ART24, a novel live biotherapeutic product in development for the prevention of CDI, is bactericidal against *C. difficile* and degrades toxins A&B *in vitro*

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Background

- ART24 is being investigated as a live biotherapeutic product (LBP) treatment to prevent recurrence of *Clostridioides difficile* infection (CDI) following successful antibacterial treatment. ART24 was identified from a large-scale screening of stool samples to detect bacteria with direct anti-*C. difficile* activity and proteolytic capability. ART24 was identified as a member of the *B. amyloliquefaciens/B. velezensis* group. We sought to determine if protease and inhibitory activities translated *in vitro* in *C. difficile* toxin degradation and *C. difficile* killing

Materials/methods

- The anti-*C.difficile* activity of ART24 was investigated using a 12-well plate liquid co-culture method incubating different ART24 to *C. difficile* ratios for 24 hours. *C. difficile* was enumerated pre- and post-incubation to assess the effect of each ratio of ART24
- Co-culture method
  - Capsules of lyophilized ART24 were resuspended 1:10 in 900 µL maximum recovery diluent (MRD), serially diluted, and plated on brain heart infusion (BHI) broth for colony-forming unit (CFU) enumeration of the viable ART24. A second 1:10 dilution series of the resuspended capsules was used in a 12-well plate liquid co-culture (3 mL working volume) assay against *C. difficile*
  - The resuspended capsules were diluted 5 times (10^{-2}–10^{-6}) and the dilutions were co-incubated anaerobically with *C. difficile* for 24 h
- Extracellular protease activity from ART24 was tested for its ability to cleave purified *C. difficile* toxins A and B. Toxin cleavage experiments were run using ART24 pH-neutralized cell-free supernatant (CFS) and isopropanol (IPA) extracts prepared from two independent cultures, and lyophilized powder
  - 10 mL of fresh ART24 grown in BHI was pelleted, the CFS removed, pH-neutralized and filter-sterilized
  - The ART24 pellets were vortexed in 2 mL IPA, centrifuged, and the IPA extracts filter-sterilized
  - ART24 CFS and IPA extracts (serially diluted 1:2 in BHI) were stored short-term at 4°C and long-term at -20°C
  - Lyophilized ART24 was reconstituted and serially diluted in PBS
  - 2.5 µL (0.2 mg/mL) aliquots of purified *C. difficile* toxin A or B was co-incubated for 2 h at 37°C with 30 µL of either ART24 IPA extract, ART24 CFS, or lyophilized ART24 and the reactions were stopped, heated, and electrophoresed
    - Toxin A or toxin B primary antibodies were used at a 1:5000 dilution

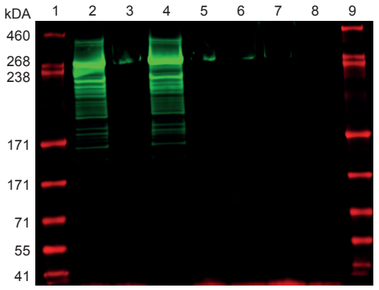
Results

- ART24 is bactericidal against *C. difficile* in liquid co-cultures. The lowest ratio (ART24:*C. difficile* CFU) that supports *C. difficile* killing (>3 log reduction in 24 h) was 275:1, with lower ratios inhibiting the growth rate of *C. difficile* without complete killing (**Table 1** and **Figure 1**)

Table 1. Effect of re-suspended lyophilized formulated ART24 on *C. difficile* growth

ART24:*C. difficile* ratio	Log10 CFU change (T24–T0)
865:1	- >3
275:1	- >3
21:1	+ 1.2
1:1	+ 2.0
0.1:1	+ 2.6
C. difficile only	+ 1.5

Figure 2. Cleavage detection of toxin A exposed to fresh ART24 pH-neutralized supernatants and IPA extracts



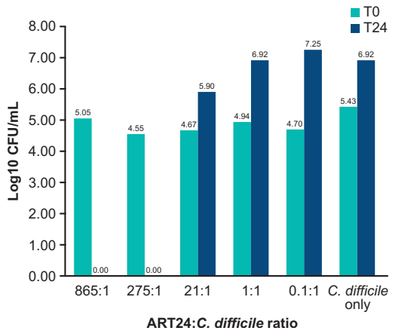
Lanes:
1&9. Novex HI Mark pre-stained protein ladder
2. Toxin A + water
3. Toxin A + IPA extracted BHI control
4. Toxin A + pH-neutralized BHI control
5. Toxin A + IPA extract CS* — Culture 1
6. Toxin A + pH-neutralized CS — Culture 1
7. Toxin A + IPA extract CS — Culture 2
8. Toxin A + pH-neutralized CS — Culture 2

