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Antibacterial and antivirulence activities of auranofin against *Clostridium difficile*

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ABSTRACT

Clostridium difficile is a deadly, opportunistic bacterial pathogen. In the last two decades, *C. difficile* infections (CDIs) have become a national concern because of the emergence of hypervirulent mutants with increased capability to produce toxins and spores. This has resulted in an increased number of infections and deaths associated with CDI. The scarcity of anticlostridial drugs has led to unsatisfactory cure rates, elevated recurrence rates and permitted enhanced colonization with other drug-resistant pathogens (such as vancomycin-resistant enterococci) in afflicted patients. Therefore, both patients and physicians are facing an urgent need for more effective therapies to treat CDI. In an effort to find new anticlostridial drugs, we investigated auranofin, an FDA-approved oral antirheumatic drug that has recently been found to possess antibacterial activity. Auranofin exhibited potent activity against *C. difficile* isolates, inhibiting growth at a concentration of 1 μg/mL against 50% of all tested isolates. Auranofin inhibited both toxin production and spore formation, a property lacking in both vancomycin and metronidazole (the primary agents used to treat CDI). Auranofin had a direct protective activity against *C. difficile* toxin-mediated inflammation and inhibited the growth of vancomycin-resistant enterococci. Auranofin is a promising candidate that warrants further investigation as a treatment option for *C. difficile* infections.

1. Introduction

Clostridium difficile is the most common hospital-acquired infectious agent [1]. *C. difficile* infection (CDI), also known as *C. difficile*-associated diarrhea (CDAD), affected nearly half a million patients in the United States alone and was associated with over 29 000 deaths in 2011; direct costs exceed $5 billion (U.S.) annually [2]. Though CDI was discovered in the 1970s, the incidence and severity of infection has increased sharply over the past two decades [3]. This upsurge in CDI has been attributed to the emergence of hypervirulent strains (e.g. the North American pulsotype 1 [NAP1], PCR-ribotype 027, and restriction endonuclease analysis [REA] group BI 8). These hypervirulent *C. difficile* strains exhibit enhanced motility and adherence, increased drug-resistance and produce higher levels of toxins (toxins A and B and binary toxin) [1,4]. *C. difficile* toxins are the main virulence factor, and are essential for the bacteria to cause disease. Furthermore, *C. difficile* forms very resistant spores that can persist in the environment and these are the springboard for disease transmission. Moreover, spores can survive in the gastrointestinal tract of infected patients until the cessation of antibiotic treatment, provoking relapse of CDI [5,6].

Three primary drugs are used to treat CDI: vancomycin, metronidazole and fidaxomicin. Vancomycin and metronidazole have been used for decades with limited efficacy and high recurrence rates [7]. Fidaxomicin is the only anticlostridial drug approved in the past three decades. However, fidaxomicin treatment is not superior to vancomycin treatment in reducing recurrence rate or in the occurrence of treatment-emergent adverse events. Additionally, reports about *C. difficile* resistance or reduced susceptibility to metronidazole, vancomycin and, to a lesser extent, fidaxomicin are emerging worldwide [8,9]. There is a critical need for new anticlostridial drugs with improved treatment outcomes.

Drug repurposing is a promising approach to find new indications for existing or abandoned drugs. Adopting an old drug with a well-studied safety and pharmacokinetic profile can circumvent some of the pitfalls and costs associated with clinical testing and regulatory approval processes for novel compounds [10-12]. Auranofin [2,3,4,6-tetra-*o*-acetyl-l-thio-β-d-glycopyranp-sato-*S*-(triethyl-phosphine)-gold] is a gold-containing anti-inflammatory

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oral drug that has been used for treatment of rheumatoid arthritis for over 30 years. The safety and pharmacokinetic profile of auranofin in humans has been well-characterized and has permitted investigation of auranofin for other clinical indications [13,14]. The present study evaluates the potential of auranofin to be repurposed as a novel anticlostridial drug to treat CDI.

In the current study, we report that auranofin has potent antibacterial activity against a wide panel of *C. difficile* strains. Additionally, auranofin can inhibit toxin production and spore formation and protect human gut cells against inflammation induced by sterile *C. difficile* toxins. These data indicate that auranofin is a promising candidate to treat CDI.

2. Materials and methods

2.1. Chemicals, media and bacterial strains

Auranofin, linezolid (Chem-Impex International, Wood Dale, IL), vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO), metronidazole (Beantown Chemical Corporation, Hudson, NH), sodium selenite (MP Biomedicals, Santa Ana, CA), and fidaxomicin (Apexbio, Houston, TX) were procured from commercial vendors. Brain heart infusion (BHI) was purchased from Becton, Dickinson and Company (Cockeysville, MD). Phosphate-buffered saline, fetal bovine serum and non-essential amino acids (NEAA) were purchased from Fisher Scientific (Waltham, MA). Yeast extract, L-cysteine, vitamin K, hemin, Dulbecco's Modified Eagle's medium (DMEM), and penicillin/streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). *C. difficile* and enterococcal isolates (Table 1) were obtained from the American Type Culture Collection (ATCC) and Biodefense and Emerging Infections Research Resources Repository (BEI Resources).

2.2. Minimum inhibitory concentration (MIC) of auranofin against C. difficile

The broth microdilution assay was employed as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines, with slight modifications [15]. A bacterial suspension equivalent to 0.5 McFarland standard was prepared and subsequently diluted in BHIS broth to $\sim 10^5$ CFU/mL. The bacterial suspension was seeded in 96-well plates containing the required concentrations of auranofin and control antibiotics (vancomycin and metronidazole). Plates were then incubated anaerobically for 48 h at 37°C. MICs reported represent the lowest concentration of each agent that suppressed the visual growth of bacteria. MIC_{50} and MIC_{90} are the minimum concentration of each agent that inhibited the visual growth of 50% and 90% of the tested isolates, respectively.

