In Vitro Investigation of Auranofin as a Treatment for *Clostridium difficile* Infection

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Abstract

**Background** *Clostridium difficile* infection is the leading cause of hospital-acquired gastrointestinal infection and incidence rates continue to rise. *Clostridium difficile* infection is becoming increasingly complex to treat owing to the rise in treatment failures and recurrent infections. There is a clear need for new therapeutic options for the management of this disease.

**Objective** This study aimed to assess auranofin, a drug approved for the treatment of arthritis, as a treatment for *C. difficile* infection. Previous investigations have demonstrated potential antimicrobial activity of auranofin against *C. difficile* and other organisms.

**Methods** The activity of auranofin was assessed by in vitro investigations of its effect on *C. difficile* M7404 growth, vegetative cell viability, and spore viability. Activity of auranofin was also compared to that of the current treatments, metronidazole and vancomycin.

**Results** Auranofin showed bactericidal activity at concentrations as low as 4.07 µg/mL, effectively reducing bacterial cell density by 50–70% and the viable vegetative cell and spore yields by 100%. The activity of auranofin was shown to be non-inferior to that of metronidazole and vancomycin.

**Conclusions** Auranofin is highly efficacious against *C. difficile* M7404 in vitro and has the potential to be an ideal therapeutic option for the treatment of *C. difficile* infection.

Key Points

Auranofin exhibits strong antimicrobial activity against *Clostridium difficile*.

Concentrations of auranofin as low as 4.07 µg/mL demonstrated bactericidal and sporicidal activity against *C. difficile*.

1 Introduction

The Gram-positive, anaerobic bacterium *Clostridium difficile* is the leading cause of healthcare-associated diarrhea [1]. *Clostridium difficile* infection (CDI) is a significant cause of morbidity and mortality among hospitalized patients [1]. *Clostridium difficile* infection can present as mild-to-moderate diarrhea through to more severe pseudomembranous colitis. Serious complications can be life threatening [1].

Infection with *C. difficile* is through the ingestion of spores via the nasogastric route, and is typically associated with antibiotic use [2]. Antibiotics deplete the intestinal microbiota, creating the ideal environment for *C. difficile* spores to germinate into vegetative cells [2–4]. The vegetative cells then colonise the gut mucosa and proliferate and produce more spores, as well as the primary virulence factors toxin A (TcdA) and toxin B (TcdB) [3–5].

Further disruption of the intestinal microbiota occurs during treatment of CDI with the antibiotics, metronidazole or vancomycin [2]. While these antibiotics have been effective in the past, reports of treatment failures as high as 38%...
indicate that these therapies are no longer adequate and new options are needed [6–8]. Newer antibiotic options such as fidaxomycin and rifaximin are recommended as alternatives to metronidazole and vancomycin [8, 9]. There have been reports of resistance to these antibiotics, further highlighting the need for other treatment options [10, 11].

One drug that has gained significant attention as a potential therapy for CDI is auranofin, a previously approved treatment for rheumatoid arthritis [11–13]. Auranofin has more recently been investigated as a treatment for a number of diseases including cancer, human immunodeficiency virus, parasitic infections such as Leishmaniasis, and infections caused by bacteria such as Staphylococcus aureus, Treponema denticola, and C. difficile [12, 13]. Auranofin is poorly absorbed via the gastrointestinal tract, thus it has the ideal pharmacokinetics to treat CDI. Up to 85% of the administered dose will reach the distal colon [14], indicating the ideal pharmacokinetics to treat CDI. Up to 85% of the administered dose will reach the distal colon [14], indicating the ideal pharmacokinetics to treat CDI. Auranofin needs to be tested against a culture in this growth phase to determine the antimicrobial activity of auranofin against a clinically relevant model and to determine the effects auranofin may have on spores.

This study further assesses auranofin as a treatment for CDI by testing the activity of auranofin on established C. difficile cultures using in vitro methods. It is hypothesized that auranofin will effectively inhibit C. difficile growth and reduce vegetative cell viability without promoting sporulation and will be non-inferior to the current treatments for CDI.