*CS = Culture Supernatant

Toxin A: 308 kDa
BHI, brain heart infusion broth; IPA, isopropanol

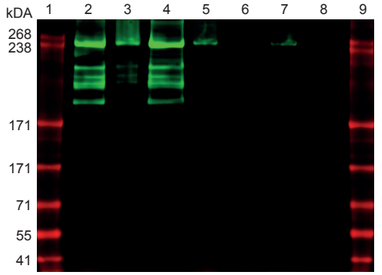
- ART24 CFS caused complete cleavage of *C. difficile* toxins A and B (**Figure 2** and **Figure 3**). Both the water and pH-neutralized BHI controls did not cause toxin cleavage. The IPA-extracted BHI control caused cleavage of toxins A and B
- Lyophilized ART24 caused complete cleavage of *C. difficile* toxins A and B (**Figure 4** and **5**)
- The amount of toxin cleavage was dependent upon the amount of lyophilized ART24 CFUs reconstituted, with lower dilutions (i.e. 10^{-2} and 10^{-3} dilutions) showing more cleavage than higher dilutions (range of 10^{-4} to 10^{-7} serial dilutions). The PBS and heat-treated ART24 controls showed no degradation

Figure 1. Effect of re-suspended lyophilized formulated ART24 on *C. difficile* growth



CFU, colony-forming unit

Figure 3. Cleavage detection of toxin B exposed to fresh ART24 pH-neutralized supernatants and IPA extracts

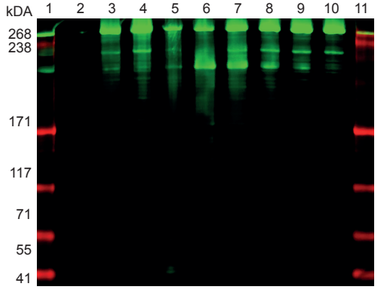


Lanes:
1&9. Novex HI Mark pre-stained protein ladder
2. Toxin B + water
3. Toxin B + IPA extracted BHI control
4. Toxin B + pH-neutralized BHI control
5. Toxin B + IPA extract CS* — Culture 1
6. Toxin B + pH-neutralized CS — Culture 1
7. Toxin B + IPA extract CS — Culture 2
8. Toxin B + pH-neutralized CS — Culture 2

*CS = Culture Supernatant

Toxin B: 270 kDa
BHI, brain heart infusion broth; IPA, isopropanol

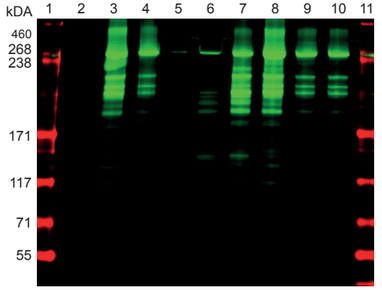
Figure 4. Cleavage detection of toxin A exposed to re-suspended lyophilized formulated ART24



Lanes:
1. Novex HI Mark pre-stained protein ladder
2. pH-neutralized supernatant (Culture 2)
3. PBS control
4. Heat-treated ART24 10^{-1}
5. ART24 10^{-2} (6.21 x 10^{6} CFU)
6. ART24 10^{-3} (6.21 x 10^{5} CFU)
7. ART24 10^{-4} (6.21 x 10^{4} CFU)
8. ART24 10^{-5} (6.21 x 10^{3} CFU)
9. ART24 10^{-6} (6.21 x 10^{2} CFU)
10. ART24 10^{-7} (6.21 x 10^{1} CFU)
11. Novex HI Mark pre-stained protein ladder

Toxin A: 308 kDa
CFU, colony-forming unit; PBS, phosphate-buffered saline

Figure 5. Cleavage detection of toxin B exposed to re-suspended lyophilized formulated ART24



Lanes:
1. Novex HI Mark pre-stained protein ladder
2. pH-neutralized supernatant (Culture 2)
3. PBS control
4. Heat-treated ART24 10^{-1}
5. ART24 10^{-2} (6.21 x 10^{6} CFU)
6. ART24 10^{-3} (6.21 x 10^{5} CFU)
7. ART24 10^{-4} (6.21 x 10^{4} CFU)
8. ART24 10^{-5} (6.21 x 10^{3} CFU)
9. ART24 10^{-6} (6.21 x 10^{2} CFU)
10. ART24 10^{-7} (6.21 x 10^{1} CFU)
11. Novex HI Mark pre-stained protein ladder

Toxin B: 270 kDa
CFU, colony-forming unit; PBS, phosphate-buffered saline

Conclusions

At projected clinically relevant concentrations, ART24 is bactericidal against *C. difficile* in liquid co-cultures and completely cleaves toxins A and B. ART24 is a promising LBP candidate in Phase 1 clinical development for the prevention of recurrent CDI.

Acknowledgments

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Disclosures

Michelle O' Donnell has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

James Hegarty has no disclosures to make in relation to this work.

Sarah Schulz has no disclosures to make in relation to this work.

Rachael Jacobson has no disclosures to make in relation to this work.

Colin Hill has received grants and personal fees from Artugen Therapeutics Ltd.; has received grants from Janssen Pharmaceuticals, Kerry Foods, and ADARE Pharmaceuticals outside of this work; and additionally has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

R. Paul Ross is a paid consultant of Artugen Therapeutics Ltd.; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

Mary C. Rea is a paid consultant of Artugen Therapeutics Ltd.; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

Ronald Farquhar is a paid consultant of Artugen Therapeutics Ltd., in which he also holds stock; and additionally has a patent pending: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

Laurent Chesnel is an employee of Artugen Therapeutics Ltd., in which he also holds stock; and additionally has a patent issued: METHODS AND COMPOSITIONS FOR THE TREATMENT OF *C. DIFFICILE*.

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