2.3. Effect of auranofin on toxin production from a toxigenic C. difficile strain

To assess whether auranofin could inhibit *C. difficile* toxin production, total amounts of toxins A and B were measured in the cell-free supernatant of a late exponential phase culture of *C. difficile* ATCC BAA-1870. Toxin levels were compared after the addition of different subinhibitory concentrations of auranofin and control anticlostridial drugs [16,17]. Briefly, *C. difficile* ATCC BAA-1870 was grown in BHIS broth, washed twice and aliquoted into 500 µL tubes. Drugs at the required concentrations were added to each tube, in triplicate, then tubes were incubated anaerobically (using BD GasPak™ EZ Container Systems) at 37°C for 6 h. One portion of each suspension was serially diluted, plated on BHIS agar and incubated anaerobically at 37°C for 24 h to detect the bacterial count. The second portion was centrifuged at 10 000 rpm for 5 min. The total concentration of *C. difficile* toxins A and B was measured in the supernatant of each tube using an enzyme-linked immunosorbent assay (ELISA) kit (Premier®, Meridian Bioscience, Inc, Cincinnati, OH) following the manufacturer's instructions. The optical density (450 nm) corresponding to the toxin concentration was measured and compared for auranofin and the control drugs.

2.4. Effect of auranofin on C. difficile spore formation

C. difficile HM-88, in stationary phase, was diluted in fresh BHIS broth and incubated anaerobically for 4-6 h at 37°C. The bacterial suspension was aliquoted into tubes and drugs were added (in triplicate) at concentrations equivalent to ½ × and 1 × MIC. Tubes were then incubated anaerobically for 5 days at 37°C. After the incubation period, each tube was divided into two parts. One part was used to count the total amount of bacteria (vegetative bacteria + spores) through serial dilutions and culturing on BHIS agar plates supplemented with 0.1% taurocholic acid. The second part was centrifuged, media was replaced with PBS and stored at 4°C overnight. The bacterial suspensions in PBS were shock heated at 70°C for 20 min to kill vegetative cells, then serially diluted and plated to determine heat-resistant spore counts.

2.5. Protection of human gut cells against the inflammatory effect of C. difficile toxins

To appraise the anti-inflammatory effect of auranofin against *C. difficile* toxin-mediated inflammation of human gut cells, a cell rounding assay was utilized [16]. Briefly, *C. difficile* ATCC BAA-1870 was grown in BHIS broth for 24 h, centrifuged and the supernatant was sterile filtered and then frozen. Human colorectal epithelial cells (Caco-2) were grown in cell culture medium (DMEM supplemented with 10% FBS, 1 × NEAA, 100 IU/mL penicillin, and 100 µg/mL streptomycin) for 5 days. Cells were then trypsinized and seeded on a 96-well plate and grown at 37°C + 5% CO_2. Once cells reached ~90% confluency, medium was removed, and the bacterial supernatant was added to the cells with or without auranofin (1 and 8 µg/mL) and control anticlostridial drugs (vancomycin, metronidazole and fidaxomicin, 1–128 µg/mL). Drugs were incubated with cells for 24 h at 37°C + 5% CO_2. Cells were then observed via a phase contrast microscope for morphological changes (cell rounding) as a result of *C. difficile* toxin-induced inflammation.

2.6. Reduction of IL-8 release from toxin-treated Caco-2 cells

To further understand the anti-inflammatory activity of auranofin, IL-8 (a key cytokine in the process of *C. difficile* toxin-induced inflammation of gut cells) was detected in cell supernatants obtained from the cell rounding assay experiment (after 24 h of incubation with *C. difficile* toxin with or without auranofin, 1 µg/mL, treatment) [18]. Supernatants were tested for IL-8 concentrations using an ELISA kit (Human IL-8 PicoKine™ ELISA Kit) according to the manufacturer's instructions.

2.7. Activity of auranofin against vancomycin-resistant enterococci (VRE)

The minimum inhibitory concentrations (MICs) of auranofin and control antibiotics were tested using the broth microdilution assay as per CLSI guidelines [19]. Briefly, enterococcal isolates were streaked on brain heart infusion (BHI) agar plates and incubated aerobically at 37 °C for about 18 h. Bacterial colonies were scraped off the agar plates and suspended in BHI broth at a concentration of $\sim 10^5$ CFU/mL. Serial dilutions of the drugs were incubated with the bacterial suspensions for 16-20 hours at 37 °C. The reported MICs are the lowest concentrations of each drug that could inhibit the bacterial growth visually [20,21].

Table 1
Bacterial strains used in this study.