2 Methods

2.1 Routine Bacterial Culture

The strain used was C. difficile M7404, which was stored in a glycerol stock at −80 °C (MHB, 30% glycerol). When removed from storage, isolates were streaked for single colonies on brain heart infusion (BHI; Oxoid, Hampshire, England) agar with 0.1% (w/v) taurocholic acid sodium salt hydrate (Sigma-Aldrich, Castle Hill, NSW, Australia) to allow for spore germination. Broth cultures were grown in Tryptone yeast extract [3]. All bacterial work, including incubation periods, were undertaken in a Don Whitley DG250 Workstation (Don Whitley Scientific, Gosford, England) agar with 0.1% (w/v) taurocholic acid sodium salt hydrate (Sigma-Aldrich, Castle Hill, NSW, Australia) to allow for spore germination. Broth cultures were grown in Tryptone yeast extract [3]. All bacterial work, including incubation periods, were undertaken in a Don Whitley DG250 Workstation (Don Whitley Scientific, Gosford, NSW, Australia) to maintain an anaerobic environment (10% H2, 10% CO2 in N2; BOC, NSW, Australia) and a temperature of 37 °C with 75% humidity [3]. All media were anaerobically conditioned prior to use by overnight incubation.

2.1.1 Preparation of Antibiotic Stock Solutions

Stock solutions of auranofin (Enzo, United Biosciences, Carindale, QLD, NSW, Australia) 4005.9 mg/L, metronidazole (Sigma-Aldrich) 171.15 mg/L, and vancomycin hydrochloride (Sigma-Aldrich) 4999.9 mg/L were prepared by dissolving a powdered antibiotic in either 100% ethanol (Chem-supply, Westlabs, Mitchell Park, VIC, Australia) for
auranofin or sterile deionised water for metronidazole and vancomycin.

2.1.2 Bacterial Growth Assays (n = 3)

For each assay, 40 mL of Tryptone yeast broth was inoculated with 1% (v/v) overnight culture of C. difficile M7404 with optical density (OD₆₀₀) of 0.2 and incubated as described above. Treatments were applied at 12 h, which is the end of the exponential growth phase. Treatment groups were auranofin at 4.07 µg/mL, 33.97 µg/mL, 169.87 µg/mL, and 339.75 µg/mL, the diluent ethanol at a percentage equivalent to that in the auranofin 33.97 µg/mL treatment (0.8%), metronidazole 2.74 µg/mL, and vancomycin 11.59 µg/mL. The control group was untreated C. difficile culture. Bacterial growth was measured using OD₆₀₀ at 0, 2, 4, 6, 8, 10, 12, 14, 24, and 48 h with an Epoch 2 microplate reader (Biotek, Millennium Biosciences, Mulgrave, VIC, Australia). Statistical analyses were performed using a two-tailed unpaired t test with a 95% confidence interval.

2.2 Viable Cell Counts and Viable Spore Counts

Cell viability was assessed by viable cell counts (VCC) performed from samples taken at 0, 12, 24, and 48 h from the growth assay described above. Samples taken at each of these timepoints were serially diluted and 100 µL of each dilution from neat to 10⁻⁵ were inoculated onto BHI agar and incubated for 48 h. Colonies on each plate were then counted and the average colony-forming unit (CFU) was calculated.

Spore viability was assessed by viable spore counts (VSC), the method of which is similar to VCC with the following changes. First, the vegetative cells were heat killed at 75 °C for 30 min, and second, the BHI agar contained 0.1% (w/v) taurocholic acid sodium salt hydrate to germinate the spores. Control samples were also inoculated onto BHI to confirm the heat killing of the vegetative cells.

2.3 Statistical Analysis

The statistical test used for each analysis is reported in the results. The relative change is defined as the difference between the final value and the initial value divided by the initial value, with a onefold change equivalent to a 100% change.

### Table 1 Optical density (OD₆₀₀) of Clostridium difficile cultures treated with auranofin

| Culture treatment | Treatment | End timepoint | Relative change |
|-------------------|-----------|---------------|-----------------|
| Auranofin 6 µM    | 0.37      | 0.087         | −0.7            |
| 0.8% ethanol      | 0.417     | 0.573         | 0.3             |
| Control           | 0.46      | 0.687         | 0.5             |
| Auranofin 50 µM   | 0.095     | 0.043         | −0.5            |
| Auranofin 250 µM  | 0.091     | 0.052         | −0.5            |
| Auranofin 500 µM  | 0.089     | 0.054         | −0.5            |
| Control           | 0.085     | 0.086         | 0.01            |

3 Results

3.1 Minimum Inhibitory Concentration of Auranofin

Experiments to determine the minimum inhibitory concentration (MIC) were consistent with those reported by AbdelKhalek et al., with the MIC for auranofin being 4.07 µg/mL (data not shown). Sub-inhibitory concentrations (< 67.84 ng/mL) did not promote sporulation (data not shown).

