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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 due to the emergence of hypervirulent mutants armed with a higher capability of producing toxins and spores. This has resulted in an increased number of infections and death 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 which has recently been found to also possess antibacterial activity. Auranofin exhibited potent activity against *C. difficile* isolates inhibiting growth at a concentration of 1 µg/ml against 50% of all the tested isolates. Auranofin inhibited both toxin production and spore formation, a property that is 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. Overall, auranofin is a promising candidate that warrants further investigation as a treatment option for *C. difficile* infections.

Keywords

C. difficile infection; anti-toxin; spores formation; auranofin; antibacterial; repurposing

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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), afflicted nearly half a million patients in the United States alone and was associated with over 29,000 deaths in 2011 resulting in a direct cost that exceeds \$5 billion (U.S.) annually [2]. Though first discovered in the 1970s, the incidence and severity of CDI has sharply increased 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 production of higher levels of toxins (toxins A and B and binary toxin) [1, 4]. *C. difficile* toxins are the main virulence factor, and they are essential for the bacteria to cause disease. Furthermore, *C. difficile* forms very resistant spores that can persist in the environment for extensive periods of time. These spores serve as 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 with regards to reducing the 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 lesser extent fidaxomicin are starting to emerge worldwide [8, 9]. Altogether, there is a critical and imperative 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-1-thio- β -D-glycopyranp-sato-*S*-(triethyl-phosphine)-gold] is a gold-containing anti-inflammatory oral drug that has been used for treatment of rheumatoid arthritis for over 30 years. Auranofin's safety and pharmacokinetic profile in humans has been well-characterized, which has permitted investigation of auranofin for other clinical indications [13, 14]. The present study evaluates auranofin's potential to be repurposed as a novel anticlostridial drug to treat CDI.

In the current study, we report that auranofin possesses potent antibacterial activity against a wide panel of *C. difficile* strains. Additionally, auranofin is capable of inhibiting toxin production, spore formation and protects human gut cells against the inflammation induced by sterile *C. difficile* toxins. Taken all together, auranofin is a promising candidate to evaluate further 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 emulating 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 hours, at 37 °C. MICs reported represent the lowest concentration of each agent that suppressed the visual growth of bacteria. MIC₅₀ and MIC₉₀ 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 auranofin's ability to 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 μ L tubes. Drugs at the required concentrations were added to each tube, in triplicates, then tubes were incubated anaerobically (using BD GasPak™ EZ Container Systems) at 37 °C for six hours. One portion of each suspension was serially diluted, plated on BHIS agar and incubated anaerobically at 37 °C for 24 hours to detect the bacterial count. The second portion was centrifuged at 10,000 rpm for five minutes. 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 hours at 37 °C. The bacterial suspension was aliquoted into tubes and drugs were added (in triplicate) at concentrations equivalent to $\frac{1}{2} \times$ and $1 \times$ MIC. Tubes were then incubated anaerobically for five 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 minutes 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 hours, 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, $1 \times$ NEAA, 100 IU/mL penicillin, and 100 µg/mL streptomycin) for five days. Cells were then trypsinized and seeded on a 96-well plate and grown at 37° C + 5% CO₂. 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 anticrostrial drugs (vancomycin, metronidazole and fidaxomicin, 1 – 128 µg/mL). Drugs were incubated with cells for 24 hours at 37° C + 5% CO₂. 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.

In order 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 hours 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 broth microdilution assay 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 hours. 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 concentration of each drug that could inhibit the bacterial growth visually [20, 21].

2.8. Statistical analysis.

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

3. RESULTS

3.1. Antibacterial activity of auranofin against *C. difficile*.

Auranofin's anticlostridial activity was evaluated against a large panel of *C. difficile* strains including hypervirulent strains (ribotype 027). As shown in Table 1, auranofin inhibited growth of the 41 tested *C. difficile* strains at concentrations ranging from 0.25 – 4 µg/mL. Auranofin inhibited 50% of the tested isolates (MIC₅₀) at a concentration of 1 µg/mL and inhibited 90% of the isolates (MIC₉₀) 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 – 2 µg/mL (MIC₅₀ = 0.5 µg/mL and MIC₉₀ = 1 µg/mL). Metronidazole was active at a range of 0.06 – 0.25 µg/mL (MIC₅₀ = 0.025 µg/mL and MIC₉₀ = 0.25 µg/mL).

