DEVELOPMENT OF NEW ANTIBACTERIAL AGENTS FOR TREATMENT OF VANCOMYCIN-RESISTANT ENTEROCOCCI AND *CLOSTRIDIOIDES DIFFICILE* INFECTIONS

by

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Dedicated to the memory of my father who taught me many moral lessons on discipline and sense of responsibility from an earlier age. It is also dedicated to my big family (my mother, brothers and sisters) and my small family (Amira and Omar) for their endless love, support and encouragement.

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ANOVA	Analysis of Variances
ATCC	American Type Culture Collection
AZM	Acetazolamide
BEI resources	Biodefense and Emerging Infections Research Resources Repository
BHI	Brain heart infusion
BHIS	Supplemented brain heart infusion
C. difficile	*Clostridioides difficile*
CAs	Carbonic anhydrases
CAIs	Carbonic anhydrase inhibitors
CARB	Combating Antibiotic-Resistant Bacteria
CDC	Centers for Disease Control and Prevention
CDT	*C. difficile* transferase (binary toxin)
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CDI	*Clostridioides difficile* infection
d-Ala-d-Ala	D-alanine-D-alanine
d-Ala-d-Lac	D-alanine-D-lactate
d-Ala-d-Ser	D-alanine-D-serine
DIHQ	Diiodohydroxyquinoline
DMSO	Dimethyl sulfoxide
ESBL	Extended spectrum β-lactamase
E. faecium	*Enterococcus faecium*
E. faecalis	*Enterococcus faecalis*
ELISA	Enzyme linked immunosorbent assay
EZM	Ethoxzolamide
FDA	Food and Drug Administration
FIC	Fractional inhibitory concentration
FMT	Fecal microbiota transplant
GIT	Gastrointestinal tract
GSH	Glutathione
GTD	Glucosyl transferase domains
GTP	Guanosine triphosphate
HI	High inoculum
ICU	Intensive care units
IDSA	Infectious diseases society of America
IgG	Immunoglobulin G
LIN	Linezolid

LSR	Lipolysis-stimulated lipoprotein receptor
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MZM	Methazolamide
NAP	North American pulsotype
PACUC	Purdue University Animal Care and Use Committee
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RBCs	Red blood cells
REA	Restriction endonuclease analysis
ROS	Reactive oxygen species
S.C	Subcutaneous
SGF	Simulated gastric fluid
SHEA	Society for healthcare epidemiology of America
SI	Standard inoculum
SIF	Simulated intestinal fluid
SRFE	Sterile rat fecal extract
TcdA	*Clostridioides difficile* toxin A
TcdB	*Clostridioides difficile* toxin B
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UTI	Urinary tract infection
VRE	Vancomycin-resistant enterococci
VRE *faecalis*	Vancomycin-resistant *Enterococcus faecalis*
VRE *faecium*	Vancomycin-resistant *Enterococcus faecium*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

ABSTRACT

The discovery of penicillin marked the beginning of the golden era of antibiotics. Antibiotics have been the valuable weapons combating an array of bacterial infections. However, each year, millions of bacterial infections occur which lead to thousands of deaths. For instance, over three million infections were reported in the United States in 2019, that were caused by antibiotic-resistant pathogens and *Clostridioides difficile.* These infections resulted in more than 50,000 deaths. *Clostridioides difficile* and vancomycin-resistant enterococci (VRE) are listed as top-threat bacteria that urgently require prompt action to fight the infections caused by both of them. Both are highly prevalent in the healthcare settings, ranking as the first and second bacterial species causing nosocomial infections. The increased incidence and severity of diseases caused by both bacteria in addition to the dearth of effective anticlostridial and anti-VRE agents have created an urgent need for development of new therapeutic agents. The process of discovering new antibiotics is time-consuming and associated with high costs and risk. Repurposing FDA-approved drugs represents an attractive venture of antibacterial drug discovery. FDA-approved drugs possess well-studied safety, pharmacology and pharmacokinetics. Hence, drug repurposing saves time and costs, and reduces the risk associated with the de novo drug discovery. In the following studies, new drugs discovered utilizing the drug repurposing approach, were investigated *C. difficile* and VRE.

Against *C. difficile*, diiodohydroxyquinoline (DIHQ) and auranofin were investigated. DIHQ exhibited potent activity against *C. difficile* isolates inhibiting growth of 90% of these isolates at the concentrations of 2 µg/mL. It demonstrated superior activity to vancomycin and metronidazole, in its killing kinetics. Furthermore, it reacted synergistically with vancomycin and metronidazole against *C. difficile in vitro.* Moreover, at subinhibitory concentrations, DIHQ was superior to vancomycin and metronidazole in inhibiting two key virulence factors of *C. difficile*, toxin production and spore formation. Additionally, DIHQ did not inhibit growth of intestinal normal microbiota. In addition, the *in vivo* activity of auranofin was investigated in *C. difficile* infection (CDI) mouse and hamster model. It efficiently protected mice and hamsters against CDI. Remarkably, at clinically achieved concentrations, auranofin was capable of preventing CDI recurrence.

For VRE, three drugs where identified with potent inhibitory activities both *in vitro* and *in vivo*, auranofin, acetazolamide and dorzolamide. Auranofin's antibacterial activity was not affected when evaluated against a higher inoculum size of VRE and it successfully reduced the burden of stationary phase VRE cells. In addition, auranofin reduced VRE production of key virulence factors including proteases, lipase and hemagglutinin. In a lethal mouse model of VRE septicemia, auranofin-treated mice were protected from the lethal VRE challenge. Interestingly, auranofin successfully reduced VRE count below the limit of detection in murine internal organs after only four days of oral or subcutaneous treatment. In addition to auranofin, two carbonic anhydrase inhibitors, acetazolamide and dorzolamide, were investigated against VRE. Acetazolamide exhibited potent activity against a wide panel of different enterococcal strains. Moreover, it outperformed linezolid in two *in vivo* VRE mouse models; murine colonization-reduction and VRE septicemia. Additionally, dorzolamide exhibited potent activity against VRE isolates. Remarkably, in combination with gentamicin, dorzolamide interacted synergistically reducing gentamicin's MICs by several folds. Moreover, dorzolamide significantly reduced the VRE burden in mice fecal samples by 99.9% after 3 days of treatment. Furthermore, dorzolamide significantly surpassed linezolid in reducing the VRE count in the cecal and ileal contents of mice.

Collectively, several drugs exhibited promising activities against *C. difficile* and VRE, *in vitro* and *in vivo*. The results presented suggest that these drugs merit further evaluation as potential candidates for treatment of infections caused by either bacterium.

CHAPTER 1. INTRODUCTION

1.1 Bacterial resistance poses a terrific global public health threat

One of the most influential scientific advances in the modern human history is the development of antibiotics. Prior to the discovery of penicillin, bacterial infections of trivial injuries resulted into deaths. After the discovery of penicillin, the search for additional antibiotics was triggered and ultimately the pharmaceutical industry was initiated [1].

Since the discovery of antibiotics, people have become heavily reliant on them and they were massively consumed. It was reported that 258 million antibiotics courses were prescribed in the United States in 2010 (over 8 prescriptions for every 10 persons, on average) [2]. Furthermore, the antibiotics produced each year for healthcare, veterinary and agricultural applications are estimated to be over 100,000 tons [3]. However, regrettably, the ubiquity of antibiotic use has led to a constant pressure over bacteria which in turn, stimulated the bacterial development of resistance to our antibiotics. A study by the Centers for Disease Control and Prevention (CDC) in 2016, reported that approximately 30% of the 154 million prescriptions administered each year in doctors' offices and emergency departments are unnecessary [4]. Moreover, the misuse of antibiotics as growth promotors for animals has further exacerbated the progression of bacterial resistance [5]. Over time, different bacterial resistance mechanisms have evolved, and bacterial resistance is now widely spread throughout both pathogenic and non-pathogenic species.

Antibiotic resistance could be referred to as "the silent Tsunami facing modern medicine". This is because a great deal of antibiotics that were once considered very efficacious, have completely or partially lost their activity against bacteria due to the development of resistance. According to the most recent report released by the CDC, more than 2.8 million antibiotic-resistant infections occur in the United States each year, and more than 35,000 people die as a result [6]. Because of bacterial infections with antibiotic resistant pathogens or *Clostridioides difficile*, about 48,700 families lose a loved one each year [6]. Thus, the CDC's report stated that "the post-antibiotic era is already here". The overall deaths from antibiotics resistance significantly decreased by 18% and deaths from antibiotic resistance in hospitals decreased by 28% since the previous report released in 2013 [7]. However, the number of people facing antibiotic resistance in the United States is still too high and infections caused by some bacterial pathogens such as

erythromycin-resistant invasive group A streptococci, drug-resistant *Neisseria gonorrhoeae*, and extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae demonstrated increases by 315%, 124%, and 50%, respectively since the previous report released in 2013 [6]. Therefore, CDC strongly recommended adopting more effective measures and more aggressive strategies to prevent the spread of bacterial resistance such as containing emerging threats through early detection and aggressive response, stopping the spread of resistant germs within and between healthcare facilities and tracking and improving appropriate antibiotic use. Besides, a study commissioned by the United Kingdom, projected that the antimicrobial resistant infections are estimated to result in more than 700,000 deaths worldwide every year. Further, based upon the current rate of resistance development, antibiotic resistant infections will be responsible for over 10 million deaths annually by 2050 [8]. Thus, based on these predicted mortality rates, antibiotics resistant infections could soon be competing cancer as one of the leading causes of death in the world [9].

Owing to the skyrocketing rise in the bacterial resistance and the increasing occurrence of untreatable infections, several policies to limit the administration of antibiotics and to reduce the unnecessary use of them, are being adopted. For instance, the European Union banned the use of all antibiotics as growth promoters in animal feed [10, 11] as well as strict regulations were implemented by the Food and Drug Administration (FDA) to reduce the unnecessary use of antibiotics in agriculture [12]. Furthermore, the National Action Plan for Combating Antibiotic-Resistant Bacteria (CARB) was released by the White House which indicated specific goals to reduce the excessive use of antibiotics besides expediting the development new antimicrobial therapies [13-15].

Despite the numerous efforts to overcome the increasing bacterial resistance and expedite the development of new antimicrobial therapeutics, there remains a dire need for developing new antimicrobial therapeutics. The discovery and approval of new antibacterials had been on a steady decline since 1990s [16, 17]. This long-term dry pipeline of antibiotic development is partly due to that the economic incentive for pharmaceutical companies to develop new antimicrobials is very low as compared to other drugs [17]. For instance, the net value of an antimicrobial agent was estimated to be about $50 million whereas that of an agent for treatment of a neuromuscular disease approximates $1 billion [18]. Consequently, antimicrobial agents are not ranked among the top selling drugs in the market [19]. Another main issue which discourage pharmaceutical companies

from investing in developing new antimicrobials is the rapid rate of developing resistance. For instance, linezolid was introduced into the market in 2000 and linezolid-resistant *Staphylococcus aureus* was isolated within one year only (2001) [7, 20, 21]. The macrolide antibiotic, levofloxacin was introduced to the market in 1996 and levofloxacin-resistant *Streptococcus pneumoniae* cases were reported in the same year [7, 22]. Ceftaroline, a newly discovered antibiotic (in 2010) belonging to the fifth generation cephalosporins that has been successfully used in treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia and infective endocarditis, encountered development of resistance after one year only from its introduction to the market [7, 23].

Due to the aforementioned reasons, antibacterial drug development has become a global priority. The time is ripe for the development of new antimicrobial therapeutics for bacterial infections. Without the immediate exploration and development of novel antibiotic therapies, a plethora of bacterial infections will be inevitably left without treatments and the world may return back to the era before the discovery of antibiotics where simple infections led to deaths.

In this thesis, two malicious infective bacteria were targeted; *Clostridioides difficile* and vancomycin-resistant enterococci (VRE). *C. difficile* is listed by the CDC as an urgent threat bacterium whereas VRE are considered as serious threat bacteria [15, 24]. Both bacteria urgently require the development of novel effective therapeutic agents against them. Both *C. difficile* and VRE are gut-associated pathogens. Gastrointestinal tract (GIT) is the site of infection for *C. difficile* while for VRE, GIT is a transient colonization site serving as an origination point for these bacteria to spread in the body and cause life-threatening infections. Furthermore, both bacteria remain in the gut controlled by the population of normal gut microbiota, which is known as colonization resistance. Dysbiosis (disturbance of the intestinal microbiome composition) can lead to colonization of the gut by *C. difficile*/VRE. In addition, both are a leading cause of healthcare-associated infections. Hospitalized patients are considered a higher risk group for infection by these bacteria due to their underlying conditions, including immunocompromised conditions, advanced age, receiving medication like chemotherapeutic agents, and undergoing surgical procedures. It was reported that patients who acquired infections during their hospital stay, remained in the hospital for prolonged times (2.5 times longer) resulting in about 3 times higher costs than the uninfected patients [25].

1.2 *Clostridioides difficile*

1.2.1 *Clostridioides difficile* epidemiology and statistics

Clostridioides difficile is a Gram-positive, spore forming, anaerobic, pathogenic bacterium [26]. It was first isolated from the faeces of healthy neonates in 1935 [27, 28]. Forty years later, *C. difficile* was first recognized as a human pathogen that caused diarrhea and pseudomembranous colitis in 1978 [29]. Nowadays, *C. difficile* infection (CDI) is the leading cause of hospital acquired infections and healthcare associated diarrhea [30]. CDI is listed, by the CDC, as an urgent threat, that requires immediate and rigorous action due to its capability of causing widespread and difficult to treat disease [6, 7]. In 2017 CDI caused 223,900 hospitalization and resulted in over 12,800 deaths with an excess of $1 billion attributable healthcare costs [6, 31]. This means that one person loses his life every 40 minutes due to CDI [32, 33]. CDI symptoms range from mild, watery diarrhea to more severe pseudomembranous colitis or pseudomembranous enterocolitis [28, 34, 35]. Other symptoms of CDI include loss of appetite, nausea, fever, leukocytosis and abdominal cramping. Chronic diarrhea results in serious complications such as dehydration, hypotension, electrolyte disruption, and renal failure. Moreover, complications of CDI are life threatening and include colonic ileus, toxic megacolon, intestinal perforation, sepsis, shock, and systemic inflammatory response syndrome [28, 34, 35].

The incidence of CDI has been steadily increasing since 2000. In the United States, the number of CDI related hospitalizations has tripled to reach about 450,000 cases annually in 2013 [36, 37]. Further, there has been an increase in the severity of the disease associated with CDI and recurrence over the last two decades, resulting in prolonged hospital stays, and increase in number of patients admitted to the intensive care units (ICU) [38-42]. Moreover, as a result of the increased incidence and severity of CDI, the mortality rate out of CDI increased which in turn, has led to the increased need to more emergency interventions like colectomies [43-46]. In addition, although CDIs are traditionally considered a healthcare-acquired infection with about 50% of the infections are reported in the healthcare settings, community-onset CDIs are on the rise, which represent about 41% of CDI cases [47]. CDI is being increasingly recognized in the community, in younger individuals, and in patients lacking the CDI traditional risk factors, such as hospitalization, age, and antibiotic exposure [34, 48-51].

Recently, the emergence of hypervirulent *C. difficile* strains has led to increase in occurrence and severity of CDI and CDI has suddenly become one of the most violent nosocomial pathogens. Examples of hypervirulent *C. difficile* strains include North American pulsotype 1 (NAP1), polymerase chain reaction (PCR)-ribotype 027, PCR-ribotype 078, and restriction endonuclease analysis (REA) group BI 8. A hypervirulent *C. difficile* strain was first encountered in Quebec province, Canada in 2002 [52]. Ribotype 027 *C. difficile* has been involved in many hospital outbreaks in Canada, the United States, Asia, Europe and Australia [28, 53, 54]. Ribotype 078 is zoonotic-associated hypervirulent *C. difficile*, which is commonly found in neonatal pigs [55]. The hypervirulent strains have a greater ability to secrete increased levels of toxins for due to a defect in the expression of a regulatory sigma factor responsible for TcdA and TcdB downregulation [56]. Consequently, this increase in toxin production contributes to the increased virulence of these hypervirulent strains, leading to more severe disease, a higher rate of treatment failure and increased mortality [53, 54].

1.2.2 *Clostridioides difficile* infection and colonization of the gut

CDI is facilitated by the ingestion of the metabolically dormant spores. Once ingested, the spores pass through the stomach to the intestine where, they begin the germination process into vegetative cells, under favorable conditions and upon detecting the presence of bile acids like taurocholates [57-59]. By the start of the germination process, the germinating spore dedicates all its energy and resources to germinate into a vegetative cell many genes are upregulated such as genes of ABC-transporters, DNA replication, cell division, stress response, ribosomal proteins, RNA polymerase, and toxin production [59, 60]. After germination, and in the presence of imbalanced gut microflora, i.e. by a broad-spectrum antibiotic use for instance, the vegetative *C. difficile* can colonize gut mucosa, and proliferate producing more spores and the toxins and establish a CDI [61, 62].