Bacterial Strain	Alternate Designation	Source	Comments
C. difficile NAP07 (CDC#2007054)	HM-88	Isolated from human feces	Reference genome for The Human Microbiome Project (HMP)
C. difficile P2	NR-32883	Isolated in 2001 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P3	NR-32884	Obtained in 2001 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P4	NR-32889	Isolated in 2001 from fecal material of a human patient with relapsing *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P5	NR-32885	Obtained in 2001 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P6	NR-32886	Isolated in 2001 from fecal material of a human patient with relapsing *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P7	NR-32887	Obtained in 2001 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P8	NR-32888	Isolated in 2001 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P13	NR-32891	Isolated in 2005 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P15	NR-32892	Isolated in 2005 from fecal material of a human patient with CDI in Western Pennsylvania, USA	Toxigenic strain
C. difficile P19	NR-32895	Obtained in 2005 from fecal material of a human patient with relapsing *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P20	NR-32896	Isolated in 2005 from fecal material of a human patient with relapsing *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P21	NR-32897	Isolated in 2005 from fecal material of a human patient with relapsing *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain
C. difficile P29	NR-32903	Isolated in 2009 from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxigenic strain and reported to be co-colonized with non-toxigenic *C. difficile*
C. difficile P30	NR-32904	Obtained in 2009 from fecal material of an asymptomatic human patient in Western Pennsylvania, USA	Non-toxigenic strain
C. difficile Isolate 1	NR-13427	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 2	NR-13428	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 4	NR-13430	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 5	NR-13431	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 6	NR-13432	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 7	NR-13433	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 9	NR-13435	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 10	NR-13436	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 11	NR-13437	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile Isolate 13	NR-13553	Obtained in 2008/2009 from a human patient from the Mid-Atlantic region of the United States	–
C. difficile 002-P50-2011	HM-746	Isolated in January 2011 from stool of a patient with diarrhea	Reference genome for the Human Microbiome Project (HMP)
C. difficile ATCC 700057	VPI 11186	–	Toxinotype tcdA-, tcdB-, Ribotype 038, Nontoxigenic
C. difficile ATCC 43598	1470	Human feces, asymptomatic neonate, Belgium	Presence of tcdB gene confirmed by PCR, Ribotype 017
C. difficile ATCC BAA 1801	3232	Human feces, adult with diarrhea, Belgium.	Non-toxigenic, Ribotype 010
C. difficile ATCC BAA 1870	4118	–	Presence of binary toxin, tcdA and tcdB genes. Toxinotype IIIb, Ribotype 027
C. difficile Isolate 20100207	NR-49278	Obtained in 2010 from stool of an elderly adult male patient with a healthcare-associated *C. difficile* infection in New York, USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20100502	NR-49277	Obtained in 2010 from stool of an older adult male patient with a community-associated *C. difficile* infection in Colorado, USA	PCR ribotype 019, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* of the PaLoc[e] operon, as well as the CDT[f]
C. difficile Isolate 20110052	NR-49281	Obtained in 2010 from stool of an elderly male patient with a healthcare-associated *C. difficile* infection in northeastern USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20110868	NR-49287	Obtained in 2011 from stool of an elderly female patient with a healthcare-associated *C. difficile* infection in southern USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20110870	NR-49288	Obtained in 2011 from stool of a young adult female patient with a healthcare-associated *C. difficile* infection in Tennessee, USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]

(*continued on next page*)

Table 1 (*continued*)

Bacterial Strain	Alternate Designation	Source	Comments
C. difficile Isolate 20110979	NR-49285	Obtained in 2011 from stool of an elderly female patient with a community-associated *C. difficile* infection in midwestern USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20110999	NR-49286	Obtained in 2011 from stool of an elderly female patient with a healthcare-associated *C. difficile* infection in western/midwestern USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20120013	NR-49283	Obtained in 2011 from stool of a young male patient with a community-associated *C. difficile* infection in northeastern USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20120184	NR-49289	Obtained in 2011 from stool of an elderly female patient with a fatal healthcare-associated *C. difficile* infection in Tennessee, USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
C. difficile Isolate 20120187	NR-49290	Obtained in 2011 from stool of an elderly male patient with a healthcare-associated *C. difficile* infection in Tennessee, USA	PCR ribotype 019, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* of the PaLoc[e] operon, as well as the CDT[f]
C. difficile Isolate 20120236	NR-49291	Obtained in 2011 from stool of an older female patient with a community-associated *C. difficile* infection in midwestern, USA	PCR ribotype 027, NAP1[a], contains *tcdA*[b], *tcdB*[c] and *tcd*[d]*C* (with 18 base pair deletion) PaLoc[e] operon and the CDT[f]
E. faecium HF50104	NR-32052	Isolated in 2008 from swine feces in Michigan, USA	Resistant to erythromycin, tetracycline and vancomycin
E. faecium Patient #3-1	NR-31912	Isolated from stool of a human patient having dominance of vancomycin-resistant *Enterococcus* in the stool but no bacteremia	Vancomycin-resistant
E. faecium E1071	NR-28978	Hospitalized person free of enterococcal infection in the Netherlands in 2000 during a hospital surveillance program	Non-infectious fecal isolate Resistant to vancomycin
E. faecium ERV165	HM-970	Isolated in 2008 from human feces in Colombia	Resistant to vancomycin
E. faecium ERV102	HM-968	Isolated in 2006 from human oral sputum in Colombia	Resistant to ampicillin, vancomycin and streptomycin

[a] NAP= North American pulsed-field gel electrophoresis type.
[b] *tcdA*= *C. difficile* toxin A gene.
[c] *tcdB*= *C. difficile* toxin B gene.
[d] *tcdC*= Anti-sigma factor gene.
[e] PaLoc= Pathogenicity locus.
[f] CDT= *C. difficile* binary toxin.

2.8. Statistical analysis

GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla CA) was utilized for statistical analyses. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed to analyse the IL-8 data from cell supernatants. Two-way ANOVA followed by Dunnett's post-hoc comparisons test were utilized to analyse the spore inhibition data.