3.2. Auranofin inhibits *C. difficile* toxin production.

After confirming the potent *in vitro* anticlostridial activity of auranofin, we next moved to test 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, when compared to the untreated control. As depicted in Fig. 1, auranofin at 1/8, 1/4 and 1/2 × 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], was found to inhibit 37.2%, 50.1% and 52.3% of toxin production at 1/8, 1/4 and 1/2 × 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's 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). Auranofintreated bacteria displayed reduced spore count, ~1.5 log₁₀ at both 1/2 and 1 × MIC. While a similar effect was observed with fidaxomicin, almost no reduction in the spore count was detected with either vancomycin or metronidazole treatment.

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

Auranofin has been previously shown to have anti-inflammatory activity, an important factor for its use in the treatment of rheumatoid arthritis. We thus moved to investigate if auranofin would also have the ability to 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. 4, healthy Caco-2 cells display normal morphologic characteristics of enterocytes. Upon treatment with *C. difficile* toxins, cell rounding and detachment occurred [24]. The goal was to inspect if drug treatment will preserve the normal Caco-2 morphology, in the presence of *C. difficile* toxins. Auranofin (1 and 8 µg/mL) protected cells against the inflammatory effect 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) all 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 auranofin's antiinflammatory effect 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. 5). However, when cells were treated with toxins along with auranofin, no significant increase in IL-8 was observed. The OD₄₅₀ is represent to the IL-8 concentration present in the supernatants.

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

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

4. DISCUSSION

Clostridium difficile infections (CDIs) are increasing in morbidity and mortality within 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 was 20% and came at a direct cost of \$4.8 billion for acute care facilities alone. Moreover, approximately 29,000 deaths occurred within 30 days of the 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 damages the intestinal microbiota permitting *C. difficile* to expand, attach to epithelial cells and produce toxins. *C. difficile* toxins afflict the colonic

epithelium leading to loss of tight junctions, enhanced mucosal permeability and ultimately 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, about 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 there remains a 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 such as the safety profile, pharmacodynamics and pharmacokinetics of these drugs is known, which will undoubtedly reduce both 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, that has a well-defined safety profile with 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 auranofin's impact 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 Jackson-Rosario *et al.*'s 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, toxins are crucial for *C. difficile* to induce inflammation and to provoke disease. As a result, non-toxigenic bacteria are not associated with disease. So it is conjectured that an inhibition of toxin production will contribute to effective treatment of CDI [17, 33]. Additionally, toxin production occurs during 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 auranofin's ability to reduce *C. difficile* toxin-mediated inflammation of the 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* to induced cell rounding similar to that was 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. 4) and suppressed production of the 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* are associated with a higher ability to form spores that are resistant to standard disinfection procedures. This accounts for the ability of these strains to 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 *Staphylococcus aureus* [35]. Given sporulation requires the synthesis of spore coat proteins, we hypothesized auranofin would be able to inhibit spore formation in *C. difficile* [38]. As anticipated, auranofin (at $\frac{1}{2} \times \text{MIC}$) inhibited *C. difficile* spore formation; in contrast, neither vancomycin or metronidazole treatment was effective. Auranofin's ability to interfere with spore formation, may translate into lower recurrence rates of CDI.

One complication of using vancomycin or metronidazole for treating CDI is the promotion of persistent colonization by vancomycin-resistant enterococci (VRE) [39], and avoidance of this problem is one goal for anticlostridial drug development [40]. When investigated against five isolates of VRE, auranofin (MIC ranged from 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, possesses potent *in vitro* antibacterial activity against *C. difficile*, is capable of inhibiting both toxin production and spore formation. Furthermore, auranofin protected 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.