C. difficile toxins, TcdA and TcdB in particular, are the primary virulence factors of *C. difficile* and the direct cause the disease pathology [62]. TcdA binds to glycoprotein 96, a member of the heat shock protein family that is expressed in the cytoplasm of human colonocytes. TcdB binds to chondroitin sulphate proteoglycan 4, that is highly expressed throughout the intestine [63]. Upon binding to their respective cell receptors, the toxins initiate clathrin-dependent endocytosis,

which results in the release of the glucosyl transferase domains (GTD). The GTD acts on the guanosine triphosphate (GTP)-binding proteins such as Rho, Rac and Cdc42. GTD glycosylates Rho GTPases in the colonic epithelial cells leading to their inactivation. GTPases inactivation results in disorders in cell signaling, actin cytoskeleton disruption, opening of tight junctions between cells, cell rounding, apoptosis, fluid accumulation, inflammation, and intestinal injury [63, 64]. TcdB has been found to be 10 times more potent than TcdA and it is essential for virulence [63].

In addition to production of TcdA and TcdB, approximately 10% of *C. difficile* strains produce a third binary toxin; *C. difficile* transferase (CDT), which belongs to the binary actin-ADP-ribosylating toxins. CDT binds to its cell-surface receptor that is lipolysis-stimulated lipoprotein receptor (LSR), which is highly expressed in the intestine. After binding to its receptor, CDT induces actin modification, inhibiting polymerization, which in turn, leads to a decrease in the cortical actin cytoskeleton and results in the formation of microtubule protrusions which extend from the cell surface. These protruding microtubules form a dense extracellular network which enhances bacterial adhesion to the cells and facilitates *C. difficile* colonization [65-68].

Several risk factors are linked with CDI. The most common risk factor is the broad-spectrum antibiotic use, which is associated with nearly all cases of CDI. It was reported that most CDI patients have received antibiotics within the three months prior to the onset of disease symptoms [28]. Most antibiotics, particularly broad-spectrum ones, have been implicated in CDI. Yet, clindamycin, cephalosporins, and fluoroquinolones like moxifloxacin posed the greatest risk [53]. Another risk factor for CDI is aging. The chance for elderly people to contract CDI is higher than other categories due to their decreased humoral immunity. Additionally, women (especially elderly women) are more prone to that disease than men mainly because of the increased rate of urinary tract infections (UTIs) in women than men which leads to increased antibiotic prescribing for them [69]. Many other risk factors are associated with contracting CDI such as immunosuppressive therapy, chemotherapy, inflammatory bowel disease, Crohn's disease, long hospital stays, admission to ICU, recent gastrointestinal surgical procedures, nasogastric tubes, gastric acid suppressors, renal impairment, contact with CDI patients, prophylactic use of antibiotics and the use of non-steroidal anti-inflammatory agents [34, 62, 70].

1.2.3 Recurrent CDI

The high economic burden of CDI is largely due to the recurrent CDI [71]. Recurrent (relapsing) CDI is the disease which initially responded to the antibiotic therapy, but it recurs after completion of the treatment course. *C. difficile* recurrence occurs because of presence of residual spores in the GIT that are resistant to the antibiotic therapy. Once treatment is discontinued, these spores will germinate into vegetative cells, thereby, secrete toxins and establish CDI. Relapsing CDI can also occur if the initial treatment merely suppressed the infection rather than clearing it. Thus, after the end of treatment course, intestinal dysbiosis induced by the antibiotic treatment course would facilitate the re-infection. Recurrent CDI, in most cases, is very serious and difficult to treat, often requiring prolonged treatment with antibiotics [72]. It was discussed that after the initial infection, 15-30% of patients encounter recurrent CDI. Most worrisome, the patient's risk of recurrent CDI increases with each episode of recurrence. After the initial episode (CDI), the risk of recurrence is approximately 20%. After the first CDI recurrence, the risk getting recurrence increases to 40%, and then increases to 60% after two or more recurrences [71-73].

1.2.4 Current and emerging therapeutics

1.2.4.1 Antibiotics

Currently, only three antibiotics are routinely used for CDI treatment which are metronidazole, vancomycin, and fidaxomicin. Metronidazole is a broad-spectrum nitroimidazole antibiotic which targets DNA and inhibits cellular enzymatic functions [74]. Metronidazole is recommended only for non-severe CDI, or when patients cannot obtain or be treated with vancomycin or fidaxomicin, as it is inexpensive [73]. It cannot be used to treat chronic infections as long-term use could result in cumulative neurotoxicity. Metronidazole is highly absorbed so only low concentrations are reached in the GIT [75, 76]. It does not inhibit *C. difficile* toxin production and spores formation and even it has been shown to increase toxin production and sporulation at subinhibitory concentrations [77]. Moreover, it possesses inhibitory activity against gut normal flora. As a result, metronidazole treatment is associated with high treatment failure and relapse [28, 78]. Further, metronidazole treatments have a risk of promoting the overgrowth of vancomycin resistant bacteria like vancomycin-resistant enterococci (VRE) [79].

Vancomycin is a broad-spectrum glycopeptide antibiotic that acts through inhibition of the later stages of peptidoglycan biosynthesis [80]. Vancomycin is recommended for severe CDI as it has superior efficacy, good pharmacokinetics (poorly absorbed) and less side effects, than metronidazole [75, 81]. However, like metronidazole, vancomycin does not inhibit *C. difficile* toxin production and spores formation and even it has been shown to increase toxin production and sporulation at subinhibitory concentrations [77, 82]. Moreover, it possesses inhibitory activity against gut normal flora. As a result, vancomycin treatment is associated with high treatment failure and relapse [28, 78]. Further, vancomycin use does increase the risk of acquiring vancomycin-resistant organisms like VRE [79].

Fidaxomicin is a macrocyclic class antibiotic that inhibits DNA transcription by targeting the bacterial RNA polymerase [83, 84]. Fidaxomicin has a better profile than vancomycin and metronidazole regarding the oral bioavailability and specificity. It is minimally absorbed from the GIT and has limited activity against the normal gut flora [83, 85, 86]. It was reported also to be able to prevent spore germination and toxin production, thereby reducing the rate of recurrence [81, 87]. However, the clinical outcome is still insufficient regarding the risk of recurrence when infected with hypervirulent *C. difficile* strains like ribotype 027 [83, 88]. In addition, the expensive cost of fidaxomicin's treatment limits its use [89, 90].

The high treatment failure rates and the emerging resistance or reduced susceptibility to these anticlostridial antibiotics [89, 91] are causes for concern and highlight the need for new effective therapies.

There are a number of emerging antibiotics that were investigated for their effectiveness against *C. difficile* both *in vitro* and *in* vivo, such as tigecycline [77], rifaximin [92], surotomycin [93, 94], ridinilazole [76, 95], and LFF571 [96]. Many of these drugs are currently undergoing clinical and pre-clinical trials as promising treatment options for CDI, but unfortunately, as for today none of them has proved effectiveness enough to be approved by the FDA.

1.2.4.2 Monoclonal antibodies and vaccines

The immune response to TcdA and TcdB has been shown to play a significant role in CDI severity and the risk of relapse. It was reported that in presence of high serum immunoglobulin G (IgG) to TcdB, the cytotoxic activity of TcdB was neutralized and CDI was recovered without

relapse [97]. This raised the interest in the development of immune based therapies primarily aiming at preventing the recurrent *C. difficile*. Toxin neutralizing antibodies against TcdA and TcdB have been recently demonstrated to enhance the outcome of antibiotic treatments regarding recurrence and mortality. Bezlotoxumab, a monoclonal antibody neutralizing TcdB was recently approved by the FDA for the treatment of severe cases of CDI especially in elderly patients at high risk of recurrence [98]. Monoclonal antibody therapeutics are currently undergoing clinical and pre-clinical trials. A fully humanized monoclonal antibody cocktail against TcdA and TcdB demonstrated strong toxins' neutralizing activity both *in vitro* and *in vivo* [97]. Additionally, a combination of two monoclonal antibodies actoxumab (anti-TcdA) and bezlotoxumab (anti-TcdB), was investigated in Phase III clinical trials. This combination effectively reduced the rate of recurrence in Phase II clinical trials by 73% among CDI patients [99]. Despite the efficacy of monoclonal antibodies as a treatment option for CDI, the antibody cannot be used alone in treatment and preferred to be coupled with an antibiotic to kill the bacteria. Another drawback is that these antibodies are highly expensive and cannot be afforded by a great majority of patients.

Although anti-CDI vaccines are limited in terms of treating CDI due to the time required for the patient's immune system to respond, they still have a great potential for CDI prevention. Several anti-CDI vaccines were developed and being investigated in clinical trials exhibiting a good premise, such as a *C. difficile* toxoid vaccine developed by Sheldon et al. that was explored in Phase I clinical trials [99], and VLA84 vaccine in Phase I clinical trials [100].

1.2.4.3 Fecal microbiota transplant

Intestinal microbiome is the main host defense against CDI. Imbalanced microbiome composition could end up into CDI. Thus, restoration of gut normal bacteria could be employed to fight against CDI. Fecal microbiota transplant (FMT) is done by implanting fecal material into the distal colon of the GIT. FMT functions to restore the intestinal microbiota to the healthy state, and thereby restoring the colonization resistance. FMT has been proven to be effective at resolving CDI, with success rates of up to 90% [101, 102]. However, one of the biggest risks associated with FMT is the possibility of introduction of pathogenic microorganism to the patient, especially in highly susceptible patients such as elderly and immunocompromised patients [103]. Thus, donors need to be screened for the pathogenic microorganisms. Yet, the screening process is expensive,

delays treatment and with that there still remains a risk of transmitting pathogens to the patients [101]. Moreover, there are some other restrictions regarding the donors such as they should have taken no antibiotics, 3 months before FMT, have no gastrointestinal symptoms, no immune disorders or other diseases. Furthermore, there is a lack of standardization of FMT protocols. Delivery of the fecal material can be via colonoscopy, nasogastric tube, retention enema or orally delivered encapsulated stool. Different methods of delivery have different levels of efficacy, and associated risks [101, 104]. Due to the risks of pathogenic infection, strict donor screening requirements, invasiveness of the procedure (colonoscopy and nasogastric tube) as well as the perceived unpleasantness, feelings of embarrassment and discomfort associated with FMT, this venture is typically reserved as a treatment for severe recurrent or refractory CDI, rather than as routine therapy [105].

1.3 Vancomycin-resistant Enterococci (VRE)

1.3.1 Emergence of VRE as a public health threat

VRE infections ensued as serious nosocomial infections owing to the propensity for VRE colonization of GIT, the ability of VRE to persist in hospital environments, increased mortality, in adition to presence of multiple resistance mechanisms [106]. Vancomycin-resistant *Enterococcus faecium* (VRE *faecium*) clinical isolate was first isolated in England and France in 1986. One-year later, VRE *faecalis* isolate was reported in the United States [107, 108]. The rise of VRE in Europe, was thought to be as a result of transmission of the vancomucin-resistant bacteria from animal food products to humans (i.e. community-acquired), where the glycopeptide antibiotic, avoparcin, was extensively used as a growth promoter in livestock [109]. On the other hand, VRE were believed to be predominated in the United States due to the increasing use of vancomycin (i.e. healthcare-associated) [110]. Thereafter, VRE rapidly spread in the United States and Europe in the 1990s, and the 2000s, respectively, and eventually a worldwide spread emerged [111]. Further, a patient case of transmission of vanA resistance genes from VRE to MRSA in 2002, resulting in the first appearance of vancomycin-resistant *Staphylococcus aureus* (VRSA), was reported. Thus, the threat of VRE infections in the healthcare and community settings highly increased [112]. Among about 54 species of enterococci, *E. faecalis* and *E. faecium* are considered the most clinically relevant species. *E. faecalis* is more common and pathogenic than *E. faecium*. However,

E. faecium exhibits more resistance to vancomycin, composing the majority of VRE infections [113].

1.3.2 Epidemiology and statistics of VRE

VRE are categogorized by the the CDC as a serious public health threat, that requires require prompt and sustained action due to its capability of causing widespread and difficult to treat infections. In 2017, VRE were responsible for around 55,000 hospitalization cases and resulted in about 5,700 deaths with about $0.6 billion attributable healthcare costs, in the United States. VRE infections account for over the third of enterococcal infections. Enterococci are the second leading causes of hospital-acquired infections [114].

Enterococci are considered normal flora in humans, animals, birds and insects [115]. In humans, whilst enterococci are most abundant in the GIT, they are normal inhabitants of the oral cavity, genital tract and biliary system [116]. Under dysbiosis conditions, resident enterococci can act as opportunistic pathogens and infect their host. They can also cause nosocomial infections. VRE nosocomial infections emante via person-to-person contact or exposure to contaminated objects. Suppresion of microbiota through antimicrobial treatment allow for VRE overgrowth, as VRE are intrinsically resistant to several antibiotics. Thereby, VRE can flourish, colonize the GIT and cause the clinical illness [117]. GIT is the major site for VRE colonization, with *E. faecalis* as the most common colonizer. However, VRE, to a lesser extent, can also colonize on the skin, in the genitourinary tract, and in the oral cavity [117]. Once VRE colonize the GIT, it often persist for long period of time. Unfortunately, efforts of VRE decolonization are typically transitory with limited success. No effective drug is currently approved for enterococcal decolonization. Several drugs have been investigated as VRE decolonizing agents, but they showed poor outcomes and encounter recurrence of VRE, days or weeks later [118, 119].

Risk factors associated with VRE infections include host characteristics such as immunosupression, malignancies, organ transplants, prolonged hospital stays, and ICU stays, proximity to another colonized or infected patient, and serious comorbid conditions such as diabetes and renal failure [120]. Another main risk factor is exposure to antimicrobials, especially broad-spectrum ones. An increased risk of VRE colonization occurs with prior exposure to antimicrobials such as vancomycin, aminoglycosides, cephalosporins, clindamycin, metronidazole, and carbapenems [121].

1.3.3 VRE infections

1.3.3.1 Bacteremia

Enterococcal Bacteremia is more common than enterococcal endocarditis, especially in debilitated patients receiving antibiotics [122]. Enterococci are the second most common cause of bacteremia that accounts for 18% of all central line associated bloodstream infections, in the United States [114]. Enterococci can translocate from the GIT and genitourinary tract leading to many bacteraemias with no identifiable source [123]. Additionally, most cases of enterococcal nosocomial bacteremias are commonly acquired from identifiable sources such as intravascular or urinary catheters, abscesses, UTIs, and contaminated hospital equipment [124]. VRE bacteremia is associated with a 2.5-fold increase in mortality as compared to vancomycin-sensitive Enterococci (VSE) bacteremia [125].

1.3.3.2 Endocarditis

Enterococci are the second most common cause of infective endocarditis, which are responsible for 5%–20% of cases [126]. *E. faecalis* is a more common cause of endocarditis than *E. faecium* [127]. It was discovered that endocarditis induced by VRE *faecalis* is associated with mitral valve infections, whereas that caused by VRE *faecium* is associated with tricuspid valve infection [127]. Moreover, other enterococcal species can also cause this disease such as *E. avium, E. casseliflavus, E. durans, E. gallinarum,* and *E. raffinosus* [114].

1.3.3.3 Urinary tract infections (UTIs)

VRE are a major cause of healthcare-associated UTIs. Enterococci are second cause of all catheter-associated UTIs in the United States, which are responsible for about 15% of such infections [114]. The increase in enterococcal UTIs could be attributed to increasing use of urethral catheters and broad-spectrum antibiotic therapy like cephalosporins [128]. Enterococcal UTIs are more common in men and are usually associated with previous antibiotic treatment, indwelling catheters, and abnormalities of the genitourinary tract. The bladder, kidney and prostate are commonly affected infection sites [129].

1.3.3.4 Intra-abdominal and pelvic infections

Enterococci are commensals of the GIT, so they are commonly isolated from pelvic and intra-abdominal infections, usually along with Gram-negative and anaerobic organisms [130]. Entercocci alone can rarely cause intra-abdominal infections or soft tissue infections, and often they are present with other microorganisms [131]. Enterococci were reported in some cases of peritonitis infections, especially with patients undergoing chronic peritoneal dialysis or suffering from liver cirrhosis [132].

1.3.3.5 Other enterococcal infections

Enterococci have been associated with skin infections, usually as a part of polymicrobial infections. They were isolated from decubitus and diabetic foot ulcers [130]. Additionally, enterococci are known to infect other sites, such as the central nervous system, lungs, ears and eyes, although these infections occur less frequently [129]. Enterococcal central nervous system infections occur in older patients with serious underlying diseases, such as malignancies, pulmonary diseases, and cardiac diseases. VRE *faecium* is a more common cause for these infections (82%) compared to VRE *faecalis* (5%) [133].