As shown in Fig. 1, when treated at stationary growth phase, C. difficile cultures treated with 4.07 µg/mL of auranofin showed a reduction in bacterial cell density of 0.7-fold. The ethanol-treated and control cultures showed a 0.3-fold and a 0.5-fold increase in bacterial cell density, respectively (data are shown in Table 1).
3.2 Treatment Concentrations of Auranofin

Treatment concentrations of 33.97 µg/mL, 169.87 µg/mL, and 339.75 µg/mL were compared to untreated control cultures. The lower concentration (33.97 µg/mL) was chosen based on the results of the MIC titration assay (data not shown), while the highest concentration (339.75 µg/mL) was determined to be the maximum dose able to be used in a mouse model. The bacterial growth of these cultures is shown in Fig. 2. All three auranofin-treated cultures showed a significant onefold reduction in bacterial cell density compared with the control, which showed a 0.01-fold increase in bacterial cell density (p < 0.01 and p = 0.005, respectively). There was no significant difference in the bacterial cell density between any of the auranofin-treated cultures. Data are shown in Table 1. The VCC and VSC from 12- and 24-h cultures are shown in Fig. 3. There was a onefold reduction in CFUs in all auranofin-treated cultures. The control culture showed a fourfold increase in CFUs. Similarly, the auranofin-treated cultures showed a onefold reduction in the spore counts, while the control showed a 0.5-fold increase (data are shown in Table 2).

3.3 Auranofin vs Metronidazole vs Vancomycin

The treatment concentrations used were based on MICs determined from microbroth dilution (data not shown). Additional timepoints of 36 and 60 h were added to accommodate the slow-acting antimicrobial activity of vancomycin. Bacterial growth is shown in Fig. 4. The auranofin- and vancomycin-treated cultures both showed a 0.7-fold decrease in bacterial cell density between treatment (8 h) and 48 h. Overall, these cultures showed a 0.6-fold decrease in bacterial cell density (8–60 h). Metronidazole had an initial 0.6-fold decrease (8–36 h), and then showed a 0.8-fold increase (36–60 h). Overall, the metronidazole-treated cultures had a 0.2-fold decrease in bacterial cell density (8–60 h). The control cultures demonstrated an overall 0.4-fold decrease in bacterial cell density (8–60 h) [data are shown in Table 3].

The VCC and VSC for 12, 24, and 48 h are shown in Fig. 5. For cell viability (VCC), auranofin-treated cultures showed a onefold decrease in CFUs. The vancomycin-treated cultures initially showed a 35-fold increase (12–24 h) followed by a 0.8-fold decrease (24–48 h) to have a 4.5-fold increase in CFUs overall. Metronidazole initially showed a onefold decrease (12–24 h) followed by a > 2000-fold increase (24–48 h) to have a 71-fold increase overall. The control group had a > 400-fold increase overall. The auranofin- and vancomycin-treated cultures had a CFU of < 30 for the VSC at all timepoints. At 48 h, the metronidazole-treated cultures had a VSC of ~ 18,000 CFUs and the control had 116,000 CFUs.