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Highlights

- Auranofin is a potent inhibitor of *Clostridium difficile* growth
- Auranofin, not vancomycin or metronidazole, blocked *C. difficile* toxin production
- Auranofin also inhibited *C. difficile* spore formation
- Auranofin protected gut cells against the *C. difficile* toxins' effect *in vitro*
- Auranofin halted vancomycin-resistant enterococci more than anticlostridial drugs

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 $1/2 \times \text{MIC}$ were incubated with a hypervirulent, toxigenic strain of *C. difficile* (strain ATCC BAA-1870). The bacterial counts were determined for each sample, and the 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 to the control antiostridial drugs, vancomycin and metronidazole.

Drugs (1/2× and 1 × 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. Auranofin's effect 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, fidaxomicin, 128 µg/mL, F. auranofin, 1 µg/mL or G. auranofin, 8 µg/mL for 24 hours 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. OD₄₅₀ 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 $\mu\text{g/mL}$) using one-way (ANOVA) followed by Dunnett's multiple comparisons test.

Table 1.

Bacterial strains used in this study.

Bacterial Strain	Alternate Designation	Source	Comments
<i>C. difficile</i> NAP07 (CDC#2007054)	HM-88	Isolated from human feces.	Reference genome for The Human Microbiome Project (HMP).
<i>C. difficile</i> P2	NR-32883	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
<i>C. difficile</i> P3	NR-32884	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.
<i>C. difficile</i> P4	NR-32889	Isolated from the fecal material of a human patient with a relapsing CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
<i>C. difficile</i> P5	NR-32885	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.
<i>C. difficile</i> P6	NR-32886	Isolated from the fecal material of a human patient with a relapsing CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
<i>C. difficile</i> P7	NR-32887	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.
<i>C. difficile</i> P8	NR-32888	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
<i>C. difficile</i> P13	NR-32891	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2005.	Toxigenic strain.
<i>C. difficile</i> P15	NR-32892	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2005.	Toxigenic strain.
<i>C. difficile</i> P19	NR-32895	It was obtained in 2005 from the fecal material of a human patient with a relapsing <i>C. difficile</i> infection in Western Pennsylvania, USA.	Toxigenic strain.
<i>C. difficile</i> P20	NR-32896	Isolated from the fecal material of a human patient with a relapsing <i>C. difficile</i> infection in Western Pennsylvania, USA in 2005.	Toxigenic strain.
<i>C. difficile</i> P21	NR-32897	Isolated from the fecal material of a human patient with a relapsing <i>C. difficile</i> infection in Western Pennsylvania, USA in 2005.	Toxigenic strain.
<i>C. difficile</i> P29	NR-32903	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2009.	Toxigenic strain and reported to be co-colonized with non-toxigenic <i>C. difficile</i> .
<i>C. difficile</i> P30	NR-32904	It was obtained in 2009 from the fecal material of an asymptomatic human patient in Western Pennsylvania, USA.	Non-toxigenic strain.
<i>C. difficile</i> Isolate 1	NR-13427	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--

Bacterial Strain	Alternate Designation	Source	Comments
<i>C. difficile</i> Isolate 2	NR-13428	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 4	NR-13430	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 5	NR-13431	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 6	NR-13432	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 7	NR-13433	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 9	NR-13435	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 10	NR-13436	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 11	NR-13437	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> Isolate 13	NR-13553	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
<i>C. difficile</i> 002-P50-2011	HM-746	Isolated from the stool of a patient with diarrhea in January 2011.	Reference genome for the Human Microbiome Project (HMP).
<i>C. difficile</i> ATCC 700057	VPI 11186	--	Toxinotype tcdA-, tcdB-, Ribotype 038, Nontoxigenic.
<i>C. difficile</i> ATCC 43598	1470	Human feces, asymptomatic neonate, Belgium	Presence of tcdB gene confirmed by PCR, Ribotype 017.
<i>C. difficile</i> ATCC BAA 1801	3232	Human feces (adult with diarrhea), Belgium.	Nontoxigenic, Ribotype 010.
<i>C. difficile</i> ATCC BAA 1870	4118	--	Presence of binary toxin, tcdA and tcdB genes. Toxinotype IIb, Ribotype 027.
<i>C. difficile</i> Isolate 20100207	NR-49278	Obtained from the stool of an elderly adult male patient with a healthcare-associated (HA) <i>C. difficile</i> infection in New York, USA, in 2010.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20100502	NR-49277	Obtained from the stool of an older adult male patient with a community-associated (CA) <i>C. difficile</i> infection in Colorado, USA, in 2010.	PCR ribotype 019, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ of the PaLoc ⁵ operon, as well as the CDT ⁶
<i>C. difficile</i> Isolate 20110052	NR-49281	Obtained from the stool of an elderly male patient with a healthcare-associated (HA) <i>C. difficile</i> infection in northeastern USA in 2010.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20110868	NR-49287	Obtained from the stool of an elderly female patient with a healthcare-associated (HA) <i>C. difficile</i> infection in southern USA, in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base