3. Results

3.1. Antibacterial activity of auranofin against C. difficile

The anticlostridial activity of auranofin was evaluated against a large panel of *C. difficile* strains, including hypervirulent strains (ribotype 027). As shown in Table 2, auranofin inhibited growth of the 41 tested *C. difficile* strains at concentrations ranging from 0.25 to 4 μg/mL. Auranofin inhibited 50% of the tested isolates (MIC_{50}) at a concentration of 1 μg/mL and inhibited 90% of the isolates (MIC_{90}) at a concentration of 2 μg/mL. The MIC of auranofin was comparable to values obtained for vancomycin, the drug of choice for treatment of severe CDI. Vancomycin was effective at a range of 0.25 to 2 μg/mL (MIC_{50} = 0.5 μg/mL and MIC_{90} = 1 μg/mL). Metronidazole was active at a range of 0.06 to 0.25 μg/mL (MIC_{50} = 0.0.25 μg/mL and MIC_{90} = 0.25 μg/mL).

3.2. Auranofin inhibits C. difficile toxin production

After confirming the potent in vitro anticlostridial activity of auranofin, we tested the inhibitory activity of auranofin against *C. difficile* toxin production. Bacteria in the late log phase were incubated with subinhibitory concentrations of auranofin and control anticlostridial drugs. Auranofin exhibited a dose-dependent inhibition of *C. difficile* toxin production compared with untreated control. As depicted in Fig. 1, auranofin at $^1/_8$, $^1/_4$ and $^1/_2 \times$ MIC inhibited 15.6%, 31.2% and 40% of total toxin production, respectively. Fidaxomicin, an anticlostridial antibiotic known to inhibit toxin production and spore formation [22], inhibited 37.2%, 50.1% and 52.3% of toxin production at $^1/_8$, $^1/_4$ and $^1/_2 \times$ MIC, respectively. As expected, no toxin inhibition was observed when *C. difficile* was treated with either vancomycin or metronidazole; on the contrary, the toxin concentration increased at certain concentrations, in agreement with previous reports [17,22,23].

3.3. Auranofin inhibits C. difficile spore formation

C. difficile HM-88, in late exponential growth phase, was incubated with subinhibitory concentrations of auranofin and control anticlostridial drugs. Total vegetative cells and heat-resistant spores were counted in each sample. Spores comprised most of the viable count in the untreated control (Fig. 2). Auranofin-treated bacteria displayed reduced spore count, ~1.5 $\log_{10}$ at both $^1/_2$ and 1 × MIC. While a similar effect was observed with fidaxomicin, almost no reduction in spore count was detected with vancomycin or metronidazole.

3.4. Protection of human gut cells against inflammatory effect of C. difficile toxins

Auranofin has been shown to have anti-inflammatory activity, which is important for the treatment of rheumatoid arthritis. We investigated whether auranofin would also protect gut cells from inflammation induced by *C. difficile* toxins. Human colorectal cells (Caco-2) were treated with sterile-filtered bacterial supernatant, with or without the addition of auranofin or control anticlostridial drugs. As shown in Fig. 3, healthy Caco-2 cells display normal morphological characteristics of enterocytes. Upon treatment with *C. difficile* toxins, cell rounding and detachment occurred [24]. The goal was to inspect if drug treatment preserved the normal Caco-2 morphology in the presence of *C. difficile* toxins. Auranofin (1 and 8 μg/mL) protected cells against the inflammatory effect

Table 2
The minimum inhibitory concentration (MIC, μg/mL) of auranofin and control anticlostridial drugs against *C. difficile* isolates.

C. difficile Strain	ID number	Auranofin	Vancomycin	Metronidazole
P2	NR-32883	0.5	0.25	0.06
P3	NR-32884	4	1	0.25
P4	NR-32889	1	2	0.125
P5	NR-32885	0.5	1	0.125
P6	NR-32886	2	1	0.125
P7	NR-32887	0.5	1	0.125
P8	NR-32888	1	0.5	0.125
P13	NR-32891	1	0.5	0.125
P15	NR-32892	1	0.5	0.06
P19	NR-32895	1	1	0.25
P20	NR-32896	4	1	0.25
P21	NR-32897	1	0.25	0.125
P29	NR-32903	1	0.25	0.06
P30	NR-32904	1	0.5	0.25
Isolate 1	NR-13427	1	1	0.25
Isolate 2	NR-13428	1	1	0.06
Isolate 4	NR-13430	2	0.25	0.06
Isolate 5	NR-13431	2	0.5	0.25
Isolate 6	NR-13432	1	0.5	0.125
Isolate 7	NR-13433	0.5	0.5	0.25
Isolate 9	NR-13435	0.25	0.5	0.06
Isolate 10	NR-13436	1	0.5	0.125
Isolate 11	NR-13437	2	1	0.25
Isolate 13	NR-13553	4	1	0.25
NAP07	HM-88	1	0.5	0.25
002-P50-2011	HM-746	0.25	0.25	0.125
ATCC 700057	VPI 11186	1	0.5	0.25
ATCC 43598	1470	0.5	0.5	0.25
ATCC BAA 1801	3232	1	0.5	0.125
ATCC BAA 1870	4118	0.5	1	0.25
Isolate 20100502	NR-49277	0.25	0.5	0.125
Isolate 20100207	NR-49278	0.25	0.25	1
Isolate 20110052	NR-49281	0.25	0.25	0.125
Isolate 20110868	NR-49287	0.25	0.25	0.25
Isolate 20110870	NR-49288	0.5	0.5	0.25
Isolate 20110979	NR-49285	0.25	0.25	0.25
Isolate 20110999	NR-49286	0.25	0.25	0.25
Isolate 20120013	NR-49283	0.25	0.25	0.125
Isolate 20120184	NR-49289	0.5	0.25	0.25
Isolate 20120187	NR-49290	0.5	0.5	0.25
Isolate 20120236	NR-49291	0.25	0.5	0.25
MIC_{50}		1	0.5	0.25
MIC_{90}		2	1	0.25

of *C. difficile* toxins and successfully preserved the normal cell morphology. Similar results were obtained with polarized cells (data not shown). On the other hand, cells treated with vancomycin, metronidazole or fidaxomicin (at a concentration ranging from 1 to 128 μg/mL) exhibited rounding and detachment after exposure to toxins, indicating these drugs were unable to protect the gut epithelial cells.

