or metronidazole, blocked toxin production efficiently, since no rounding of the fibroblasts occurred in the presence of supernatants from C. difficile cultures treated with REP3123 (Figure 1c). Cytotoxicity titration assays of serially diluted culture supernatants indicated that toxin levels were reduced at least 100-fold upon exposure to REP3123, but other agents, such as vancomycin and metronidazole, had no effect compared with drug-free control cultures (data not shown).

Effect of REP3123 on sporulation of C. difficile

Since sporulation requires the synthesis of spore coat proteins, REP3123 was evaluated for its ability to interfere with this process in C. difficile clinical isolates. To measure sporulation on solid media, C. difficile was grown on agar containing 0.06–2 mg/L REP3123, vancomycin or metronidazole, which is a range that spans from subinhibitory concentrations to levels just above their MICs. The four strains evaluated in this study were susceptible to these agents with MIC ranges of 0.5–1 mg/L (REP3123), 1–2 mg/L (vancomycin) and 0.25–1 mg/L (metronidazole). The total viable counts and the number of spores were determined after 4 days, and the percentages of spores were calculated (Table 1). Spores were detected in samples of all non-treated cultures, although the sporulation capacity varied among the four strains tested, ranging from 0.8% to 17.3% after 4 days. Sporulation was greatly affected on media containing antibacterial agents at subinhibitory concentrations. After treatment with REP3123, all strains showed reductions in spore production at 0.5× MIC (<1% spores) and at 0.25× MIC (<2% spores). This is in stark contrast to the results obtained after treatment with metronidazole, where three of the four strains that were tested displayed a marked increase in spore production after exposure to sub-MIC levels of the drug. Similarly,
In vitro and in vivo efficacy of REP3123 against C. difficile

<table>
<thead>
<tr>
<th></th>
<th>Inoculum</th>
<th>No drug (DMSO)</th>
<th>REP3123 (4 mg/L)</th>
<th>Vancomycin (4 mg/L)</th>
<th>Metronidazole (4 mg/L)</th>
<th>Toxin A</th>
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<tr>
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<td>1d</td>
<td>2d</td>
<td>5d</td>
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<td>Toxin B</td>
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Figure 1. (a) Western blot analysis of supernatants collected from high cell density (>10^7 cfu/mL) C. difficile RMA 18386 (BI/NAP1/027) cultures after exposure to 4 mg/L REP3123, vancomycin or metronidazole for 1, 2 and 5 days. (b) ELISA and western blot analysis of supernatants collected from C. difficile ATCC 43255 (VPI 10463) cultures exposed to REP3123, vancomycin, metronidazole, clindamycin or amoxicillin/clavulanate for 4 h, 18 h, 2 days and 6 days. (c) Morphological changes (rounding) of human IMR-90 fibroblasts due to clostridial toxins. Normal cells appear filamentous. Supernatants from drug-free control cultures or from cultures exposed to 1 mg/L of drugs for 5 days were diluted 1000-fold, added to fibroblast monolayers and morphology was recorded after 18 h.

Treatment with subinhibitory concentrations of vancomycin promoted spore formation in two strains under these conditions. In fact, in strains ATCC 43596 and MB903, spores accounted for 100% of the total viable counts detected in samples from cultures grown in the presence of 0.12–0.5 × MIC of metronidazole or vancomycin. The microscopic evaluation of C. difficile ATCC 43596 cells and spores after Wirtz–Conklin staining with malachite and safranin is illustrated in Figure 2, with vegetative cells appearing as rods and spores as spheres. Exposure to sub-MIC levels of REP3123 inhibited the formation of spores, and the cells appeared to remain in the vegetative state. At 0.5 × MIC, REP3123 was superior to vancomycin and metronidazole in suppressing spore formation. REP3123 inhibited spore formation in C. difficile ATCC 43596 in a concentration-dependent manner, with absence of spores at 0.5 × MIC, few spores at 0.25 × MIC and more spores at 0.12 × MIC (data not shown).