1.3.4 Antibiotic resistance of enterococci

The rapid emergence of multi-drug resistance in enterococci in the last decades, has certified them as major nosocomial pathogens. Besides their intrinsica resistance to many antimicrobial agents, Enterococci are incredibly efficient in acquiring antibiotic resistance determinants through plasmids and transposons transfer and genetic mutation [134]. The mechanisms of enterococcal resistance to several classes of antibiotics are summarized below.

1.3.4.1 β-lactam antibiotics

Enterococci are intrinsically resistant to β-lactams through two mechanisms, the production of low-affinity penicillin binding protein 5 (PBP5), or the production of β-lactamases [135]. Therefore, β-lactams, which are known to be bactericidal, exert a bacteriostatic activity against most enterococci. Thus, for infections requiring the bactericidal activity such as endocarditis or septicemia, a combination of a β-lactam with an aminoglycoside is recommended

[135]. Overproduction of low- affinity PBP5 is more common in *E. faecium* than *E. faecalis*. Thus, most VRE *faecium* strains in the United States are highly resistant to ampicillin, while most VRE *faecalis* strains are susceptible to it [136]. Furthermore, enterococci can acquire resistance to β-lactams via the acquisition of transferable plasmids encoding for β-lactamase, that hydrolyzes and inactivates β-lactams [132]. In addition, resistance to β-lactams can be also mediated via superoxide dismutase which alleviates oxidative stress induced by β-lactams and prevents apoptosis [137].

1.3.4.2 Aminoglycosides

Low level resistance of enterococci to aminoglycosides are mediated by the decreased cellular permeability of these antibiotics,. Combined with β-lactams, aminoglycosides can overcome the decreased uptake as β-lactams increase the entry of them into the cell. High level resistance to aminoglycosides can be acquired via two mechanisms: 1) modification of the ribosomal attachments sites through methylation of 16S rRNA by enterococcal methyltransferase [137, 138], and 2) the production of aminoglycoside-modifying enzymes such as 6'-acetyltransferase and 2"-phosphotransferase-6'-acetlytransferase [138].

1.3.4.3 Glycopeptides

Glycopeptides (e.g. vancomycin and teicoplanin), exert their antibacterial activity by binding with high affinity to the D-alanine-D-alanine (d-Ala-d-Ala) terminal of peptidoglycan precursors leading to inhibition of cell wall biosynthesis. Resistance to glycopeptides is mediated through the modification of the terminal (d-Ala-d-Ala) into low-affinity precursors such as D-alanine-D-lactate (d-Ala-d-Lac) or D-alanine-D-serine (d-Ala-d-Ser) [139]. Nine operons that are responsible for developing this resistance, have been identified (VanA, VanB, VanC, VanD, VanE, VanG, VanL, VanM and VanN). VanA, VanB, VanD and VanM change the precursor to d-Ala-d-Lac, resulting in a 1,000-fold decrease in affinity for vancomycin. VanC, VanE, VanG, VanL, and VanN change the precursor to d-Ala-d-Ser, resulting in a 7-fold decrease in affinity for vancomycin [140]. VanA is the most common resistance operon and it is responsible for most of the human cases of VRE around the world. It can be transferred via plasmids to other cells confering glycopeptide resistance. VanA encodes for 7 enzymes; (VanA, VanH, VanX, VanY,

VanR, VanS and VanZ). VanA and VanH are responsible for the assembly of the modified dipeptide. VanX and VanY hydrolyze the original dipeptide. VanR and VanS have regulatory function, while VanZ supports resistance through an unclear mechanism [137, 141].

1.3.4.4 Other antibiotics

Resistance to sulfamethoxazole/trimethoprim is mediated through the ability of enterococci to utilize exogenous folate [132]. In addition, the expression of *lsa* gene encoding for efflux pumps in *E. faecalis* is responsible for natural resistance to clindamycin and streptogramins (quinupristin/dalfopristin). Resistance to quinupristin/dalfopristin can be mediated through target alteration [137]. Methylation of the 23S rRNA of the 50S ribosomal subunit, encoded by *ermB* gene, results in resistance to macrolides and lincosamides while its methylation mediated by *cfr* gene results in oxazolidinones (e.g. linezolid) resistance [141]. Further, resistance to oxazolidinones (e.g. linezolid) can also be triggered via mutation in the genes encoding 23S rRNA or ribosomal proteins [138].

1.3.5 Therapeutic options for treatment of VRE infections

1.3.5.1 Oxazolidinones (linezolid)

It is the only drug approved by FDA for the treatment of VRE infections. Although resistance to linezolid is rare, it has been documented in several cases [142]. The treatment outcomes of linezolid in systemic VRE infections such as bacteremia and endocarditis are not satisfactory and treatment failures have been reported [143]. Moreover, linezolid showed limited activity in VRE decolonization owing to its rapid absorption from the GIT, its low concentration in the stool, or its limited activity against a high bacterial inoculum (~10^8 CFU) [144, 145]. Additionally, linezolid's treatment is associated with serious side effects including bone marrow toxicity and neurotoxicity [146].

1.3.5.2 β-lactams/aminoglycosides combination

This combination is utilized if bactericidal activity is required as in case of treatment of bacteremia and endocarditis [147]. It was reported that ampicillin/ceftriaxone could be considered

an alternative treatment option in treatment of endocarditis, as it showed similar activity to that of ampicillin/gentamicin, but with less nephrotoxicity [148].

1.3.5.3 Quinupristin/dalfopristin

This combination has bactericidal activity against various Gram-positive bacteria, but is bacteriostatic against VRE *faecium*, and ineffective against *E. faecalis* due to efflux pumps [149]. Although this combination was approved for the treatment of VRE, this indication was removed due to a failure to show a clinical benefit and its treatments are associated with high toxicity including myalgias and arthralgias [150]. VRE *faecium* resistance to the combination is mediated by target modification, drug inactivation, or active efflux [116].

1.4 The utilized strategy for development of new drugs against *C. difficile* and VRE infections

The lack of effective therapeutic options against both *C. difficile* and VRE highlights the critical need for developing new drugs against them. However, the de novo drug discovery is a very long process with a cost exceeding hundreds of millions of Dollars. Furthermore, the failure rate for a novel antibiotic development is estimated to be up to 95% [151]. In addition, the high possibility of developing resistance against the newly discovered antibiotic after being used in the market remains a huge nightmare that can drastically discourage the pharmaceutical companies from investing in the discovery of new antibiotics. This can be reflected by the fact that out of the 42 antibiotics currently undergoing clinical trials, only 11 of them are targeting pathogens on the World Health Organization's (WHO) threats list, because these pathogens are highly associated with development of resistance.

Drug repurposing in an attractive venture for antibacterial drug discovery that saves time and cost associated with drug innovation [152-160]. Repurposing is utilizing an FDA-approved drug or a clinical molecule outside the scope of its original indication, to treat a different disease, which is bacterial infection in this case. FDA-approved drugs and clinical molecules have well-characterized profiles of safety, pharmacokinetics and biological activity. Recently, both pharmaceutical companies and research institutions are extensively exploring repurposing approach for drug development, especially against the bacterial serious threats [161, 162]. Drug repurposing is advantageous to de novo drug discovery in terms of time, cost and risk. The rate of

failure of repurposing is much less than that of drug discovery as repurposed drugs have acceptable safety profile in humans which would allow them to pass the safety assessment in phase I clinical trials. Moreover, the time frame for repurposing is about 10-17 years whilst that for de novo drug discovery is 10-17 years. Furthermore, de novo drug discovery is estimated to cost about $800 million- $1 billion whereas drug repurposing is estimated to save $120 million of the costs associated with drug discovery, due to bypassing several preclinical steps [163].

Several drugs have been repurposed from their original use and are currently used for new indication. For example, sildenafil which was initially discovered for treatment of angina pectoris and was later repurposed for the management of erectile dysfunction [161, 162]. In contrast, no repurposed agent has been approved by the FDA till now, for treatment of bacterial infections. However, several repurposed drugs were proposed elsewhere to be repurposed as antibacterial agents [152-160, 164, 165].

In the following studies, several FDA-approved drugs were identified, through drug repurposing approach, and investigated for their activity against *C. difficile* and VRE. Details about each study will be discussed in the following chapters.

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CHAPTER 2. REPURPOSING THE ANTIAMOEBIC DRUG DIIODOHYDROXYQUINOLINE FOR TREATMENT OF *CLOSTRIDIOIDES DIFFICILE* INFECTIONS

A version of this chapter has been reprinted with permission. Abutaleb, N. S. & Seleem, M. N. (2020). Repurposing the antiamoebic drug diiodohydroxyquinoline for treatment of *Clostridioides difficile* infections. *Antimicrob Agents Chemother,* 64(6), e02115-19. doi: 10.1128/AAC.02115-19

2.1 Abstract

Clostridioides difficile, the leading cause of nosocomial infections, is an urgent health threat worldwide. The increased incidence and severity of disease, the high recurrence rates, and the dearth of effective anticlostridial drugs have created an urgent need for new therapeutic agents. In an effort to discover new drugs for treatment of *Clostridioides difficile* infections (CDIs), we investigated a panel of FDA-approved antiparasitic drugs against *C. difficile* and identified diiodohydroxyquinoline (DIHQ), an FDA-approved oral antiamoebic drug. DIHQ exhibited potent activity against 39 *C. difficile* isolates, inhibiting growth of 50% and 90% of these isolates at the concentrations of 0.5 µg/mL and 2 µg/mL, respectively. In a time-kill assay, DIHQ was superior to vancomycin and metronidazole, reducing a high bacterial inoculum by 3-$\log_{10}$ within six hours. Furthermore, DIHQ reacted synergistically with vancomycin and metronidazole against *C. difficile in vitro.* Moreover, at subinhibitory concentrations, DIHQ was superior to vancomycin and metronidazole in inhibiting two key virulence factors of *C. difficile*, toxin production and spore formation. Additionally, DIHQ did not inhibit growth of key species that compose the host intestinal microbiota, such as *Bacteroides*, *Bifidobacterium* and *Lactobacillus* spp. Collectively, our results indicate that DIHQ is a promising anticlostridial drug that warrants further investigation as a new therapeutic for CDIs.

Key words: *Clostridium difficile* infections (CDIs), spores, toxins, synergy, diiodohydroxyquinoline.

2.2 Introduction

Clostridioides difficile is a Gram-positive, spore-forming obligate anaerobic bacterium [1]. The incidence of *C. difficile* infections (CDIs) has increased dramatically and is now the leading cause of antibiotic- and healthcare-associated diarrhea in the United States [2, 3]. In addition, community-onset CDIs are on the rise. CDI is being increasingly recognized in the community, in younger individuals and in patients lacking the CDI traditional risk factors for, such as hospitalization, age and antibiotic exposure [4, 5].

Fidaxomicin was the only new antibiotic that has been approved in the last 30 years for the treatment of CDIs. Currently, only two drugs are recommended for treatment of both non-severe and severe CDIs, vancomycin and fidaxomicin. According to The Infectious Diseases Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) guidelines, metronidazole, which was previously recommended as a first-line therapeutic option for CDIs in adults, is no longer recommended and its use is now restricted to non-severe cases of CDI when patients are unable to obtain or be treated with vancomycin or fidaxomicin [6]. The available treatment options are inadequate in efficacy and associated with high recurrence rates [7-9]. Moreover, it was reported that about 22% of patients treated with metronidazole and 14% of those treated with vancomycin, will experience treatment failure and about 25 – 30% of patients treated with either metronidazole or vancomycin will go through CDI recurrence [10]. Moreover, resistance or reduced susceptibility to these antibiotics is emerging [8, 9]. Taken together, there is a critical and an unmet need for new effective drugs against *C. difficile.*

Drug discovery is a time-consuming and expensive venture. Developing a new drug can take 10 to 15 years from early stage discovery to receiving regulatory approval and can cost more than two billion dollars [11]. Repurposing FDA-approved drugs, particularly those that are off-patent, for new indications represents a promising approach that can significantly reduce the cost and time associated with drug innovation [12-14]. Antiparasitic drugs are a class of medications used for the treatment of parasitic diseases caused by parasites such as helminths, amoeba, and protozoa [15]. Most of them are poorly absorbed from the gastrointestinal (GI) tract, which is ideal for targeting intestinal pathogens, such as *C. difficile*. Consequently, in this study, we screened a small panel of antiparasitic drugs against *C. difficile* with the aim of discovering a new anticlostridial drug. From the initial screening against *C. difficile*, diiodohydroxyquinoline (DIHQ) emerged as the most potent candidate. DIHQ was subsequently evaluated against a wider panel of

C. difficile clinical isolates, examined for the ability to eliminate a high inoculum of *C. difficile* in a time-kill assay, investigated for the ability to inhibit *C. difficile* toxin production and spore formation, and tested for its ability to be used in combination with both vancomycin and metronidazole. Finally, we evaluated DIHQ's effect on growth of key bacterial species that comprise the human intestinal microbiota that help to curb *C. difficile* colonization.

2.3 Materials and Methods

2.3.1 Bacterial strains, chemicals and media.

All experiments were performed following relevant guidelines and regulations of the Purdue University Institutional Biosafety Committee. *C. difficile* isolates were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and the American Type Culture Collection (ATCC) (table 2.1S). DIHQ, albendazole, mebendazole (Tokyo Chemical Industry), praziquantel, cambendazole, ricobendazole, thiabendazole (Cayman Chemicals), paromomycin sulfate (Alfa Aesar), pyrantel pamoate (Acros Organics), pyrimethamine (MP Biomedicals), fidaxomicin (Cayman Chemicals), metronidazole (Alfa Aesar), and vancomycin hydrochloride (Gold Biotechnology) were purchased from commercial vendors. Brain heart infusion broth and MRS broth were purchased from Becton, Dickinson and Company and phosphate buffered saline (PBS) was purchased from Fisher Scientific. Yeast extract, L-cysteine, vitamin K and hemin were all obtained from Sigma-Aldrich.

2.3.2 Screening a small panel of antiparasitic drugs against *C. difficile*

The minimum inhibitory concentrations (MICs) of all drugs was determined against two *C. difficile* clinical strains, as previously described [16-18]. Briefly, 0.5 McFarland bacterial solution was prepared and diluted in brain heart infusion supplemented (BHIS) broth (inoculum size ~5 x 10^5 CFU/mL). Drugs were added and serially diluted before plates were incubated anaerobically at 37°C for 48 hours. MICs reported are the lowest drug concentration that completely suppressed the growth of bacteria, as observed visually.

2.3.3 Antibacterial activity of DIHQ against a wide panel of *C. difficile* strains

The MICs of DIHQ and control antibiotics (vancomycin, metronidazole and fidaxomicin) was determined against 39 clinical isolates of *C. difficile* using the broth microdilution method [16]. MIC_{50} and MIC_{90} are the minimum concentration of each agent that inhibited growth of 50% and 90% of the tested isolates, respectively. The minimum bactericidal concentration (MBC) of these drugs was tested by plating 5 µL from wells with no growth onto BHIS agar plates. The MBC was categorized as the lowest concentration that reduced bacterial growth by 99.9% [19].

2.3.4 Time-kill assay

To examine the killing kinetics of DIHQ, a time-kill assay against *C. difficile* ATCC BAA-1870 and *C. difficile* NR-49277 was performed, as described previously [20]. *C. difficile* cells in logarithmic growth phase were diluted to ~10^6 CFU/mL and exposed to concentrations equivalent to 5 × MIC of DIHQ, metronidazole, or vancomycin (in triplicates) in BHIS broth. Aliquots (100 µL) were collected from each treatment after 0, 4, 6, 8, 12 and 24 hours for viable CFU/mL determination.

2.3.5 Combination testing of DIHQ and standard anticlostridial drugs (metronidazole and vancomycin) against *C. difficile*

To evaluate the interactions between DIHQ and vancomycin or metronidazole, a standard checkerboard assay was utilized [21, 22]. The fractional inhibitory concentration index (ΣFIC) was calculated for each interaction against nine *C. difficile* clinical isolates. Interactions where the ΣFIC index was ≤ 0.5 were categorized as synergistic (SYN). An ΣFIC value of $> 0.5 – 1.25$ was considered additive (ADD), and ΣFIC value of $>1.25 – 4$ was considered indifference. ΣFIC values > 4 were categorized as antagonistic [23].

2.3.6 The effect of DIHQ on *C. difficile* toxin inhibition

To investigate DIHQ's effect on *C. difficile* toxin production, toxin A and toxin B levels were measured, as described previously [16, 24]. Briefly, drug concentrations equivalent to ¼ × MIC and ½ × MIC were added to a late exponential phase culture of a hypervirulent toxigenic strains (*C. difficile* ATCC BAA-1870, *C. difficile* ATCC 43255and *C. difficile* NR-49277), and

incubated anaerobically at 37°C for eight hours. 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 (tgc BIOMICS®, Dunwoody, GA) following the manufacturer's instructions. The (OD_{450}-OD_{620}) values, corresponding to the toxin concentration, were determined for DIHQ and control drugs.