3.5. Reduction of IL-8 release from toxin-treated Caco-2 cells

After confirming auranofin can mitigate *C. difficile* toxin-mediated inflammation, we investigated if the anti-inflammatory effect of auranofin was due to inhibition of IL-8. IL-8 is a major proinflammatory cytokine released upon exposure of gut epithelial cells to *C. difficile* toxins [18]. IL-8 was measured in the Caco-2 supernatants after incubation with *C. difficile* toxins with or without auranofin (1 μg/mL). Toxin treatment resulted in a significant increase in IL-8 concentration in the cell supernatant (Fig. 4). However, when cells were treated with toxins along with auranofin, no significant increase in IL-8 was observed. The OD_{450} represents the IL-8 concentration in the supernatants.

3.6. Activity of auranofin against vancomycin-resistant enterococci (VRE)

To evaluate if auranofin treatment can prompt an overgrowth of vancomycin-resistant enterococci (VRE), the MIC of auranofin was determined against five VRE strains (Table 3). Auranofin inhibited growth of VRE at concentrations ranging from 0.25 to 0.5 μg/mL. Fidaxomicin also inhibited VRE growth, albeit at higher concentrations (MIC 1–2 μg/mL). In contrast, vancomycin and metronidazole were not effective against all the tested VRE strains.

4. Discussion

CDIs are associated with increasing morbidity and mortality in the healthcare setting. The U.S. Centers for Disease Control and Prevention (CDC) reported about half a million CDI cases in 2011, an increase from 333 000 cases reported in 2007. More worrisome is the relapse rate of 20% and a direct cost of $4.8 billion for acute care facilities alone. Moreover, approximately 29 000 deaths occurred within 30 days of initial diagnosis, with much higher rates reported among elderly patients [2]. CDI in the healthcare setting is most likely attributed to administration of broad-spectrum antibiotics that subsequently damage the intestinal microbiota permitting *C. difficile* to expand, attach to epithelial cells and produce toxins. *C. difficile* toxins affect the colonic epithelium, leading to loss of tight junctions, enhanced mucosal permeability and intense inflammation and neutrophilic infiltration [25].

Treatment options for CDI, including for infections caused by hypervirulent strains, are very limited. Only three drugs are currently in use, vancomycin and metronidazole, both discovered in the mid-20th century, and fidaxomicin, approved in 2011. However, there are several limitations with these drugs. For example, it is estimated that about 22% and 14% of patients treated with metronidazole or vancomycin, respectively, will experience treatment failure. Moreover, 25–30% of patients treated with either drug will suffer from CDI recurrence [7,17]. Unfortunately, treatment outcome remains unsatisfactory even with the introduction of fidaxomicin, where relapsing CDI occurs [26,27]. This highlights the need to identify new, effective agents to treat CDI.

Antibacterial drug discovery is a very lengthy and expensive process. Repurposing FDA-approved drugs for new indications is a promising approach for drug discovery. Due to extensive preclinical and clinical investigation, key parameters of these drugs, such as the safety profile, pharmacodynamics and pharmacokinetics, are known, thus reducing the time and cost associated with drug development [28–31]. Auranofin represents one drug we have been extensively investigating to repurpose as an antibacterial agent. Auranofin is an antirheumatic drug approved by the FDA in 1985 with a well-defined safety profile and limited reports of adverse reactions [13]. One of the attractive traits of auranofin is its low oral absorbability as only 15-25% of the administered dose is absorbed and about 85% of the drug is excreted in feces [13]. Therefore, auranofin is an attractive drug for development against gut pathogens.

Although Jackson-Rosario et al. reported auranofin is active in vitro against *C. difficile* [32], a detailed investigation of auranofin against a wide panel of *C. difficile* isolates and the impact of auranofin on key virulence factors expressed by *C. difficile* has yet to be undertaken. In this study, we evaluated auranofin against 41 different *C. difficile* strains, including hypervirulent (NAP1, ribotype 027) and clinical toxigenic isolates. In agreement with the Jackson-Rosario et al. study [32], auranofin inhibited growth of *C. difficile* at a low concentration (0.25–4 μg/mL), which was comparable to the standard anticlostridial drugs, vancomycin and metronidazole.

We next sought to investigate the inhibitory activity of auranofin against *C. difficile* toxin production. As mentioned above, tox-