The effect of REP3123, vancomycin and metronidazole on sporulation was also examined in broth, by adding the
Table 1. Effect of antibacterial agents on the sporulation rate of C. difficile after 4 days on agar

<table>
<thead>
<tr>
<th>Treatment and concentration (mg/L)</th>
<th>% Spores after 4 days on Brucella agar</th>
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<tbody>
<tr>
<td></td>
<td>ATCC 43596</td>
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<tr>
<td>REP3123</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>4.0</td>
</tr>
<tr>
<td>0.12</td>
<td>0.3</td>
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<tr>
<td>0.25</td>
<td>0.0</td>
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<tr>
<td>0.5</td>
<td>NG</td>
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<tr>
<td>1</td>
<td>NG</td>
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<tr>
<td>2</td>
<td>NG</td>
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<tr>
<td>Vancomycin</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>100</td>
</tr>
<tr>
<td>0.12</td>
<td>100</td>
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<tr>
<td>0.25</td>
<td>100</td>
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<tr>
<td>0.5</td>
<td>100</td>
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<tr>
<td>1</td>
<td>NG</td>
</tr>
<tr>
<td>2</td>
<td>NG</td>
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<tr>
<td>Metronidazole</td>
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<tr>
<td>0.06</td>
<td>100</td>
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<tr>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>NG</td>
</tr>
<tr>
<td>0.5</td>
<td>NG</td>
</tr>
<tr>
<td>1</td>
<td>NG</td>
</tr>
<tr>
<td>2</td>
<td>NG</td>
</tr>
<tr>
<td>No drug</td>
<td>10</td>
</tr>
</tbody>
</table>

NG, no growth (since antibacterial agent is at or above its MIC for this strain).

antibacterial agents at inhibitory concentrations (10 mg/L) to late-exponential, high cell density cultures of C. difficile ATCC 43596 (Table 2). At the start of drug exposure, the total viable count was 2.6×10⁸ cfu/mL, and the spore count was 6×10⁵ cfu/mL (0.002%). In the untreated control culture, the percentage of spores increased by two orders of magnitude to 0.32% in 4 days and further increased by another two orders of magnitude by 7 days, reaching 75% of spores. The presence of the bacteriostatic agent REP3123 (10 mg/L) caused the cells to remain in a vegetative state and inhibited the increase of spores at 4 days, when still only 0.002% of the total viable counts were spores, and only 2% spores were present at 7 days. The same concentrations (10 mg/L) of vancomycin and, in particular, metronidazole, were less effective in blocking sporulation, resulting in 5% and 25% spores at 7 days. C. difficile cultures exposed to REP3123 contained the fewest spores, both in absolute numbers and as percentages of total viable counts, compared with cultures exposed to the other agents or compared with the drug-free control culture.

Efficacy of REP3123 in a hamster gut infection model of C. difficile

In vivo efficacy of REP3123 has been evaluated in a hamster GI infection treatment model. The hamster is the standard species for non-clinical evaluation of compounds that are active against C. difficile, and the model has shown a high level of reproducibility with the infected, untreated control and the positive vancomycin control (data not shown). The untreated C. difficile gut infection is rapidly lethal to the hamsters; therefore, an increase in survival was the primary endpoint of the model. Secondary endpoints were the gross assessment of GI appearance and the histopathology of hamster caecum.

The Kaplan–Meier survival curves for two dose levels (0.5 and 5 mg/kg) of REP3123 or vancomycin are shown in Figure 3. In the infected, but untreated group, all animals expired by Day 3, exhibited weight losses of 2–8 g and typical GI tract inflammation was noted. Treatment with 0.5 mg/kg of REP3123 resulted in 62% survival, compared with 75% survival at the higher dose of 5 mg/kg. The few deaths in the REP3123-treated group occurred with little prior evidence of morbidity between Days 4 and 15. C. difficile was recovered from the caecum of these animals and was identical to the challenge strain (ATCC 43596) based on susceptibility testing (resistance to clindamycin and rifampicin) and REA/PFGE typing (data not shown). The remaining hamsters in the REP3123-treated group survived until study termination at Day 33 and had gained up to 22 g in weight, with one exception in both the 0.5 and 5 mg/kg groups that had no weight change. The surviving animals had healthy gross GI appearance, and the histopathology of REP3123-treated hamsters was similar to that of a healthy, uninfected animal (data not shown). Treatment with 5 mg/kg of vancomycin showed the pattern typically observed in this model, which is survival up to Day 12–14, followed by 100% deaths by Day 17. Weight loss among these animals ranged from no change to a loss of 7 g, and inflamed GI tracts were observed in all of the animals. The lower dose of vancomycin (0.5 mg/kg) was not efficacious since five of eight hamsters died by Day 3; two additional animals died on Day 9, with weight losses up to 41 g.