2.3.7 The effect of DIHQ on *C. difficile* spore formation

The spore inhibition assay was performed as described in a previous report [16, 25]. Briefly, Log-phase cultures of *C. difficile* strains (ATCC BAA-1870, ATCC 43255, and NR-49277) were diluted in BHIS to an initial density of ~10^6 CFU/mL. Afterwards, the bacterial suspension was split into microcentrifuge tubes and drugs were added (in triplicates) at concentrations equal to ½ × MIC or 1 × MIC. Tubes were then, incubated anaerobically for five days at 37°C. Thereafter, an aliquot from each tube was diluted and plated on BHIS agar plates supplemented with 0.1% taurocholic acid, to count the total bacterial counts (vegetative bacteria + spores). The remaining solution was centrifuged and the pellet was suspended in PBS, stored overnight at 4°C, and subsequently shock-heated at 70°C for 30 minutes to kill the vegetative cells. The resulting solution was serially diluted and plated to determine the heat-resistant spore counts.

2.3.8 *In vitro* antibacterial evaluation of DIHQ against normal microflora.

The broth microdilution assay was utilized to determine the MICs of DIHQ against commensal organisms that compose the human gut microflora, as described elsewhere [26, 27]. A bacterial solution equivalent to 0.5 McFarland standard was prepared and diluted in BHIS broth (for *Bacteroides*and *Bifidobacterium*) or in MRS broth (for *Lactobacillus*) to achieve a bacterial concentration of about 5 × 10^5 CFU/mL. Drugs were added and serially diluted with media containing bacteria. Plates were incubated for 48 hours at 37°C before recording the MIC by visual inspection of growth.

2.4 Results

2.4.1 Screening a small panel of antiparasitic drugs against *C. difficile*

A small panel of poorly absorbed antiparasitic drugs was initially screened against two clinical *C. difficile* strains to determine their anticlostridial activity. As presented in Table 2.1, the antiparasitic drugs were inactive (up to 128 µg/mL) against both *C. difficile* strains, with the exception of DIHQ. DIHQ exhibited potent activity against both *C. difficile* strains (MIC ranged from 0.5 to 1 µg/mL). It was as potent as vancomycin, the drug of choice for treatment of *C. difficile* infections.

Table 2.1 Initial screening (MICs in μg/mL) of antiparasitic drugs against *C. difficile* ATCC BAA-1870 and *C. difficile* ATCC 43255

Drug Name	***C. difficile* ATCC-BAA-1870 MIC**	***C. difficile* ATCC 43255 MIC**
Albendazole	>128	>128
Mebendazole	>128	>128
Ricobendazole	>128	>128
Thiabendazole	>128	>128
Cambendazole	>128	>128
Praziquantel	>128	>128
Pyrantel Pamoate	>128	>128
Paromomycin	>128	>128
Pyrimethamine	>128	>128
DIHQ	1	0.5
Vancomycin	1	1
Metronidazole	0.125	0.25

2.4.2 Antibacterial susceptibility testing of DIHQ against additional *C. difficile* clinical strains

After the initial testing against two *C. difficile* strains, we evaluated the anticlostridial activity of DIHQ against a wider panel of *C. difficile* clinical isolates. As presented in Table 2, DIHQ inhibited growth of all 39 clinical isolates at concentrations ranging from 0.06 μg/mL to 4 μg/mL. DIHQ inhibited 50% of the tested isolates (MIC_{50}) at a concentration of 0.5 μg/mL and inhibited 90% of the isolates (MIC_{90}) at a concentration of 2 μg/mL. Interestingly, DIHQ's MIC values were comparable to the MIC values of vancomycin, which inhibited 50% and 90% of the strains at 0.5 μg/mL and 1 μg/mL respectively. Metronidazole, the drug of choice for anaerobic bacterial infections [28], was effective at a range of 0.06 – 0.5 μg/mL with MIC_{50} and MIC_{90} values of 0.125 μg/mL and 0.25 μg/mL respectively. On the other hand, fidaxomicin inhibited 50% and 90% of the tested strains at concentrations of 0.015 μg/mL and 0.06 μg/mL respectively.

To determine if DIHQ exhibits bacteriostatic or bactericidal activity against *C. difficile*, we also determined the minimum bactericidal concentration against all 39 isolates. The MBC values for DIHQ were equal to or two-fold higher than the corresponding MIC values for all 39 isolates indicating that DIHQ is a bactericidal agent. A similar result was observed for vancomycin and metronidazole.

Table 2.2 The minimum inhibitory concentration (MIC in µg/mL) and minimum bactericidal concentration (MBC in µg/mL) of DIHQ and control anticlostridial drugs against clinical *Clostridioides difficile* isolates

***C. difficile* Strains**	**Drugs**							
	DIHQ		**Vancomycin**		**Metronidazole**		**Fidaxomicin**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
ATCC BAA-1870	1	1	1	1	0.125	0.125	0.03	0.03
ATCC 43255	0.5	0.5	1	1	0.25	0.25	0.015	0.015
ATCC 43598	1	1	1	1	0.125	0.125	0.015	0.015
ATCC 9689	0.125	0.25	0.5	1	0.125	0.25	0.03	0.03
ATCC 1801	2	4	1	1	0.25	0.25	0.06	0.125
ATCC 700057	0.5	0.5	0.5	0.5	0.125	0.125	0.007	0.007
I1	1	1	1	1	0.125	0.125	0.007	0.007
I2	2	2	1	1	0.125	0.125	0.007	0.007
I4	1	1	0.5	0.5	0.125	0.125	0.015	0.015
I6	0.5	0.5	0.5	0.5	0.25	0.25	0.06	0.06
I9	0.06	0.06	0.5	0.5	0.06	0.06	0.03	0.03
I10	1	1	0.5	1	0.25	0.25	0.015	0.03
I11	1	1	1	1	0.25	0.25	0.015	0.015
I13	2	2	1	1	0.25	0.5	0.03	0.03
P1	0.5	0.5	0.5	0.5	0.125	0.125	0.003	0.007
P2	0.25	0.25	1	1	0.125	0.25	0.03	0.03
P3	2	2	1	1	0.25	0.25	0.015	0.03
P5	2	4	0.5	0.5	0.25	0.25	0.03	0.03

Table 2.2 continued

P6	0.5	0.5	1	1	0.25	0.25	0.003	0.003
P7	0.5	0.5	0.5	0.5	0.125	0.125	0.06	0.06
P8	0.125	0.125	1	1	0.125	0.125	0.015	0.015
P11	0.25	0.25	1	1	0.125	0.25	0.03	0.03
P13	0.5	0.5	0.5	0.5	0.125	0.25	0.015	0.015
P15	1	1	1	1	0.25	0.25	0.06	0.06
P19	0.5	1	1	1	0.25	0.25	0.03	0.06
P20	1	1	1	1	0.25	0.25	0.015	0.015
P24	1	2	0.5	0.5	0.125	0.25	0.015	0.03
P30	0.5	0.5	0.5	0.5	0.5	0.5	0.007	0.007
HM-88	4	8	0.5	0.5	0.125	0.125	0.03	0.03
HM-89	1	1	1	1	0.125	0.125	0.03	0.03
HM-745	1	1	1	1	0.5	0.5	0.06	0.125
NR-49277	0.5	0.5	1	1	0.125	0.125	0.03	0.03
NR-49278	0.25	0.5	0.25	0.5	0.25	0.5	0.007	0.015
NR-49281	0.25	0.25	0.25	0.25	0.125	0.125	0.007	0.007
NR-49284	0.125	0.125	0.25	0.5	0.125	0.125	0.015	0.015
NR-49285	1	1	0.5	0.5	0.25	0.5	0.015	0.03
NR-49286	0.25	0.5	0.25	0.5	0.125	0.125	0.007	0.015
NR-49288	0.25	0.5	0.5	1	0.25	0.25	0.007	0.015
NR-49290	0.25	0.5	0.5	1	0.125	0.25	0.015	0.015
MIC_{50}	**0.5**		**0.5**		**0.125**		**0.015**	
MIC_{90}	**2**		**1**		**0.25**		**0.06**	

2.4.3 Evaluation of the killing kinetics of DIHQ and control anticlostridial drugs against *C. difficile*

In order to confirm the bactericidal activity of DIHQ against *C. difficile*, we examined how rapidly the drug reduced the burden of a high inoculum of *C. difficile* via a standard time-kill assay.

As presented in Fig. 2.1, DIHQ exerted a rapid bactericidal activity against *C. difficile.* DIHQ required only six hours to generate a 3-$\log_{10}$ reduction in CFU/mL and completely eradicated the bacteria (below the detection limit, 100 CFU/mL), within 24 hours. Interestingly, DIHQ was superior to both metronidazole and vancomycin in the *in vitro* time-kill assay. Metronidazole produced a 3-$\log_{10}$ reduction in *C. difficile* CFU/mL after 12 hours. Vancomycin exhibited slow reduction of *C. difficile* count and only reduced the bacterial burden by 1.7-$\log_{10}$ CFU/mL within 24 hours. Time kill kinetics results against *C. difficile* NR-49277 (a hypervirulent strain) were included in the SI file (Fig. 2.4S).

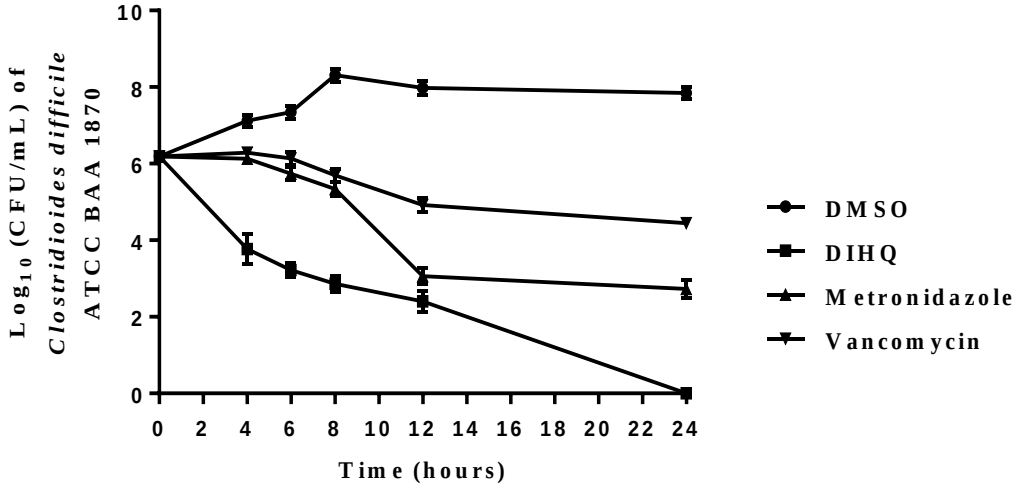

Figure 2.1 Time-kill analysis of DIHQ, metronidazole, and vancomycin (at 5 × MIC) against *Clostridioides difficile* ATCC BAA-1870 over a 24-hour incubation period at 37 ℃.

DMSO (solvent of the drugs) served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each drug studied.

2.4.4 Interactions between DIHQ and metronidazole and vancomycin against *C. difficile* clinical isolates

Combination therapy is one of the most effective therapeutic choices for different infections to increase the therapeutic outcomes and decrease relapse rates. To ascertain whether DIHQ has potential to be combined with standard anticlostridial drugs against *C. difficile*, the checkerboard assay was used. As depicted in Tables 2.3 and 2.4, DIHQ exhibited a synergistic interaction with metronidazole more frequently than with vancomycin. When combined with metronidazole, DIHQ possessed a synergistic relationship against 7 out of 9 tested strains with a

fractional inhibitory concentration (FIC) index that ranged from 0.18 to 0.37 (Table 2.3). On the other hand, DIHQ exhibited a synergistic interaction with vancomycin against 5 out of 9 tested strains with an FIC index range similar to that of metronidazole (Table 2.4).

Table 2.3 Interactions between DIHQ and metronidazole against *C. difficile* clinical isolates.

***C. difficile* strains**	**MIC (µg/ml)**				**[1]ΣFIC Index**	**Interpretation***
	Metronidazole		**DIHQ**			
	Alone	**Combined with DIHQ**	**Alone**	**Combined with metronidazole**		
ATCC BAA-1870	0.125	0.015	1	0.125	0.25	SYN
ATCC 43255	0.125	0.015	1	0.25	0.375	SYN
ATCC 9689	0.25	0.03	0.125	0.06	0.62	ADD
ATCC 1801	0.25	0.03	4	1	0.37	SYN
P11	0.25	0.03	0.5	0.125	0.37	SYN
P19	0.5	0.06	0.5	0.06	0.24	SYN
P30	0.5	0.06	1	0.06	0.185	SYN
I6	0.25	0.03	2	0.125	0.188	SYN
I9	0.125	0.015	0.06	0.06	1.125	ADD

[1]ΣFIC; fractional inhibitory concentration

[*]ΣFIC index $\leq$ 0.5 is considered synergistic (SYN); ΣFIC index > 0.5 – 1.25 is considered additive (ADD); ΣFIC index >1.25 – 4 is considered indifference (IND); ΣFIC index > 4 is considered antagonistic

Table 2.4 Interactions between DIHQ and vancomycin against *C. difficile* clinical isolates.

***C. difficile* strains**	**MIC (µg/ml)**				**[1]ΣFIC Index**	**Interpretation***
	Vancomycin		**DIHQ**			
	Alone	**Combined with DIHQ**	**Alone**	**Combined with vancomycin**		
ATCC BAA-1870	1	0.125	1	0.06	0.185	SYN
ATCC 43255	1	0.125	1	0.125	0.25	SYN
ATCC 9689	1	0.125	0.125	0.06	0.625	ADD
ATCC 1801	0.5	0.06	4	2	0.625	ADD
P11	1	0.125	0.5	0.5	1.125	ADD
P19	1	0.125	0.5	0.125	0.375	SYN
P30	1	0.125	1	0.125	0.25	SYN
I6	0.5	0.06	2	0.5	0.375	SYN
I9	1	0.125	0.06	0.06	1.125	ADD

[1]ΣFIC; fractional inhibitory concentration

*ΣFIC index $\leq$ 0.5 is considered synergistic (SYN); ΣFIC index > 0.5 – 1.25 is considered additive (ADD); ΣFIC index >1.25 – 4 is considered indifference (IND); ΣFIC index > 4 is considered antagonistic.

2.4.5 DIHQ inhibits *C. difficile* toxin production

Toxins are the main virulence factor of *C. difficile.* As a result, inhibition of toxin production will contribute to effective treatment of CDI. Therefore, we tested the toxin-inhibitory activity of DIHQ against a toxigenic *C. difficile* strain. DIHQ exhibited a dose-dependent inhibition of *C. difficile* toxins A and B and was effective at subinhibitory concentrations. As shown in Fig. 2.2, DIHQ inhibited nearly 17.8% and 27%of total toxin production, at ¼ × MIC and ½ × MIC respectively. Fidaxomicin, which is known to inhibit toxin production [24], inhibited 31.9% and 46.2% of toxin production at ¼ × MIC and ½ × MIC, respectively. No toxin inhibition was observed with either vancomycin or metronidazole, in agreement with previous reports [10, 29]. Toxin inhibition results against 2 other *C. difficile* strains were included in the SI file (Figures 2.5S & 2.6S).

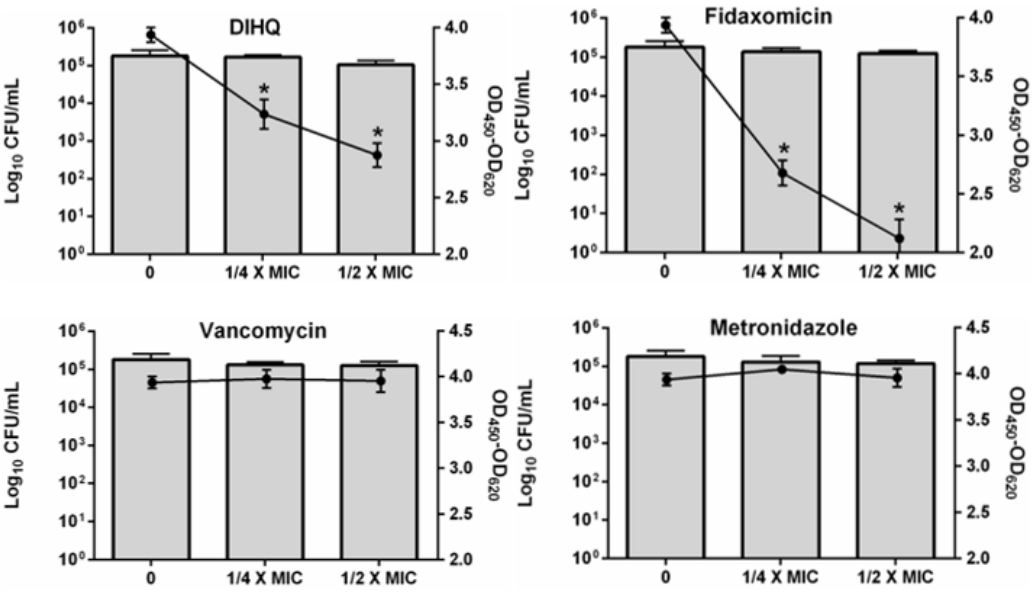

Figure 2.2 Toxin inhibition activity of DIHQ and control anticlostridial drugs (vancomycin, metronidazole, and fidaxomicin) against *C. difficile* ATCC BAA-1870.