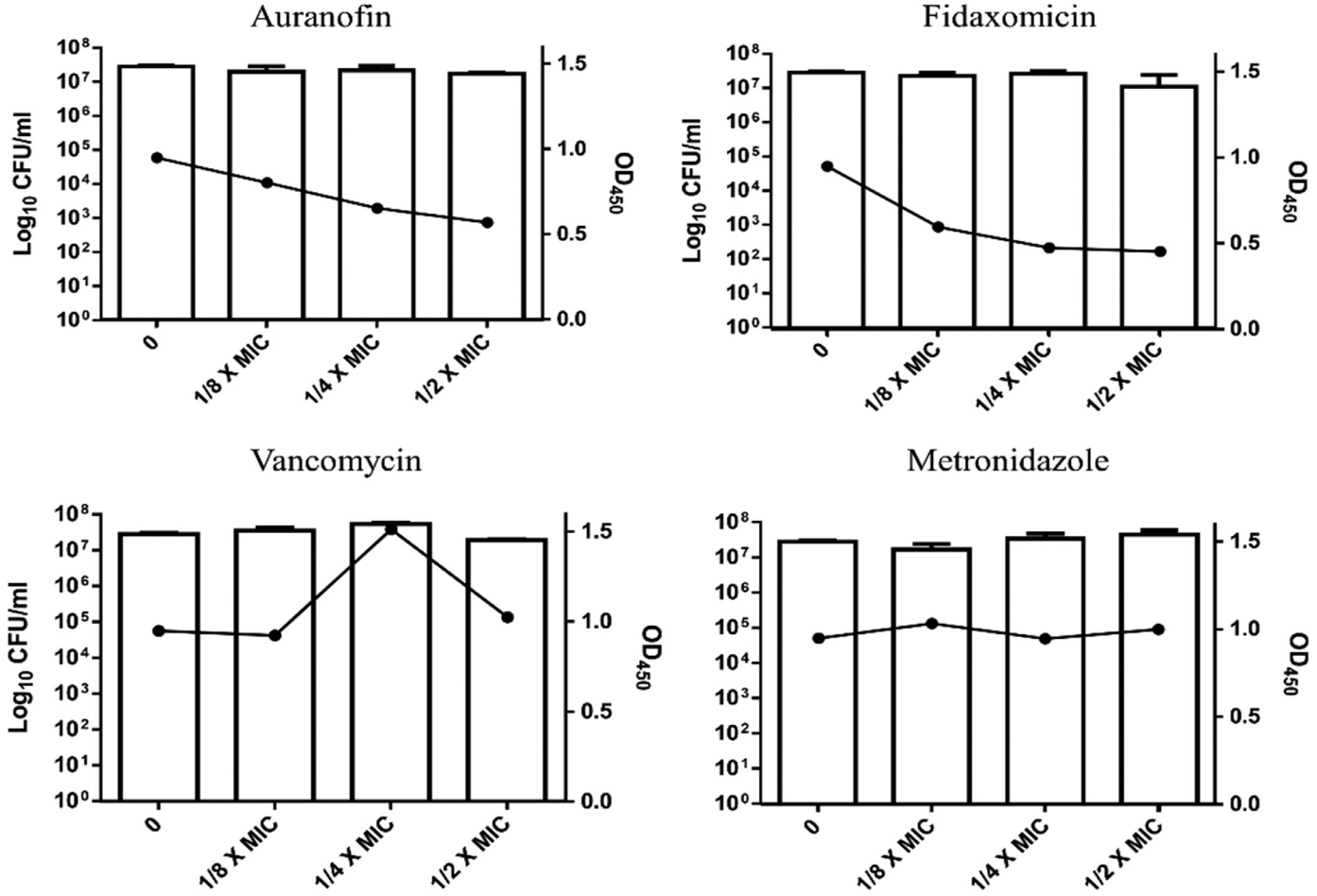

Fig. 1. Toxin inhibition activity of auranofin and control anticlostridial drugs (vancomycin, metronidazole and fidaxomicin) against *C. difficile*. Drugs at concentrations of $^{1}/_{8}\times$, $^{1}/_{4}\times$ and $^{1}/_{2}\times$ MIC were incubated with a hypervirulent, toxigenic strain of *C. difficile* (strain ATCC BAA-1870). Bacterial counts were determined for each sample, and toxin levels were assessed in the supernatant using enzyme-linked immune fluorescent assay (ELISA). Error bars represent standard deviation values from triplicate samples for each treatment.