Bacterial Strain	Alternate Designation	Source	Comments
			pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20110870	NR-49288	Obtained from the stool of a young adult female patient with a healthcare-associated (HA) <i>C. difficile</i> infection in Tennessee, USA, in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20110979	NR-49285	Obtained from the stool of an elderly female patient with a community-associated (CA) <i>C. difficile</i> infection in midwestern USA in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20110999	NR-49286	Obtained from the stool of an elderly female patient with a healthcare-associated (HA) <i>C. difficile</i> infection in western/midwestern USA in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20120013	NR-49283	Obtained from the stool of a young male patient with a community-associated (CA) <i>C. difficile</i> infection in northeastern USA, in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20120184	NR-49289	Obtained from the stool of an elderly female patient with a fatal healthcare-associated (HA) <i>C. difficile</i> infection in Tennessee, USA, in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>C. difficile</i> Isolate 20120187	NR-49290	Obtained from the stool of an elderly male patient with a healthcare-associated (HA) <i>C. difficile</i> infection in Tennessee, USA, in 2011.	PCR ribotype 019, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ of the PaLoc ⁵ operon, as well as the CDT ⁶
<i>C. difficile</i> Isolate 20120236	NR-49291	Obtained from the stool of an older female patient with a community-associated (CA) <i>C. difficile</i> infection in midwestern, USA, in 2011.	PCR ribotype 027, NAP1 ^I , contains <i>tcdA</i> ² , <i>tcdB</i> ³ and <i>tcdC</i> ⁴ (with 18 base pair deletion) PaLoc ⁵ operon and the CDT ⁶
<i>E. faecium</i> HF50104	NR-32052	Isolated from swine feces in Michigan, USA in 2008.	Resistant to erythromycin, tetracycline and vancomycin.
<i>E. faecium</i> Patient #3-1	NR-31912	Isolated from the stool of a human patient having dominance of vancomycin-resistant <i>Enterococcus</i> in the stool but no bacteremia.	Vancomycin-resistant
<i>E. faecium</i> 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.
<i>E. faecium</i> ERV165	HM-970	Isolated from human feces in Colombia, in 2008.	Resistant to vancomycin.
<i>E. faecium</i> ERV102	HM-968	Isolated from human oral sputum in Colombia, in 2006.	Resistant to ampicillin, vancomycin and streptomycin.

^I NAP= North American pulsed-field gel electrophoresis type

² *tcdA*= *C. difficile* toxin A gene

³*tcdB*= *C. difficile* toxin B gene

⁴*tcdC*= Anti-sigma factor gene

⁵PaLoc= Pathogenicity locus

⁶CDT= *C. difficile* binary toxin.

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Table 2.

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

<i>C. difficile</i> 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

<i>C. difficile</i> Strain	ID number	Auranofin	Vancomycin	Metronidazole
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

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
<i>Enterococcus faecium</i> HF50104 NR-32052	0.5	2	256	>256	0.125
<i>Enterococcus faecium</i> Patient #3-1 NR-31912	0.5	2	>256	>256	0.25
<i>Enterococcus faecium</i> NR-28978	0.5	2	128	>256	0.5
<i>Enterococcus faecium</i> ERV165 HM-970	0.25	1	256	>256	0.5
<i>Enterococcus faecium</i> ERV102HM-968	0.5	1	>256	>256	0.25