Drugs, at concentrations of ¼ × MIC or ½ × MIC were incubated with the hypervirulent toxigenic strain *C. difficile* ATCC BAA-1870. The bacterial counts (represented by gray bars) were determined for each sample, and the toxin levels (represented by the connected lines) were assessed in the supernatant using an enzyme linked immune fluorescent assay (ELISA). Error bars represent standard deviation values from triplicate samples for each treatment. The data were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons. Asterisks (*) denote statistical significant difference between the results obtained in fidaxomicin- or DIHQ- treated samples as compared to the untreated samples.

2.4.6 DIHQ inhibits *C. difficile* spore formation

Spore formation is a key virulence factor of *C. difficile* that is responsible for both the rapid spread and high recurrence rate of infection [30, 31]. We investigated the efficacy of DIHQ to inhibit *C. difficile* ATCC BAA-1870 spores formation. As shown in Fig. 2.3, DIHQ-treated bacteria displayed a significantly reduced spore count by nearly 0.74-$\log_{10}$ and 1.24-$\log_{10}$ at ½ × MIC and 1 × MIC, respectively. Fidaxomicin, which is capable of inhibiting spore formation [32], significantly reduced *C. difficile* spore formation by nearly 1.02-$\log_{10}$ reduction (at ½ × MIC) and 1.8-$\log_{10}$ reduction (at 1 × MIC). On the other hand, almost no reduction in the spore count was observed when vegetative cells were exposed to either vancomycin or metronidazole. Spores inhibition results against 2 other *C. difficile* strains were included in the SI file (Figures 2.7S & 2.8S).

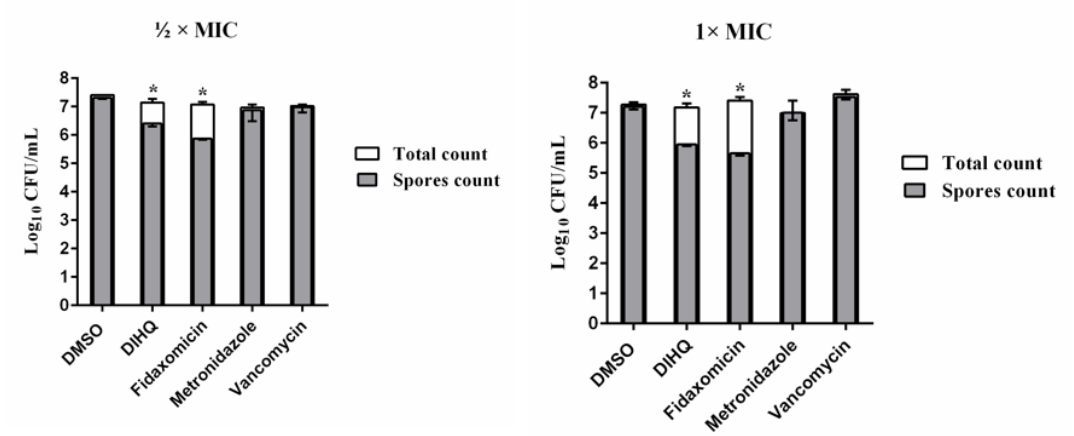

Figure 2.3 Spore inhibition activity of DIHQ against *C. difficile* ATCC BAA-1870 compared to the control anticlostridial drugs, fidaxomicin, vancomycin, and metronidazole.

DMSO (solvent of the drugs) served as a negative control. Drugs (½ × MIC and 1 × MIC) were incubated with bacteria for five days followed by serial dilution and plating to count both total bacterial count and spores count. Error bars represent standard deviation values from triplicate samples for each treatment. Asterisks (*) denote significant difference between the total count and the spore count for each test agent analyzed via a two-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P<0.05$).

2.4.7 *In vitro* antimicrobial evaluation of DIHQ against normal microflora

Administration of antibiotics, particularly those that are broad-spectrum, can lead to alteration of the normal intestinal microbial composition which results in gut colonization by opportunistic pathogens like *C. difficile* [33]. Consequently, it was imperative to determine whether DIHQ may possess a deleterious effect on commensal organisms that are part of the normal gut microbiota. We tested the antibacterial activity of DIHQ and the control anticlostridial drugs against representative bacteria that comprise the human gut microbiome, including species of *Lactobacillus*, *Bacteroides* and *Bifidobacterium.* As presented in Table 2.5, DIHQ did not inhibit growth of species of *Bacteroides* and *Bifidobacterium*, up to the maximum tested concentration of 128 µg/mL (except *Bifidobacterium longum* (MIC=64-128 µg/mL), *B. adolescentis* and *B. angulatum* (MIC = 128 µg/mL)). This was in contrast to metronidazole, which inhibited growth of all species of *Bacteroides* and *Bifidobacterium* tested at concentrations that ranged from ≤ 1 µg/mL up to 2 µg/mL. DIHQ exhibited weak antibacterial activity against both *Lactobacillus casei* (MIC = 16 µg/mL) and *L. crispatus* (MIC = 64 µg/mL). DIHQ did not inhibit growth of *L. reuteri* (MIC > 128 µg/mL). Fidaxomicin did not inhibit growth of *Lactobacillus* and

Bacteroides strains tested (with the exception of *B. dorei* HM-719) up to a concentration of 128 µg/mL. Though, they exhibited a similar activity to metronidazole against *Bifidobacterium* strains.

Table 2.5 Antibacterial activity (MIC in µg/mL) of DIHQ and control anticlostridial drugs against human normal gut microbiota.

Bacterial Strains	DIHQ	Vancomycin	Metronidazole	Fidaxomicin
Lactobacillus reuteri HM-102	>128	>128	>128	>128
Lactobacillus casei ATCC 334	16	>128	16	>128
Lactobacillus crispatus HM-371	64	4	>128	>128
Bacteroides dorei HM-719	>128	64	≤1	128
Bacteroides dorei HM-717	>128	128	≤1	>128
Bacteroides dorei HM-718	>128	128	≤1	>128
Bacteroides dorei HM-29	>128	64	≤1	>128
Bacteroides fragilis HM-711	>128	128	≤1	>128
Bacteroides fragilis HM-709	>128	64	2	>128
Bacteroides fragilis HM-710	>128	64	2	>128
Bacteroides fragilis HM-714	>128	128	≤1	>128
Bifidobacterium adolescentis HM-633	128	≤1	≤1	≤1

Table 2.5 continued

Bacterial Strains	DIHQ	Vancomycin	Metronidazole	Fidaxomicin
Bifidobacterium angulatum HM-1189	128	≤1	≤1	≤1
Bifidobacterium breve HM-411	>128	≤1	2	2
Bifidobacterium breve HM-1120	>128	≤1	≤1	≤1
Bifidobacterium longum HM-845	64	≤1	≤1	≤1
Bifidobacterium longum HM-847	128	≤1	≤1	≤1

2.5 Discussion

The current treatment options for CDI are limited and still result in unsatisfactory outcomes. Only three drugs are currently available for treatment; vancomycin, metronidazole, and fidaxomicin. Metronidazole is no longer recommended as a first-line therapy for CDI due to high recurrence rate and increased resistance. However, metronidazole is still recommended for use in treating non-severe cases of CDI where patients have limited access to, or cannot successfully be treated with vancomycin or fidaxomicin [6]. In addition, the available treatment options suffer from several limitations such as increasing recurrence rates and treatment failure in addition to the increased risk of the emergence of resistant mutants and promotes overgrowth of other opportunistic enteric pathogens like vancomycin-resistant enterococci (VRE) [10, 34]. Fidaxomicin has a comparable efficacy to vancomycin but suffers from the same pitfall of recurrence (although it occurs at a lower rate) [35]. Fecal microbiota transplantation (FMT) has been evaluated as a non-antibiotic treatment option for CDI yet, it has many restrictions and strict regulations. Additionally, the significantly higher cost associated with FMT makes it unaffordable for the majority of CDI patients [36]. Without a doubt, there is an urgent need to identify new effective drugs to treat CDI.

Ideally, an effective anticlostridial drug should exhibit potent, bactericidal activity against *C. difficile* with limited activity against intestinal microbiota, and experience limited absorption from the GI tract (when administered orally) to accumulate at a sufficient concentration at the target site. Consequently, we screened a small panel of antiparasitic drugs against *C. difficile.* We chose these drugs because they are commercially available, administered orally, poorly absorbed from the GI tract, and were not screened before against *C. difficile.* Out of these drugs, DIHQ, an antiamoebic drug that was first introduced for use in humans in the early 1960s, possesses desirable qualities as an anticlostridial drug. DIHQ is a poorly absorbed halogenated hydroxyquinoline, acting as a chelator for ferrous ions that are essential for amoebic metabolism [37]. DIHQ acts as a luminal amoebicide, but its exact mechanism of action is unknown [38, 39].

In this study, we evaluated DIHQ against a large panel of 39 *C. difficile* strains, including hypervirulent (NAP1, ribotype 027) and clinical toxigenic isolates. DIHQ inhibited growth of *C. difficile* isolates at very low, clinically-achievable concentrations. Interestingly, DIHQ was as potent as vancomycin against the *C. difficile* isolates tested with an MIC_{50} of 1 µg/mL. Antibiotics exhibiting bactericidal activity are hypothesized to contribute to better clinical outcomes than bacteriostatic agents [40]. Thus, we determined the MBC for DIHQ against *C. difficile*. DIHQ was found to be bactericidal, similar to the standard anticlostridial drugs vancomycin and metronidazole. Next, we examined how rapidly DIHQ can reduce the burden of a high *C. difficile* inoculum via a time-kill kinetics assay. DIHQ exhibited a more rapid bactericidal activity against *C. difficile* compared to both metronidazole and vancomycin. Similar to previous reports, metronidazole exhibited bactericidal activity against *C. difficile* generating 3-$\log_{10}$ reduction after 12 hours [41]. Vancomycin, in agreement with previous reports [41, 42], exhibited a slow reduction of *C. difficile* count (1.7-$\log_{10}$ CFU/mL) after 24 hours. Rapid bactericidal activity contributes to reducing the emergence of bacterial resistance to antibacterial drugs [43], and it is highly desirable for *C. difficile* infections to prevent the progression of the disease.

Combination therapy has become a standard in several diseases. This strategy is now often used in the healthcare setting to gain the advantages of combined drugs such as different mechanisms of action, lower toxicity, potential synergism and less probability of development of resistance to both agents [44, 45]. Buggy *et al.,* in 1987 used a combination of vancomycin + rifampicin for treatment of CDI patients and found this combination resolved infections and decreased the rate of recurrence [46]. Vancomycin/metronidazole combination was also, proposed

for CDI although it was as effective as vancomycin alone [47]. Nevertheless, combination therapy is a promising strategy for treating CDI, particularly for severe infections where one agent may not be effective. A previous study found that the therapeutic outcomes of DIHQ + metronidazole combination is better than metronidazole alone for treatment of intestinal amoebiasis [48]. This encouraged us to evaluate the combination of DIHQ with standard anticlostridial drugs, vancomycin and metronidazole, against nine different *C. difficile* strains. Interestingly, DIHQ exhibited a synergistic relationship in 7 out of 9 tested strains (when combined with metronidazole) and in 5 out of 9 tested strains (when combined with vancomycin). Since the treatment course of CDI is long, combination therapy is highly desirable to decrease the probability of developing resistance, to lower the toxicity potential of the administered drugs, and to curb recurrence of infection.

After confirming DIHQ's *in vitro* activity against *C. difficile*, both alone and in combination with metronidazole and vancomycin, we moved to investigate if DIHQ can interfere with expression of key virulence factors used by *C. difficile* to promote infection. First, we evaluated the ability of DIHQ to inhibit *C. difficile* toxin production. Toxigenic *C. difficile* strains are capable of inducing inflammation and provoking pseudomembranous colitis. TcdA and TcdB can inactivate host GTPases, including Rac, Rho and CDC42 resulting in rearrangement of the actin cytoskeleton, intense inflammation, enormous fluid secretion, disruption of mucosal layer barrier function and finally necrosis and apoptosis of the colonic mucosal layer [49, 50]. Therefore, agents capable of inhibiting *C. difficile* toxin production may contribute to effective treatment of CDI by limiting damage to the host's intestinal mucosa. Fidaxomicin is the only currently available anticlostridial drug with antitoxin activity [24]. We tested the toxin inhibition activity of DIHQ, at subinhibitory concentrations, against a hypervirulent toxigenic *C. difficile* strain. DIHQ (at ½ × MIC) inhibited the total toxin production of *C. difficile* by 28.5%. A similar effect was observed with fidaxomicin (37% reduction in toxin production at ½ × MIC) but not with vancomycin or metronidazole.

As DIHQ exhibited an ability to partially inhibit *C. difficile* toxin production, we moved to investigate the drug's ability to inhibit a second key virulence factor, spore formation. Spores are metabolically dormant and highly resistant to standard disinfection procedures. That is why they can persist for long periods and spread in the environment. Once ingested by susceptible hosts, spores germinate, in response to bile acids in the small intestine, into vegetative cells that produce

toxins and cause disease [51]. In addition, persistent spores in the intestine are the reason behind recurrence where they can germinate in the intestine after the conclusion of treatment [52]. The ability of DIHQ to inhibit *C. difficile* spore formation was tested. DIHQ partially inhibited *C. difficile* spore formation at subinhibitory concentration. Neither vancomycin nor metronidazole reduced the spores count. This result suggests that DIHQ may contribute to lower CDI recurrence rates by inhibiting *C. difficile* spore formation, though this needs to be confirmed in appropriate CDI animal models.

Finally, we sought to investigate DIHQ's effect on growth of major bacterial species that compose the healthy intestinal flora. Disrupting intestinal microbiota increases the susceptibility of hosts to *C. difficile* colonization. Thus, it is critically important for new anticlostridial drugs to show a minimal activity against the normal microbiome. Unlike vancomycin, fidaxomicin and metronidazole, DIHQ exhibited a limited activity against the tested representative members of the human normal intestinal microbiota.

It is worth mentioning that the recommended dose of DIHQ for treatment of amoebiasis in adults is 650 mg/kg, 3 times daily for 20 days, which is a much greater course than that would be expected for *C. difficile* treatment [39]. It can also, be used for children in a dosage of 30-40 mg/kg daily in divided doses, for 20 days, with a maximum of 2 g/day without reported toxicity; though it is not well-tolerated in children and should be avoided, if possible [53]. Moreover, DIHQ has rare, mild and self-limiting side effects such as nausea, vomiting, stomach upset, abdominal cramps, diarrhea, headache, and dizziness. Reported toxicity cases of DIHQ are rare and associated with administering high doses for a prolonged period. Seizures and encephalopathy were reported in a nine year-old boy treated with a very high dose of DIHQ (420 mg, 3 times daily, for 20 days) [54]. Furthermore, few cases of neuropathy and optic atrophy in children, after prolonged administration of high doses of hydroxyquinolines (namely clioquinol) for prolonged periods, were reported [55].

In conclusion, the current study highlights that DIHQ, an FDA-approved antiamoebic drug, has a potent *in vitro* antibacterial activity against *C. difficile* and exhibits a more rapid bactericidal activity compared to both metronidazole and vancomycin. DIHQ interacted synergistically with both vancomycin and metronidazole against most strains of *C. difficile* tested *in vitro*. In addition to its antibacterial activity, DIHQ also exhibited antivirulence properties, namely partial inhibition of both toxin production and spore formation by *C. difficile*. Furthermore, DIHQ exhibited a

minimal effect against important commensal organisms that comprise the intestinal microbiome. Future studies will need to be conducted to validate the *in vitro* findings *in vivo* in suitable animal models of CDI in order to further develop DIHQ as a novel anticlostridial drug.

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2.7 Supplemental material

Table 2.6S Bacterial strains used in the study.