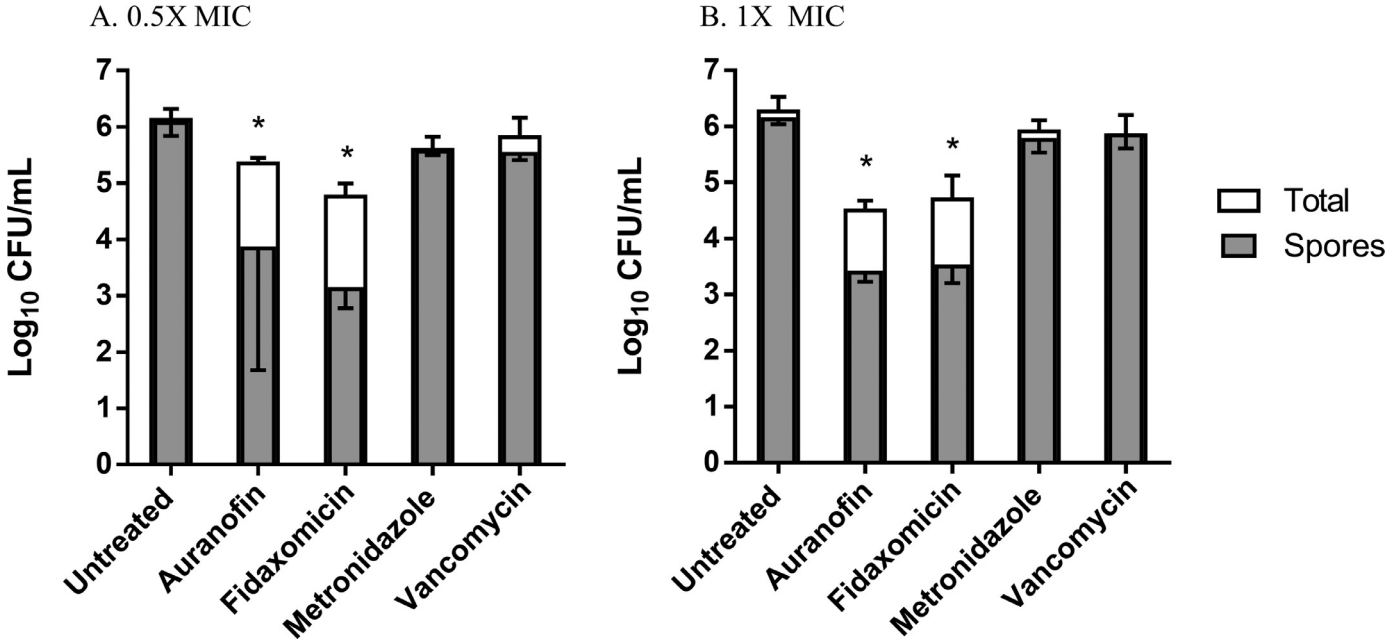

Fig. 2. Spore inhibition of activity of auranofin against *C. difficile* compared with control anticlostridial drugs, vancomycin and metronidazole. Drugs ($^{1}/_{2}\times$ and $1\times$ MIC) were incubated with bacteria for five days followed by serial dilution and plating to count both total bacterial count and heat-resistant spores. Error bars represent standard deviation values from triplicate samples for each treatment. (*) denotes significant difference between the total and the spore counts.

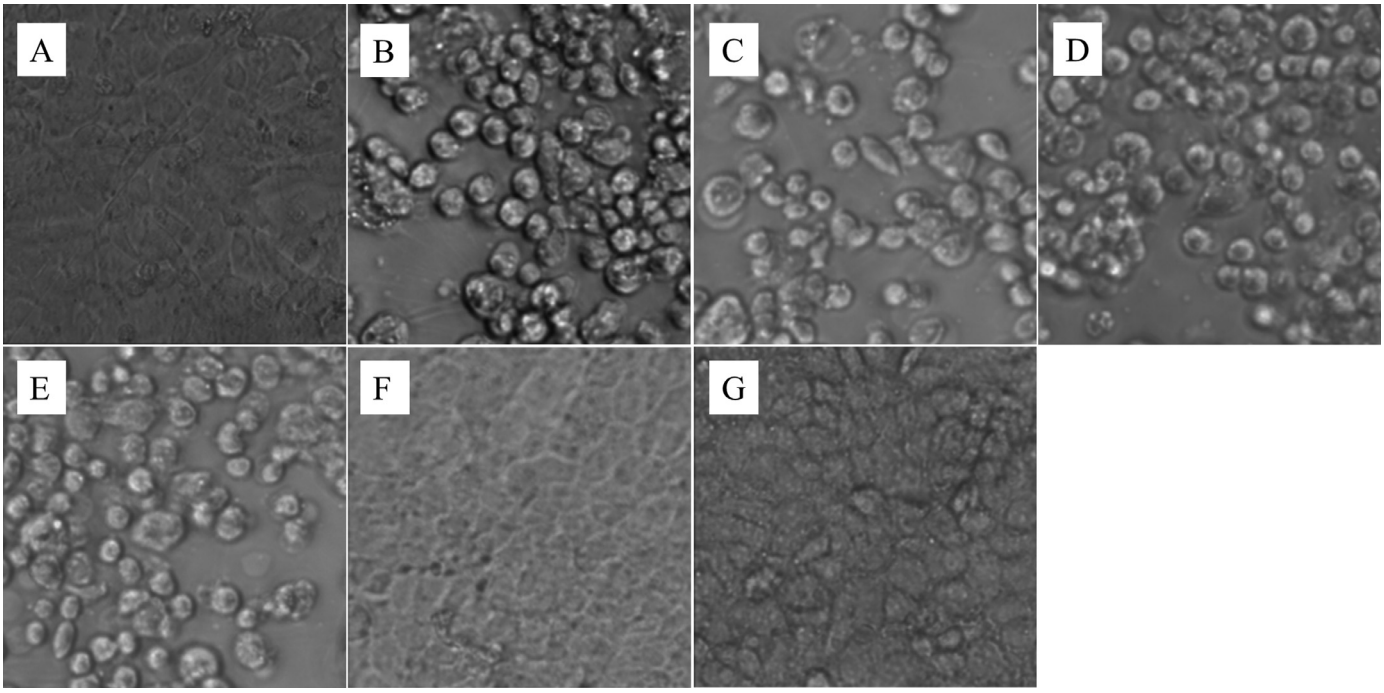

Fig. 3. Effect of auranofin against *C. difficile* toxin-mediated inflammation of gut epithelial cells. Human colorectal (Caco-2) epithelial cells were incubated with filtered *C. difficile* culture supernatant plus B. DMSO, 2.5%, C. vancomycin, 128 μg/mL, D. metronidazole, 128 μg/mL, E. fidaxomicin, 128 μg/mL, F. auranofin, 1 μg/mL or G. auranofin, 8 μg/mL for 24 h and observed under the microscope. Reference wells were not treated with *C. difficile* supernatant but still treated with 2.5% DMSO (panel A). Cell rounding is an indication of inflammation.