Discussion

CDI is a major cause of morbidity and mortality among the hospitalized elderly, and accounts for 20% to 25% of all cases of antibiotic-associated diarrhoea. The epidemiology of CDI has changed in recent years with the emergence of more virulent and drug-resistant BI/NAP1/027 strains that cause increased severity of disease. High attributable mortality rates of up to 16.7% have been reported during recent outbreaks in Quebec, Canada. Standard treatment regimens for CDI are oral metronidazole or vancomycin, but there is need to develop novel CDI agents with improved properties. REP3123 is a fully synthetic compound that is highly active against C. difficile (MIC range, 0.25–1 mg/L) without the perturbation of the normal gut flora. It was determined that REP3123 had a very low bioavailability of 0.6% based on plasma pharmacokinetic measurements in healthy, uninfected hamsters (data not shown). Therefore, maximal activity was achieved in the gut, and systemic exposure was low and, as expected, not required for efficacy. REP3123 is especially attractive as a potential novel CDI agent because of its two main physiological effects on C. difficile, the inhibition of toxin production and sporulation. Regardless of the potential levels of toxins naturally produced by different strains of C. difficile, REP3123 was found to be a very effective inhibitor of toxin production in high

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density, stationary phase cultures that represent the precise conditions when toxin is typically produced at maximal rates. By targeting methionyl-tRNA synthesis, REP3123 exerts its global mode of action through the inhibition of protein synthesis, which explains why this agent blocks clostridial toxin production even in non-dividing cells in the stationary phase.

Table 2. Effect of antimicrobial agents on the number of viable cells and number of spores of C. difficile ATCC 43596 in broth

<table>
<thead>
<tr>
<th>Antimicrobial agent (10 mg/L)</th>
<th>t=4 days (cfu/mL)</th>
<th>t=7 days (cfu/mL)</th>
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<tbody>
<tr>
<td></td>
<td>total</td>
<td>spores</td>
</tr>
<tr>
<td>No druga</td>
<td>1×10⁸</td>
<td>3.2×10⁵</td>
</tr>
<tr>
<td>REP3123</td>
<td>2×10⁸</td>
<td>4.0×10⁵</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.2×10⁸</td>
<td>1.2×10⁴</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>4×10⁶</td>
<td>3.2×10⁴</td>
</tr>
</tbody>
</table>

a At time of drug addition (t=0): total viable cells, 2.6×10⁸ cfu/mL; spores, 6×10³ cfu/mL (0.002%).

Figure 2. Microscopic evaluation of C. difficile cultures grown for 4 days on Brucella agar in the absence of drug or in the presence of REP3123, vancomycin or metronidazole at 0.5× MIC. Vegetative cells (bacilli) and spores (spheres) were distinguished by Wirtz–Conklin staining (5% Malachite Green/0.5% safranin).

Figure 3. Kaplan–Meier survival plots of hamsters that were pre-treated with clindamycin, inoculated with C. difficile ATCC 43596 and treated with REP3123 or vancomycin at 0.5 and 5 mg/kg.
Vancomycin inhibits cell wall synthesis in growing cells and metronidazole causes non-specific radical-mediated DNA damage; neither activity influences protein synthesis, therefore, these agents are unable to prevent toxin and spore formation. At best, metronidazole and vancomycin may reduce the number of viable *C. difficile* cells somewhat through their potentially bactericidal activity. However, neither vancomycin nor metronidazole at concentrations well above their MICs (10 mg/L) were truly bactericidal against stationary phase cells, since the drop in total viable cell counts was less than two orders of magnitude after 7 days. In fact, metronidazole and vancomycin promoted spore formation at least in some of the strains, at concentrations above their MICs as well as at sub-MIC levels. This may present a problem in a hospital setting during an outbreak, since spores are extremely difficult to eradicate and are the main source of new infections and recurrence.