Bacterial Strains	Source/Description
C. difficile ATCC BAA-1870 (4118)	*tcdA*[a], *tcdB*[b] and CDT[c] genes ribotype 027 and NAP[d]
C. difficile ATCC 43255 (VPI 10463)	An abdominal wound. Ribotype 087, *tcdA* and *tcdB* genes
C. difficile ATCC 43598 (1470)	Asymptomatic neonate feces in Belgium Ribotype 017, *tcdB* gene.
C. difficile ATCC 9689 (90556-M6S)	Ribotype 001, *tcdA* and *tcdB* genes.
C. difficile ATCC BAA-1801 (3232)	Human feces of an adult in Belgium. Non-toxigenic, Ribotype 010.
C. difficile ATCC 700057 (VPI 11186)	Ribotype 038, non-toxigenic.
C. difficile Isolate 1 (NR-13427)	Human patient in USA, 2008/2009.
C. difficile Isolate 2 (NR-13428)	Human patient in USA, 2008/2009.
C. difficile Isolate 4 (NR-13430)	Human patient in USA, 2008/2009.
C. difficile Isolate 6 (NR-13432)	Human patient in USA, 2008/2009.
C. difficile Isolate 9 (NR-13435)	Human patient in USA, 2008/2009.
C. difficile Isolate 10 (NR-13436)	Human patient in USA, 2008/2009.
C. difficile Isolate 11 (NR-13437)	Human patient in USA, 2008/2009.
C. difficile Isolate 13 (NR-13553)	Human patient in USA, 2008/2009.
C. difficile P1 (NR-32882)	Human patient in USA with a relapsing *C. difficile* infection, 2001. Toxigenic
C. difficile P2 (NR-32883)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P3 (NR-32884)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P5 (NR-32885)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic

Table 2.6S continued

C. difficile P6 (NR-32886)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P7 (NR-32887)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P8 (NR-32888)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P11 (NR-32890)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P13 (NR-32891)	Human patient in Western Pennsylvania, USA, 2005. Toxigenic
C. difficile P15 (NR-32892)	Human patient in Western Pennsylvania, USA, 2005. Toxigenic
C. difficile P19 (NR-32895)	Human patient in Western Pennsylvania, USA, 2005. Toxigenic
C. difficile P20 (NR-32896)	Human patient in Western Pennsylvania, USA, 2005. Toxigenic
C. difficile P21 (NR-32897)	Human patient in Western Pennsylvania, USA, 2005. Toxigenic
C. difficile P24 (NR-32900)	Human patient in Western Pennsylvania, USA, 2001. Toxigenic
C. difficile P30 (NR-32904)	Asymptomatic human patient in Western Pennsylvania, USA, 2009. Non-toxigenic
C. difficile NAP07 (CDC#2007054) (HM-88)	Human feces Reference genome for the Human Microbiome Project (HMP).
C. difficile NAP08 (CDC#2007019) (HM-89)	Human feces Reference genome for the Human Microbiome Project (HMP).
C. difficile 70-100-2010 (HM-745)	Human feces, 2010. Reference genome for the Human Microbiome Project (HMP).
C. difficile Isolate 20100502 (NR-49277)	Stool of an older patient in Colorado, USA, 2010. Ribotype 019, NAP1 *tcdA*, *tcdB*, *tcdC*[e] of the PaLoc[f] operon, and CDT.

Table 2.6S continued

C. difficile Isolate 20100207 (NR-49278)	Stool of an older patient in New York, USA, 2010. Ribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20110052 (NR-49281)	Stool of an older patient in northeastern USA, 2010. Ribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20120015 (NR-49284)	Stool of an older patient in New York, USA, 2011. Ribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20110979 (NR-49285)	Stool of an older patient in midwestern USA, 2011. Ribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20110999 (NR-49286)	Stool of an older patient in western/midwestern USA, 2011. Rribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20110870 (NR-49288)	Stool of an older patient in Tennessee, USA, 2011. Ribotype 027, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
C. difficile Isolate 20120187 (NR-49290)	Stool of an older patient in Tennessee, USA, 2011 Ribotype 019, NAP1 *tcdA, tcdB, tcdC* of PaLoc operon and the CDT.
Lactobacillus reuteri HM-102 (CF48-3A)	Feces of a healthy Finnish child. Reference genome for the Human Microbiome Project (HMP).
Lactobacillus casei ATCC 334	Isolated from dairy products (emmental cheese).
Lactobacillus crispatus HM-371 (EX849587VC02)	The mid-vaginal wall of a human patient, Virginia, March 2010 Reference genome for the Human Microbiome Project (HMP).
Bacteroides dorei HM-719 (CL02T12C06)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bacteroides dorei HM-717 (CL02T00C15)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bacteroides dorei HM-718 (CL03T12C01)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).

Table 2.6S continued

Bacteroides dorei HM-29 (5_1_36/D4)	Inflamed biopsy tissue taken from the terminal ileum of a male patient with Crohn's disease in Alberta, Canada. Reference genome for the Human Microbiome Project (HMP).
Bacteroides fragilis HM-711 (CL05T00C42)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bacteroides fragilis HM-709 (CL07T00C01)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bacteroides fragilis HM-710 (CL07T12C05)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bacteroides fragilis HM-714 (CL03T12C07)	Healthy adult human feces, Massachusetts, USA. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium adolescentis HM-633	From the fecal sample of a healthy two-year-old infant in Aberdeen, Scotland, United Kingdom, 1996. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium angulatum HM-1189	Human stool in Guelph, Ontario, Canada. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium breve HM-411	Human mid-vaginal wall in Richmond, Virginia, USA, 2010. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium breve HM-1120	A clinical vaginal swab collected from a woman that tested positive for bacterial vaginosis (Nugent score = 8) at the Washington University School of Medicine in St. Louis, Missouri, USA, 2011. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium longum HM-845	A one-year-old human patient. Reference genome for the Human Microbiome Project (HMP).
Bifidobacterium longum HM-847	A one-year-old human patient. Reference genome for the Human Microbiome Project (HMP).

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

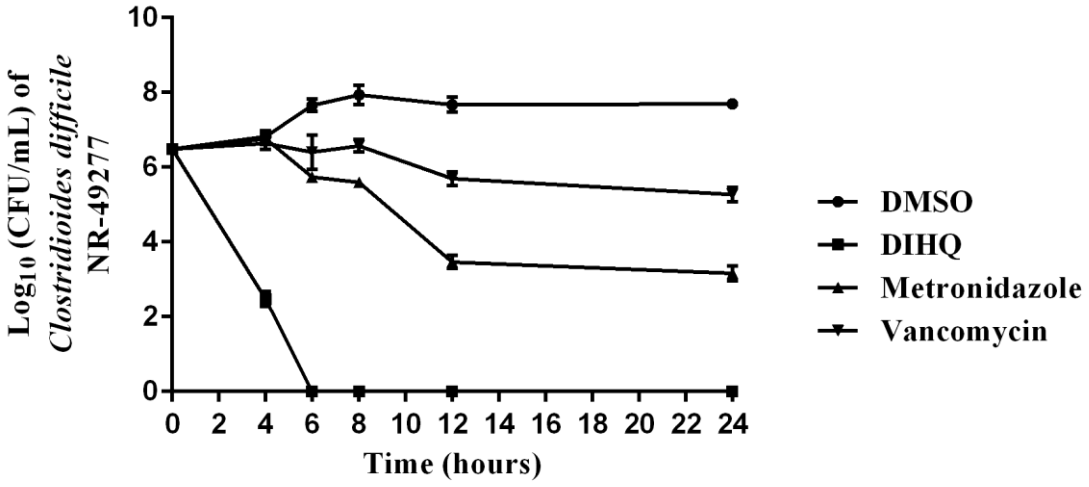

Figure 2.4S Time-kill analysis of DIHQ, metronidazole, and vancomycin (at 5 × MIC) against *Clostridioides difficile* NR-49277 over a 24-hour incubation period at 37 ℃.

DMSO (solvent of the drugs) served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each drug studied

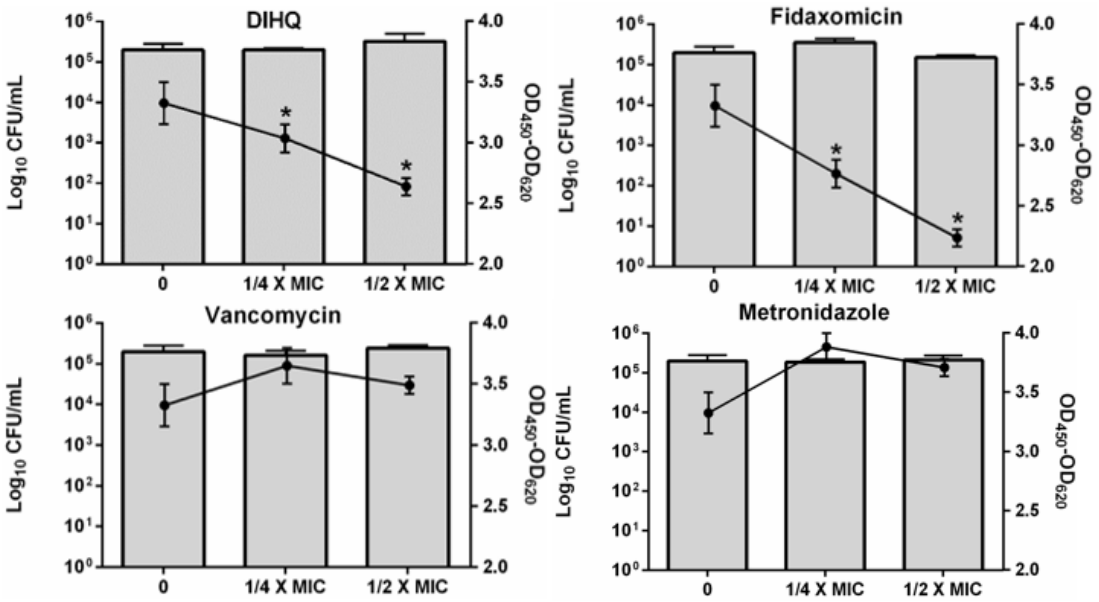

Figure 2.5S Toxin inhibition activity of DIHQ and control anticlostridial drugs (vancomycin, metronidazole, and fidaxomicin) against *C. difficile* ATCC 43255.

Drugs, at concentrations of ¼ × MIC or ½ × MIC were incubated with the hypervirulent toxigenic strain *C. difficile* ATCC 43255. The bacterial counts (represented by gray bars) were determined for each sample, and the toxin levels (represented by the connected lines) were assessed in the supernatant using an enzyme linked immune fluorescent assay (ELISA). Error bars represent standard deviation values from triplicate samples for each treatment. The data were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons. Asterisks (*) denote statistical significant difference between the results obtained in fidaxomicin- or DIHQ- treated samples as compared to the untreated samples.

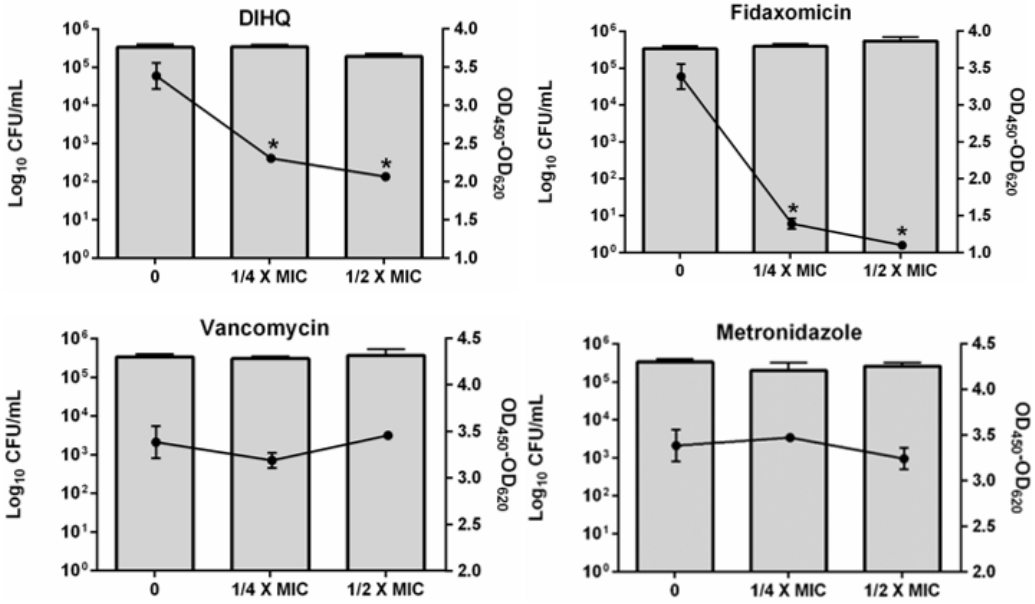

Figure 2.6S Toxin inhibition activity of DIHQ and control anticlostridial drugs (vancomycin, metronidazole, and fidaxomicin) against *C. difficile* NR-49277.

Drugs, at concentrations of ¼ × MIC or ½ × MIC were incubated with the hypervirulent toxigenic strain *C. difficile* NR-49277. The bacterial counts (represented by gray bars) were determined for each sample, and the toxin levels (represented by the connected lines) were assessed in the supernatant using an enzyme linked immune fluorescent assay (ELISA). Error bars represent standard deviation values from triplicate samples for each treatment. The data were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons. Asterisks (*) denote statistical significant difference between the results obtained in fidaxomicin- or DIHQ- treated samples as compared to the untreated samples.

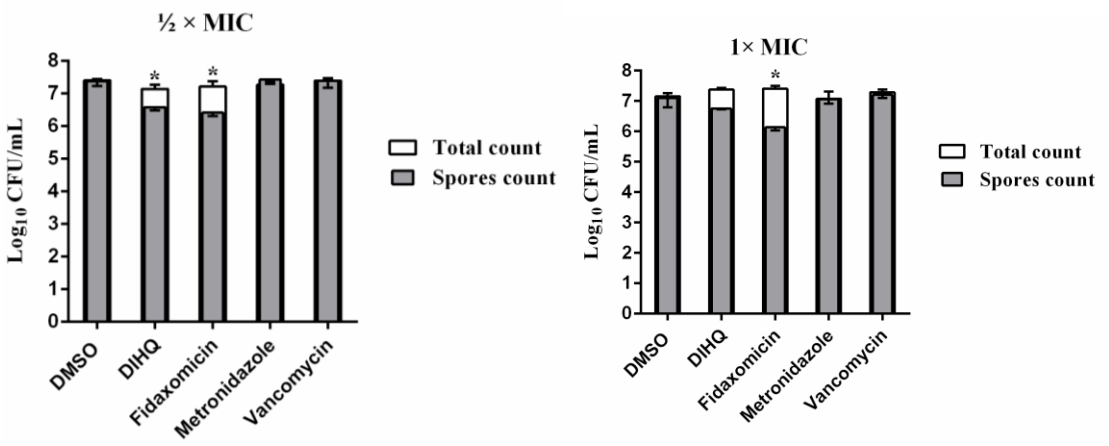

Figure 2.7S Spore inhibition activity of DIHQ against *C. difficile* ATCC NR-49277 compared to the control anticlostridial drugs, fidaxomicin, vancomycin, and metronidazole.

DMSO (solvent of the drugs) served as a negative control. Drugs (½ × MIC and 1 × MIC) were incubated with bacteria for five days followed by serial dilution and plating to count both total bacterial count and spores count. Error bars represent standard deviation values from triplicate samples for each treatment. Asterisks (*) denote significant difference between the total count and the spore count for each test agent analyzed via a two-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P<0.05$).

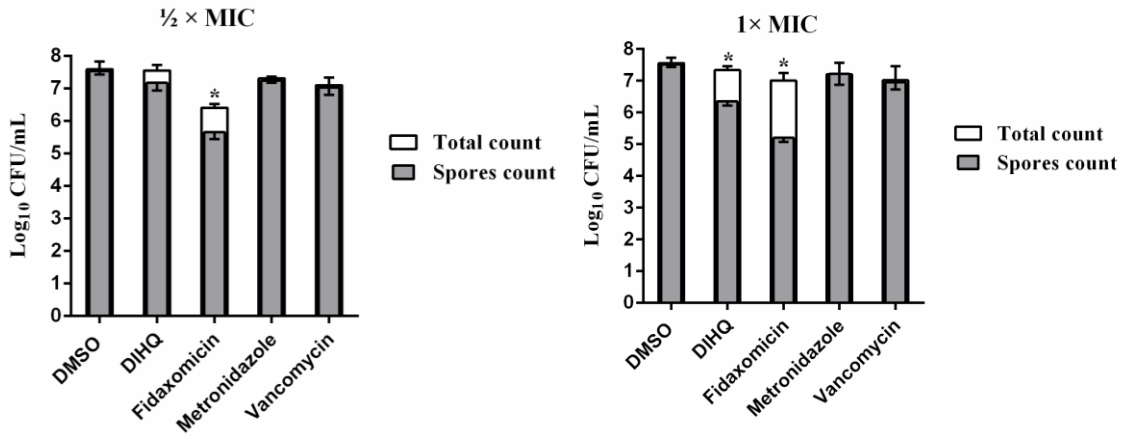

Figure 2.8S Spore inhibition activity of DIHQ against *C. difficile* ATCC NR-49277 compared to the control anticlostridial drugs, fidaxomicin, vancomycin, and metronidazole.