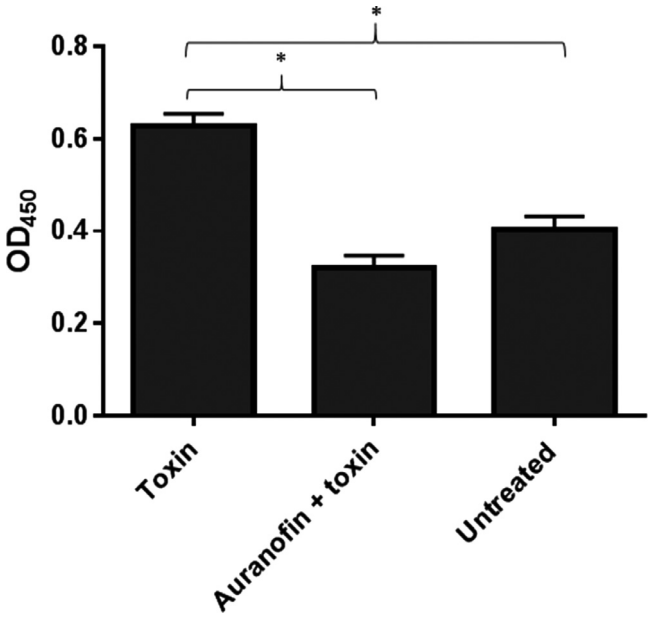

Fig. 4. Auranofin-mediated IL-8 inhibition from gut cells treated with *C. difficile* toxins. IL-8 level was assessed in Caco-2 cells treated with supernatant containing *C. difficile* toxins, with or without the addition of auranofin. OD_{450} coincides with the level of IL-8 in the cell supernatant. Error bars represent standard deviation values from triplicate samples used for each test agent. An asterisk (*) indicates significant difference ($P < 0.05$) between cells exposed to supernatant containing toxin alone and cells treated with supernatant containing auranofin (1 μg/mL) using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

ins are crucial for *C. difficile* to induce inflammation and to provoke disease. As a result, non-toxigenic bacteria are not associated with disease. Inhibition of toxin production may therefore contribute to effective treatment of CDI [17,33]. Additionally, toxin production occurs during the stationary phase of bacterial growth where antibiotics are not as effective. Furthermore, toxin production is increased by environmental stress; therefore, some anticlostridial drugs (e.g. vancomycin and metronidazole) induce *C. difficile* toxin production [34]. Auranofin was previously reported to inhibit protein synthesis, virulence factors and toxin production in *Staphylococcus aureus* [35,36]. Therefore, we tested the activity of auranofin at subinhibitory concentrations against a hypervirulent, toxigenic strain of *C. difficile*. Auranofin inhibited the total toxin production in *C. difficile*. A similar effect was observed with fidaxomicin but not with vancomycin or metronidazole. We also tested whether auranofin reduced *C. difficile* toxin-mediated inflammation of gut epithelial cells, given auranofin exhibits potent anti-inflammatory activity. Exposure of human colonic epithelial cells (Caco-2) to supernatant containing toxins produced by *C. difficile* induced cell rounding as reported previously [18]. Inclusion of a very low concentration of auranofin (1 μg/mL) with the *C. difficile* culture supernatant protected Caco-2 cells from the deleterious effect of *C. difficile* toxins (Fig. 3) and suppressed production of inflammatory cytokine IL-8.

In addition to toxin production, *C. difficile* utilizes spore formation as a key virulence factor in its pathogenesis. Hypervirulent and epidemic strains of *C. difficile* form spores that are resistant to standard disinfection procedures; therefore, these strains spread more efficiently throughout the environment. Furthermore, persistent *C. difficile* spores can germinate in the intestine after the conclusion of treatment, leading to relapse [33,37]. Auranofin was reported to inhibit several major pathways involved in protein biosynthesis in *S. aureus* [35]. Given sporulation requires the synthesis of spore coat proteins, we hypothesized auranofin would inhibit spore formation in *C. difficile* [38]. As anticipated, auranofin (at ½ × MIC) inhibited *C. difficile* spore formation; in contrast, neither vancomycin or metronidazole treatment was effective. The interference of spore formation by auranofin may translate into lower CDI recurrence rates.

Table 3
The minimum inhibitory concentration (MIC, μg/mL) of auranofin and control drugs against vancomycin-resistant *Enterococcus faecium* isolates.

Strain	Auranofin	Fidaxomicin	Vancomycin	Metronidazole	Linezolid
*Enterococcus faecium*HF50104 NR-32052	0.5	2	256	>256	0.125
*Enterococcus faecium*Patient #3-1 NR-31912	0.5	2	>256	>256	0.25
*Enterococcus faecium*NR-28978	0.5	2	128	>256	0.5
*Enterococcus faecium*ERV165 HM-970	0.25	1	256	>256	0.5
*Enterococcus faecium*ERV102HM-968	0.5	1	>256	>256	0.25

One complication of using vancomycin or metronidazole for treating CDI is the promotion of persistent colonization by VRE [39]; avoiding this problem is one goal for anticlostridial drug development [40]. When investigated against five isolates of VRE, auranofin (MIC 0.25–0.5 μg/mL) was superior to metronidazole, vancomycin, and fidaxomicin. Furthermore, auranofin has been reported to reduce VRE carriage and shedding in a mouse model of VRE colonization [20].

In conclusion, we report auranofin, an FDA-approved antirheumatic drug, has potent in vitro antibacterial activity against *C. difficile*, and inhibits both toxin production and spore formation. Furthermore, auranofin protects gut epithelial cells from the deleterious effect of *C. difficile* toxin-mediated inflammation. Additionally, auranofin has dual activity against *C. difficile* and VRE and should not promote VRE colonization. Further investigation is required to determine the activity of auranofin in animal models of CDI.

Declarations

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Competing Interests

There are not any Conflicts of Interest for all authors.

Ethical Approval

Not required

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