4 Discussion

Clostridium difficile infection is an ongoing problem in the healthcare setting owing to the high rates of treatment failure and recurrent disease [3]. The threat of emerging resistance to antibiotics is also of great concern [10, 11]. This study aimed to demonstrate that auranofin could be repurposed as a treatment for CDI. This was achieved using an in vitro model that reflected an established infection. Concentrations of auranofin as low as 4.07 µg/mL significantly reduced the bacterial cell density of C. difficile cultures after they were treated at the beginning of the stationary growth phase. This is consistent with the observations made by AbdelKhalek et al., albeit at a later, more clinically relevant timepoint. Cultures treated with 33.97 µg/mL or higher yielded no viable vegetative cells after the 24-h timepoint, indicating that auranofin has bactericidal activity against C. difficile. This bactericidal activity makes auranofin a strong candidate for treatment of CDI.
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Fig. 3 Number of viable vegetative cells and spores from Clostridium difficile cultures treated with auranofin. Vegetative cell viability assessed from C. difficile cultures at a 12 h post-inoculation and b 24 h post-inoculation. Spore viability was assessed from C. difficile cultures at c 12 h post-inoculation and d 24 h post-inoculation. Treatment concentration of auranofin 33.97 mg/L is shown by filled triangles, treatment concentration of 169.87 mg/L is shown by unfilled triangles, treatment concentration of auranofin 339.75 mg/L is shown by unfilled circles, and untreated cultures (control) are shown by filled squares. *Significant difference between cultures treated with auranofin 33.97 mg/L and the control, **significant difference between cultures treated with auranofin 169.87 mg/L and the control, x indicates a significant difference between cultures treated with auranofin 339.75 mg/L and the control. *p < 0.05; **p < 0.005, n=3. CFU colony-forming unit.
Clostridium difficile spores are the transmissible element capable of causing widespread disease and are associated with recurrent infections [3, 4]. Auranofin was able to reduce the number of viable spores in C. difficile cultures after treatment. This suggests that auranofin may have sporicidal activity and can kill the spores, or has sporistatic activity and is able to prevent the germination of the spores into the vegetative form. This finding was beyond the expectations of the hypothesis and is highly significant. If auranofin were sporicidal, this would improve treatment outcomes, as there would be clearance of both forms of the bacteria, reducing the risk of the recurrence of CDI. It would also reduce transmission of the organisms, thus limiting the spread of disease. It would be of interest to further investigate the extent of the effect auranofin has on C. difficile spores, particularly as the spores are highly resilient to the current therapies.

When compared to the current therapies, metronidazole and vancomycin, auranofin was found to be non-inferior. There was no significant difference in the bacterial growth of C. difficile cultures treated with vancomycin or auranofin at any timepoint. Auranofin-treated cultures had less viable cells and spores from 24 h onwards than the cultures treated with either of the current therapies. Metronidazole-treated cultures initially showed a reduction in bacterial growth, but recovered after 24 h, indicating that a single dose of metronidazole was not able to sustain activity. This observation was reflected in the VCC and VSC, with high numbers of viable vegetative cells and spores from 48-h cultures.

Further in vitro work is necessary to elucidate the mechanism by which auranofin is acting on C. difficile spores, followed by an in vivo animal model of CDI. It is also important to elucidate interactions between auranofin and the inflammation mediated by C. difficile toxins. AbdelKhalek et al. found that auranofin reduces the levels of the pro-inflammatory cytokine interleukin-8 by caco-2 cells exposed to C. difficile toxins. Further work is needed in this area to fully understand this anti-inflammatory activity of auranofin. Additionally, the impact of auranofin on the intestinal microbiota should be investigated because intestinal dysbiosis due to antibiotic therapy is a key risk factor for recurrent CDI [3].

Table 2 Viable cell and viable spore counts of Clostridium difficile cultures treated with auranofin

| Culture treatment | 12 h (treatment) | 24 h | Relative change |
|-------------------|------------------|------|----------------|
| VCC               |                  |      |                |
| Auranofin 50 µM   | 56.6 × 10⁶       | 53.33| − 1            |
| Auranofin 250 µM  | 71.3 × 10⁶       | 75.3 × 10⁴| − 1          |
| Auranofin 500 µM  | 11.8 × 10⁶       | 1800 | − 1            |
| Control           | 16.5 × 10⁶       | 84.6 × 10⁶| 4             |
| VSC               |                  |      |                |
| Auranofin 50 µM   | 10,000           | 126  | − 1            |
| Auranofin 250 µM  | 4866             | 33.33| − 1            |
| Auranofin 500 µM  | 7666             | <30  | − 1            |
| Control           | 7666             | 11,866| 0.5           |

VCC viable cell counts, VSC viable spore counts

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5 Conclusions

This study has demonstrated that auranofin exhibits strong antimicrobial activity against the human pathogen C. difficile. Concentrations as low as 4.07 µg/mL were shown to be effective in reducing bacterial growth and vegetative cell viability, as well as reducing the number of spores produced by the bacteria. With up to 85% of the orally administered dose reaching the distal colon [14], it is highly likely that therapeutic concentrations of auranofin can be reached at the site of infection with a dose that is low enough to minimize adverse effects. In conclusion, auranofin presents a strong candidate as a new CDI treatment and with further investigation could be considered as a therapeutic option for the treatment of CDI.

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Compliance with Ethical Standards

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Conflict of interest Christine Roder and Eugene Athan have no conflicts of interest that are directly relevant to the content of this article.

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