DMSO (solvent of the drugs) served as a negative control. Drugs (½ × MIC and 1 × MIC) were incubated with bacteria for five days followed by serial dilution and plating to count both total bacterial count and spores count. Error bars represent standard deviation values from triplicate samples for each treatment. Asterisks (*) denote significant difference between the total count and the spore count for each test agent analyzed via a two-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P<0.05$).

CHAPTER 3. AURANOFIN, AT CLINICALLY ACHIEVABLE DOSE, PROTECTS MICE AND PREVENTS RECURRENCE FROM *CLOSTRIDIOIDES DIFFICILE* INFECTION

A version of this chapter has been reprinted with permission. Abutaleb, N. S., & Seleem, M. N. (2020). Auranofin, at clinically achievable dose, protects mice and prevents recurrence from *Clostridioides difficile* infection. *Sci Rep,* 10(1), 7701. doi: 10.1038/s41598-020-64882-9

3.1 Abstract

Clostridioides difficile is the leading cause of nosocomial infections and a worldwide urgent public health threat. Without doubt, there is an urgent need for new effective anticlostridial agents due to the increasing incidence and severity of *C. difficile* infection (CDI). The aim of the present study is to investigate the *in vivo* efficacy of auranofin (rheumatoid arthritis FDA-approved drug) in a CDI mouse model and establish an adequate dosage for treatment. The effects of increased *C. difficile* inoculum, and pre-exposure to simulated gastric intestinal fluid (SGF) and simulated intestinal fluid (SIF), on the antibacterial activity of auranofin were investigated. Auranofin's *in vitro* antibacterial activity was stable in the presence of high bacterial inoculum size compared to vancomycin and fidaxomicin. Moreover, it maintained its anti-*C. difficile* activity after being exposed to SGF and SIF. Upon testing in a CDI mouse model, auranofin at low clinically achievable doses (0.125 mg/kg and 0.25 mg/kg) significantly protected mice against CDI with 100% and 80% survival, respectively. Most importantly, auranofin (0.125 mg/kg and 0.25 mg/kg) significantly prevented CDI recurrence when compared with vancomycin. Collectively, these results indicate that auranofin could potentially provide an effective, safe and quick supplement to the current approaches for treating CDI.

Key words: *Clostridioides difficile* infection (CDI), recurrence, auranofin, repurposing.

3.2 Introduction

Clostridioides difficile is the worldwide leading cause of nosocomial infections and antibiotic-associated diarrhea [1]. A recent report released by the Centers for Disease Control and Prevention (CDC) stated that about 223,900 patients were hospitalized with *C. difficile* infections

(CDI) in the United States in 2017, which was associated with around 12,800 mortality cases and in excess of $1 billion healthcare cost [2].

CDI symptoms range from mild to severe watery diarrhea to more severe life threatening complications such as pseudomembranous colitis, toxic megacolon, colon perforation, sepsis, systemic inflammatory response syndrome and shock [3]. Disease manifestations are attributed to the toxin-mediated damage elicited by the two major toxins TcdA and TcdB. These toxins catalyze inactivation of host GTPases (Rac, Rho and CDC42) and perturbation of actin cytoskeleton, ultimately causing intense inflammation, loss of tight junctions of the intestinal mucosal layer, enormous fluid secretion, cell rounding and finally necrosis and apoptosis of the colonic mucosal cells [4, 5]. The incidence and severity of CDI has increased dramatically due to the overuse of antibiotics and the emergence of hypervirulent epidemic strains such as, but not limited to, pulsed-field gel type North American pulsotype 1 (NAP1) or PCR ribotype 027, which were responsible for several outbreaks globally [6, 7]. Moreover, the clinical management of CDI is hindered by the ability of *C. difficile* to produce spores which are highly resistant to environmental conditions, antibiotics and disinfection processes. Spores can persist on unsuitable environments for long periods and spread in the environment [8]. Once ingested by susceptible hosts, these spores germinate, in response to bile acids in the gut, into vegetative cells that colonize in the intestine, produce toxins and establish infection [9]. Consequently, *C. difficile* spores serve as the major cause CDI dissemination and recurrence.

Even though the overuse of antibiotics is responsible for CDI, the management of CDI requires antibiotic administration. Currently, only two drugs are approved for treatment of both non-severe and severe CDI; vancomycin and fidaxomicin. While metronidazole is not FDA-approved for treatment of CDI, it was previously recommended as a first-line therapeutic option for CDI in adults. It use is now restricted to non-severe CDI cases when patients are unable to obtain or be treated with vancomycin or fidaxomicin [10]. Vancomycin or metronidazole treatments are limited by the high treatment failure (22% with metronidazole, and 14% with vancomycin), and the high recurrence rate (25-30%) [7, 11]. Furthermore, fidaxomicin has lower recurrence rate due to its less disturbance effect on gut microbiota; yet, its high cost restricts its use [12-14]. Further compounding the CDI problem is the emerging resistance or reduced susceptibility to these antibiotics [13, 15]. Thus, the critical and the unmet need for developing new anti-CDI therapeutics cannot be overemphasized.

Auranofin is an FDA-approved anti- rheumatoid arthritis drug, with a well-studied safety profile for human use [16, 17]. Recently, auranofin has gained interest in repurposing for treatment of bacterial and parasitic infections [18-23]. Furthermore, it is undergoing Phase II clinical trials for the treatment of amoebic dysentery, giardiasis (NCT02736968) and tuberculosis (NCT02968927). Auranofin possesses strong antibacterial and antifungal activities [17, 22-25]. We previously demonstrated that auranofin has a potent anticlostridial activity with strong inhibition of both toxins and spores and production *in vitro* [24]. We hypothesized that auranofin's potent antibacterial and antivirulence activity against *C. difficile* would be beneficial in treating mice infected with *C. difficile* in an *in vivo* CDI mouse model. The main objective of the present study was to investigate the *in vivo* efficacy of auranofin treatment in a CDI mouse model and to study the ability of auranofin to prevent CDI recurrence. In addition, this study established the doses needed to achieve 100% protection in CDI mouse model and prevent recurrence. The impact of increasing *C. difficile* inoculum, and its pre-exposure to simulated gastric fluid and simulated intestinal fluid, on the antibacterial activity of auranofin were also investigated.

3.3 Materials and Methods

3.3.1 Bacterial strains, media and reagents

All experiments were performed following the relevant guidelines and regulations of the Purdue University Institutional Biosafety Committee. *C. difficile* strains (Table 3.1) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) (Manassas, VA, USA), and the American Type Culture Collection (ATCC) (Manassas, VA, USA). Brain heart infusion broth was purchased from Becton, Dickinson and Company (Cockeysville, MD, USA). Hemin and vitamin K were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Yeast extract, sucrose and L-cysteine were purchased from Fisher Scientific (Fail Lawn, NJ, USA). Phosphate buffered saline (PBS) (Corning, Manassas, VA, USA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA), monobasic potassium phosphate (KH_2PO_4) (Macron chemicals, Center Valley, PA, USA), vancomycin hydrochloride, gentamicin sulfate, kanamycin monosulfate, taurocholic acid (Chem-

Impex, Wood Dale, IL, USA), metronidazole (Alfa Aesar, Ward Hill, MA, USA), and colistin sulfate, fidaxomicin (Cayman Chemical, Ann Arbor, MI, USA) were purchased commercially.

Table 3.1 *C. difficile* strains used in the study

***C. difficile* Strains**	**Source/Description**
ATCC BAA-1870 (4118)	*tcdA*[a], *tcdB*[b] and CDT[c] genes. Ribotype 027 and NAP[d].
ATCC 43255 (*VPI 10463)*	Abdominal wound. *tcdA* and *tcdB*, ribotype 087.
NR-49277 (20100502)	Stool sample, Colorado, 2010. *tcdA*, *tcdB*, *tcdC*[e], and CDT. Ribotype 019, NAP1.
NR-49278 (20100207)	Stool sample, New York, 2010. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 027, NAP1.
NR-49281 (20110052)	Stool sample, northeastern USA, 2010. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 027, NAP1.
NR-49284 (20120015)	Stool sample, New York, USA, 2011. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 027, NAP1.
NR-49285 (20110979)	Stool sample, midwestern USA, 2011. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 027, NAP1.
NR-49286 (20110999)	Stool sample, western/midwestern USA, 2011. *tcdA*, *tcdB*, *tcdC* and CDT. Rribotype 027, NAP1.
NR-49288 (20110870)	Stool sample, Tennessee, USA, 2011. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 027, NAP1.
NR-49290 (20120187)	Stool sample, Tennessee, USA, 2011. *tcdA*, *tcdB*, *tcdC* and CDT. Ribotype 019, NAP1.

[a]*tcdA*, toxin A gene; [b]*tcdB*, toxin B gene; [c]CDT, binary toxin; [d]NAP, North American pulsed-field gel electrophoresis type; [e]*tcdC*, Anti-sigma factor gene.

3.3.2 Evaluation of the effect of *C. difficile* inoculum size on the antibacterial activity of auranofin

The broth microdilution assay was used to determine the impact of *C. difficile* inoculum size on the minimum inhibitory concentrations (MICs) of auranofin and control antibiotics, as described previously [24, 26, 27]. Briefly, standard inoculum (SI: ~5×10^5 CFU/mL) and high inoculum (HI: ~5×10^7 CFU/mL) of each *C. difficile* strain were prepared in brain heart infusion supplemented broth (BHIS) and tested against auranofin and control antibiotics. Plates were then, incubated anaerobically at 37°C for 48 hours. MICs reported are the lowest drug concentration that completely suppressed the growth of bacteria, as observed visually.

3.3.3 Activity of auranofin after exposure to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described earlier [28, 29]. Briefly, SGF (pH=1.2) was prepared by dissolving NaCl (2 g) and pepsin (3.2 g) in 7 mL of concentrated HCl and deionized water was subsequently added to make up a final volume of 1 L. Then, the pH was adjusted to 1.2. To prepare SIF (pH=6.8), 6.8 g of KH_2PO_4 was dissolved in 250 mL of water, and 77 mL of 0.2 N NaOH and 500 mL of deionized water were added. Afterwards, 10 g of pancreatin was added, and the pH of the resulting solution was adjusted to 6.8.

The broth microdilution assay [24, 26, 27] was used to determine the MICs of auranofin and control antibiotics in presence of SGF and SIF. Briefly, auranofin and control drugs were incubated with each of SGF and SIF for 2, 4 and 24 hours. After the corresponding times, broth microdilution assay was performed to determine the MICs of the tested drugs.

3.3.4 Preparation of *C. difficile* spores for mice infection

C. difficile spores were prepared as described earlier [30]. Briefly, *C. difficile* ATCC 43255 was inoculated onto BHIS agar and incubated anaerobically for 5 days. Spores were collected anaerobically using PBS containing 10% bovine serum albumin, heated at 70°C for 20 minutes to get rid of vegetative cells and counted by dilution and plating onto BHIS supplemented with 0.1% taurocholic acid. Spores were then, stored at 4℃ overnight before infecting mice.

3.3.5 *In vivo* efficacy of auranofin in a CDI mouse model

3.3.5.1 CDI mouse model

The study was reviewed, approved and performed following the guidelines of the Purdue University Animal Care and Use Committee (PACUC) and according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in individually ventilated autoclaved cages and received sterile food and water ad libitum throughout the duration of the experiment. CDI mouse model was performed as described previously [31] with modifications. Since disruption of microbiota depends on mice drinking

naturally, we performed three modifications: 1) increasing the concentrations of antibiotics to ensure microbiota disruption, 2) adding 7.5% sucrose to the drinking water containing antibiotics to overcome the very bitter taste of the antibiotics in drinking water, as mice are expected to decrease their rate of water consumption due to its bitter taste, and 3) extending the duration of administering antibiotic cocktail in drinking water to 5 days to ensure microbiota disruption. Eight-week-old female pathogen-free C57BL/6 mice (Jackson, ME, USA) were pre-treated with an antibiotic cocktail in sterile drinking water to disrupt the mice normal intestinal microflora, reducing the colonization resistance and facilitating infection with the toxigenic strain of *C. difficile*. The cocktail contained kanamycin (1.2 mg/mL), gentamicin (0.105 mg/mL), colistin (2550 U/mL), metronidazole (0.645 mg/mL), vancomycin (0.135 mg/mL) and sucrose (75 mg/mL) for 5 days. Afterwards, mice were switched to regular autoclaved water for 2 days and they received a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day prior to *C. difficile* challenge.

For infection, mice were restrained and infected intragastrically with 1.3×10^6 spores of *C. difficile* ATCC 43255 via oral gavage using a ball tipped metal feeder. Number of spores used were re-counted after infection to confirm the infected dose.

3.3.5.2 *In vivo* efficacy of different doses of auranofin in a CDI mouse model

Following infection, mice were randomly allocated into groups (n=5) for treatment. Two hours post-infection, three groups were treated orally with auranofin (0.125 mg/kg, 0.25 mg/kg and 0.5 mg/kg), one group was treated with vancomycin (10 mg/kg) via oral gavage, and one group was treated orally with the vehicle (10% DMSO in PBS). Treatments were continued once daily for five days and mice were checked (6 times daily) for disease signs (including weight loss, behavioral changes, hunched posture, decreased activity, wet tail and diarrhea).

3.3.5.3 *In vivo* efficacy of auranofin in *C. difficile* recurrence

In order to investigate the activity of auranofin in preventing *C. difficile* recurrence, mice were infected, as described above and two groups were treated orally with auranofin (0.125 mg/kg and 0.25 mg/kg), one group was treated with vancomycin (10 mg/kg) via oral gavage, and one

group was treated orally with the vehicle (10% DMSO in PBS) for 5 days. Treatments were stopped after 5 days, and mice were monitored (6 times daily) for disease signs and recurrence of infection till the 20th day. Then, mice were humanely euthanized at 21st day post-infection using CO_2 asphyxiation.

3.3.6 Statistical analyses

The survival data were analyzed by Log-rank (Mantel-Cox) test utilizing GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

3.4 Results and Discussion

3.4.1 The effect of *C. difficile* inoculum size on the antibacterial activity of auranofin

C. difficile is known to colonize the intestinal tract in large populations. Additionally, a higher inoculum (~10^6 CFU/mL) is often used to infect animals in *in vivo* CDI models. It was reported that the bacterial burden recovered from cecal and fecal contents of infected mice had averages of ~10^6 to 10^7 CFU/g [32-34]. The dependence of the antibacterial activity of anticlostridial drugs on the inoculum effect is an important consideration, especially for a weakly absorbed drug like auranofin (85% of the administered dose is not absorbed and recovered in feces) [35]. After being administered orally, auranofin will be localized in the gut, and target the colonizing *C. difficile* populations. However, the standard antibacterial susceptibility assays typically evaluate test agents at a lower inoculum size (~10^5 CFU/mL). Thus, we evaluated the impact of the high *C. difficile* inoculum (HI, ~5 × 10^7 CFU/mL), compared with the standard inoculum (SI, 5 × 10^5 CFU/mL), on the antibacterial activity of auranofin. Upon testing against SI, auranofin exhibited a potent *in vitro* activity against the *C. difficile* strains tested with MIC values ranging from 0.25 – 1 µg/mL (Table 3.2), in agreement with a previous study [24]. Furthermore, auranofin's antibacterial activity was identical to or one-fold higher, as the inoculum size increased from 10^5 CFU/mL to 10^7 CFU/mL (Table 3.2), suggesting that its activity was not impacted by increasing the inoculum size. Its MIC_{90} was not affected by the increase in the inoculum size. Fidaxomicin MICs, in agreement with a previous study [36] were not affected by increasing the *C. difficile* inoculum size (MICs of HI were equal to or one-fold higher than SI MICs except the MICs against *C. difficile* NR-49278 that increased by three-fold). Additionally, its MIC_{90} with the

HI was the same as that of the SI. Conversely, vancomycin's activity was negatively impacted by the increased inoculum size (MIC increased three-fold against *C. difficile* NR-49278, *C. difficile* NR-49281, and *C. difficile* NR-49284), in accordance with a previous report [36]. Additionally, its MIC_{90} with the HI was one-fold higher than that of the SI.

Table 3.2 MICs (μg/mL) of auranofin and control antibiotics against *C. difficile* clinical isolates at standard and high inocula.