Few useful models are available to test the pre-clinical efficacy of agents that are active against *C. difficile*. The hamster model of clindamycin-induced CDI mimics aspects of CDI in humans after antimicrobial therapy and has been widely used to test the virulence of both epidemic and non-epidemic strains of *C. difficile*. An *in vitro* triple-stage chemostat model that simulates the human gut has also been used successfully, and results obtained from both models were concordant in past studies. The untreated *C. difficile* gut infection is rapidly lethal to the hamsters and, therefore, an increase in survival is the primary endpoint of the model. The appearance of the infected GI tract, particularly in the colon and caecum, is characterized by inflammation, redness/haemorrhage and a fluid-filled, enlarged appearance. The GI tract deteriorates very rapidly and gut motility decreases. The enlarged colon is evocative of the toxic megacolon observed in humans with severe *C. difficile* infection. However, unlike the clinical condition, hamsters rarely exhibit diarrhoea (wet tail) and frequently die of the infection precipitously without signs of wet tail when infected with highly virulent *C. difficile* strains. In the hamster model, treatment with vancomycin typically protects animals during the dosing intervals and for ~1 week thereafter, but then all animals succumb to the disease within a few days. The doses of vancomycin in this study were somewhat low (5 and 0.5 mg/kg), but other studies found that treatment with higher doses of vancomycin (50 mg/kg) still resulted in relapse of CDI in hamsters. This finding suggests that *C. difficile* remains in the gut, presumably as spores, which can germinate in the absence of a protective normal gut flora once the drug treatment has been stopped, causing renewed disease. This is reminiscent of relapse and recurrence of CDI in human patients observed weeks or months post-antibiotic therapy. In fact, it was shown in the human gut chemostat model that total *C. difficile* counts were reduced but the numbers of spores remained unchanged during vancomycin instillation. The efficacy of REP3123 in the hamster model was unique in the way that the majority of animals survived until the end of the study (Day 33). These data suggest that spores were either absent or were unable to germinate in REP3123-treated hamsters. As shown in this study, REP3123 was capable of blocking sporulation *in vitro*, and in hamsters, the formation of spores may be prevented during the drug treatment phase (Days 1–5) and for some short period thereafter. Regarding germination, REP3123, but also vancomycin, inhibited the outgrowth of spores to colonies *in vitro* at drug concentrations of 0.5× MIC or above (data not shown). In the hamster, it is unlikely that such an inhibitory effect on germination would last through to the conclusion of the study at Day 33. Therefore, the most likely explanation for the prolonged survival of REP3123-treated hamsters is that *C. difficile* was cleared from these animals and did not form spores during the treatment. Three hamsters in the REP3123-treated groups perished between Days 4 and 8, possibly due to a lethal amount of toxin that had accumulated before treatment was started. Two REP3123-treated hamsters died on Days 14 and 15, which is the period when all vancomycin-treated animals expired, suggesting that some spores had remained in these animals or that re-infection occurred. All other REP3123-treated hamsters survived through to Day 33, which is in marked contrast to the control groups. Moreover, REP3123 demonstrated a narrow spectrum of activity, with limited activity against beneficial constituents of the normal gut flora, but it is unclear whether this property of REP3123 plays a role in the hamster model, since the animals were primed with clindamycin and the microbial flora in animals that died or survived was not assessed during this study.

In conclusion, REP3123 was superior compared with vancomycin and metronidazole in attenuating toxin production and sporulation *in vitro*, which could have beneficial consequences, respectively, in reducing the virulence (cytotoxicity) and persistence of the organism in the environment. REP3123 was also superior to vancomycin *in vivo* in the hamster GI infection model. Further investigations are warranted to determine whether the favourable microbiological profile of REP3123 translates into a reduction of disease severity and relapse rates, which may lead to faster clinical response and overall improved outcomes in CDI patients.

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### References

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