***C. difficile* strains**	**Auranofin**		**Vancomycin**		**Fidaxomicin**	
	SI	**HI**	**SI**	**HI**	**SI**	**HI**
ATCC BAA 1870	1	1	1	1	0.03	0.03
ATCC 43255	0.5	0.5	1	2	0.015	0.015
NR-49277	0.5	0.5	1	2	0.03	0.03
NR-49278	0.25	0.25	0.25	1	0.007	0.007
NR-49281	0.25	0.25	0.25	1	0.007	0.015
NR-49284	0.25	0.25	0.25	1	0.015	0.015
NR-49285	0.5	0.5	0.5	1	0.015	0.03
NR-49286	0.25	0.25	0.25	0.25	0.007	0.007
NR-49288	0.25	0.5	0.5	1	0.007	0.03
NR-49290	0.25	0.25	0.5	1	0.015	0.015
MIC_{90}	**0.5**	**0.5**	**1**	**2**	**0.03**	**0.03**

SI, standard inoculum ($\sim 5 \times 10^5$ CFU/mL); HI, high inoculum ($\sim 5 \times 10^7$ CFU/mL); MIC_{90}, the concentration of the test agent that inhibited the growth of 90% of the tested strains.

3.4.2 The effect of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) on the antibacterial activity of auranofin

It is important to analyze the stability of drugs, especially those intended for oral administration, in harsh conditions of the gastrointestinal tract (GIT). The stability of a drug in gastric and intestinal fluids provides evidence whether it is prone to degradation process by the effect of GIT fluids prior to absorption [37]. Drugs stability in presence of the GIT fluids can be

investigated by incubating the drug in simulated gastric fluid (for 1-2 hours) and simulated intestinal fluids (for 3-4 hours) to mimic the *in vivo* drug exposure to these fluids [29, 37].

To investigate the effect of SGF and SIF on the antibacterial activity of auranofin against *C. difficile,* auranofin, and vancomycin and fidaxomicin (control antibiotic) were incubated with SGF and SIF for 2, 4 and 24 hours and their MICs against 2 clinical *C. difficile* strains were determined. As depicted in Table 3.3A, after incubation with SGF, the MICs of auranofin did not increase against *C. difficile* ATCC BAA 1870, even after 24 hours exposure, and increased by one-fold only against *C. difficile* ATCC 43255 after 24 hours exposure. This result suggests that auranofin was stable after exposure to the gastric pH and was not affected by the enzymes of gastric fluids. Similarly, vancomycin and fidaxomicin MICs (after exposure to SGF) were similar to or one-fold higher than their corresponding MICs in absence of SGF. Furthermore, auranofin MICs, after incubation with SIF up to 24 hours, were equal to or one-fold higher than its MIC without incubation with SIF (Table 3.3B), suggesting that auranofin was not affected by exposure to the intestinal fluids. The antibacterial activity of vancomycin and fidaxomicin also, were not affected by incubation with SIF (MICs are equal to or one-fold higher than their corresponding MICs without exposure to SIF) (Table 3.3B). This result came in coincidence with a previous report [36].

Table 3.3 MICs (µg/mL) of auranofin and control antibiotics against *C. difficile* clinical isolates after incubation with: **A)** simulated gastric fluid (SGF), **B)** simulated intestinal fluid (SIF), for the corresponding times (hours).

A) Simulated gastric fluid (SGF)

***C. difficile* strains**	**Auranofin**				**Vancomycin**				**Fidaxomicin**			
	0h	**2h**	**4h**	**24h**	**0h**	**2h**	**4h**	**24h**	**0h**	**2h**	**4h**	**24h**
ATCC BAA 1870	1	1	1	1	1	1	1	2	0.03	0.03	0.03	0.03
ATCC 43255	0.5	0.5	0.5	1	1	1	1	1	0.015	0.015	0.015	0.015

B) Simulated intestinal fluid (SIF)

***C. difficile* strains**	**Auranofin**				**Vancomycin**				**Fidaxomicin**			
	0h	**2h**	**4h**	**24h**	**0h**	**2h**	**4h**	**24h**	**0h**	**2h**	**4h**	**24h**
ATCC BAA 1870	1	1	1	2	1	1	1	2	0.03	0.03	0.03	0.06
ATCC 43255	0.5	1	1	1	1	1	1	2	0.015	0.015	0.015	0.03

3.4.3 *In vivo* efficacy of auranofin in a CDI mouse model

The potent antibacterial and antivirulent activities of auranofin against *C. difficile* [24] in addition to its stability in SGF and SIF prompted us to investigate its efficacy in a CDI mouse model and its potential to protect mice from CDI recurrence. In our study, CDI was established first before treatment. Three groups of mice were treated with (0.125 mg/kg, 0.25 mg/kg and 0.5 mg/kg) of auranofin. Two additional groups were used, positive control (vancomycin) and negative control (vehicle) groups. Mice were treated with the corresponding drugs for 5 days and monitored for disease symptoms.

As shown in Fig. 3.1, vancomycin (10 mg/kg) protected 100% of mice up to 5 days, as previously reported [31, 33]. In addition, auranofin, at low clinically achievable concentration (0.125 mg/kg), was able to protect 100% of the mice against *C. difficile* during the 5-days treatment period. Interestingly, the higher doses (0.25 mg/kg and 0.5 mg/kg) protected only 80% and 40% of mice, respectively. There was no significant difference in survival between vancomycin-treated, and auranofin (0.25 mg/kg)- treated groups. This effect (lower dose more effective) could be due to the potent anticommensal activity of auranofin against the human gut intestinal microbiota [38],

which could increase by increasing the administered dose leading to establishment of *C. difficile* colonization in the intestine and higher mortality. This activity was also, reported for niclosamide against *C. difficile* where the lower dose (2 mg/kg) protected mice from CDI more effectively than higher doses (10 mg/kg and 50 mg/kg) [39]. The effectiveness of lower dose of auranofin could also be explained by their potent anti-inflammatory activity. It was reported that the anti-inflammatory drug, indomethacin, increased the severity of *C. difficile* infection in mice [40]. Additionally, in previous study investigating the efficacy of auranofin against vancomycin-resistant enterococci peritonitis, lower doses of auranofin provided the best protection (100%) [41].

Moreover, figure 3.2 depicts the mean relative daily weight for all mice groups. The control group (vehicle-treated) showed weight loss starting day 2 after infection and their weight continued to decrease till day 4. Conversely, vancomycin-treated mice did not show weight loss till day 5. Similarly, auranofin-treated mice maintained a stable body weight with a minor weight reduction till day 5 (Fig. 3.2).

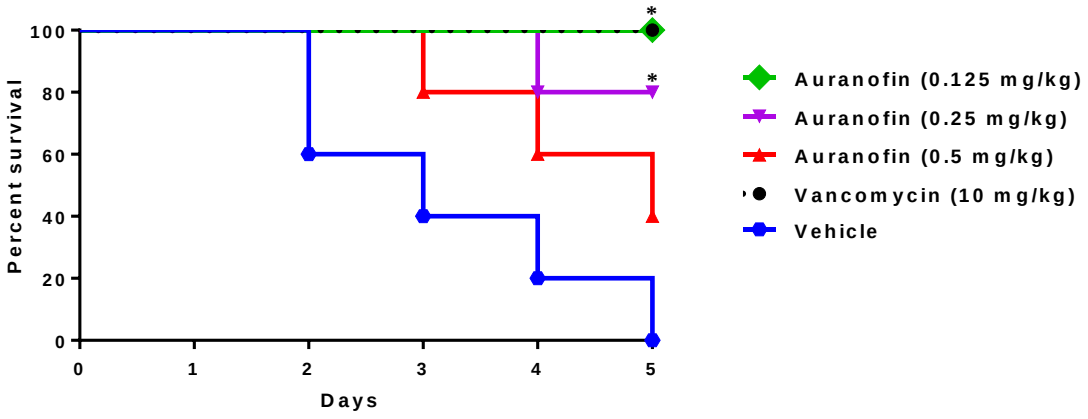

Figure 3.1 Auranofin protects mice against CDI.

Mice were treated with auranofin (0.125 mg/kg, 0.25 mg/kg, and 0.5 mg/kg), vancomycin (10 mg/kg), or the vehicle for 5 days after infection with *C. difficile* spores. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) test. Asterisks (*) denote statistical significant difference between mice treated with either auranofin, or vancomycin in comparison with the vehicle-treated mice.

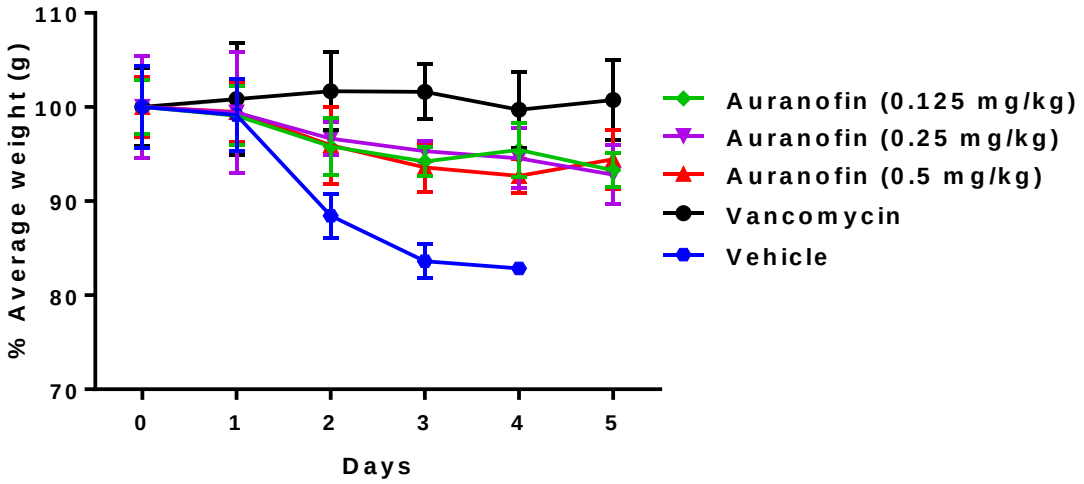

Figure 3.2 Average relative weight of all surviving mice.

Infected mice were treated with auranofin (0.125 mg/kg, 0.25 mg/kg, and 0.5 mg/kg), vancomycin (10 mg/kg), or the vehicle for 5 days and weighed daily till the end of the experiment. The data are presented as percent relative weight (mean ± standard deviation) for each group.

Symptomatic recurrence of CDI occurs in approximately 20% of patients and is challenging to treat [42-46]. In addition to subsequent prolongation of *C. difficile* shedding and transmission, 1 out of every 5 patients experienced *C. difficile* recurrence episode died within 30 days of diagnosis [47]. Then, we sought to investigate this promising activity of auranofin in preventing *C. difficile* recurrence. Mice were infected and treated for 5 days and mice were monitored for survival and possible *C. difficile* recurrence until the 20th day. Vancomycin-treated mice, in accordance with a previous study [31], were susceptible to *C. difficile* recurrence where 60% of mice died after stopping vancomycin treatment. In contrast, auranofin (0.125 mg/kg and 0.25 mg/kg), significantly protected mice from CDI recurrence with 100% and 80% survival, respectively after 20 days (Fig. 3.3).

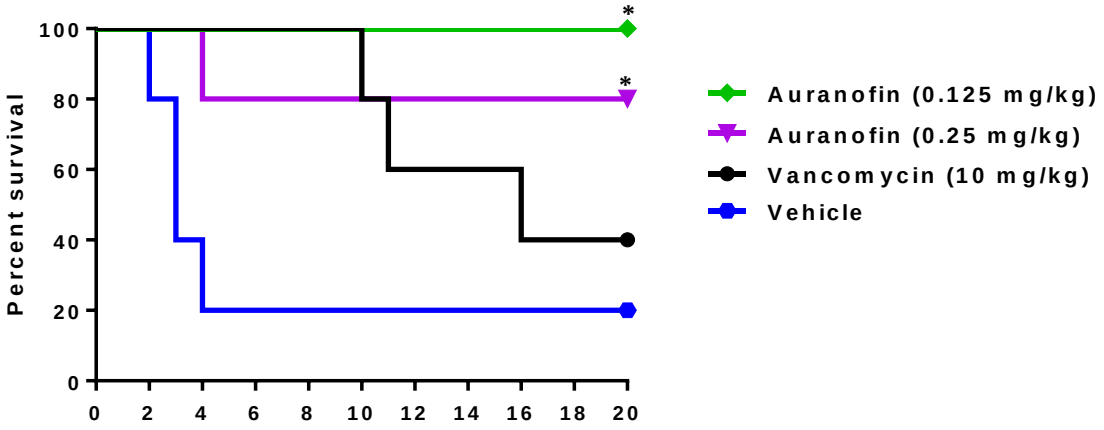

Figure 3.3 Efficacy of auranofin against CDI recurrence.

Mice were treated with auranofin (0.125 mg/kg, and 0.25 mg/kg), vancomycin (10 mg/kg), or the vehicle for 5 days after infection with *C. difficile* spores and the treatments were stopped afterwards. Mice were monitored for survival. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) test. Asterisks (*) denote statistical significant difference between mice treated with either auranofin, or vancomycin in comparison with vehicle-treated mice.

Additionally, the relative body weight results (Fig. 3.4) showed that vehicle-treated group started to lose weight on day 2 and the weight loss continued till day 4. Afterwards, the surviving mice showed clinical recovery and started to gain weight till they returned to the normal weight. Vancomycin-treated mice, in coincidence with a previous report [31], maintained their weight till the start of recurrence after the treatment discontinuation. By day 9, mice started to lose weight which continued to decrease until day 12. Thereafter, the average weight of surviving mice (40%) started to increase till they reached the normal weight. In contrast, auranofin (0.125 mg/kg and 0.25 mg/kg) groups maintained a stable body weight along the duration of the experiment

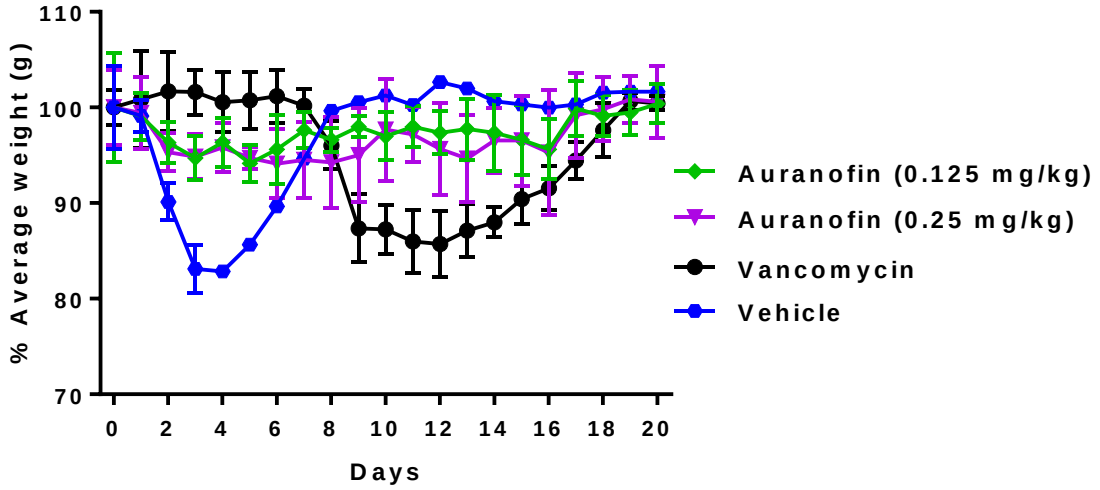

Figure 3.4 Average relative weight of all surviving mice in *C. difficile* recurrence experiment. Infected mice were treated with auranofin (0.125 mg/kg, 0.25 mg/kg, and 0.5 mg/kg), vancomycin (10 mg/kg), or the vehicle for 5 days and treatments were stopped thereafter. Mice were weighed daily till the end of the experiment. The data are presented as percent relative weight (mean ± standard deviation) for each group.

A point worth noting, auranofin doses used in this study are achievable clinically. The recommended long term dosing regimen of auranofin in adult patients is 6-9 mg daily, and 0.1-0.25 mg/kg/day for children, in a single dose or divided doses [48, 49]. Consequently, the most effective dose in this study, (0.125 mg/kg), is within range of doses administered clinically to humans. In addition, the therapeutic benefits and toxicity profile of auranofin have been monitored in clinical trials in more than 5,000 rheumatoid arthritis patients taking the drug and some of whom were monitored for more than 7 years. Auranofin did not show any evidence of cumulative toxicity and it was approved by the FDA for long-term treatment of rheumatoid arthritis in 1985 [50]. Furthermore, auranofin is approved for long-term treatment of rheumatoid arthritis, a much greater course than would be expected for anticlostridial therapeutics.

In conclusion, this study investigated the effectiveness of auranofin, at clinically achievable doses, as a CDI therapeutic. Auranofin's *in vitro* antibacterial activity was stable in the presence of high bacterial inoculum size compared to vancomycin and fidaxomicin. Moreover, it maintained its anti-*C. difficile* activity after being exposed to SGF and SIF. Interestingly, it significantly protected mice against CDI at low doses (0.125 mg/kg and 0.25 mg/kg). Most importantly,

auranofin (0.125 mg/kg and 0.25 mg/kg) significantly prevented CDI recurrence. These results indicate that auranofin warrants further investigation as a new CDI treatment option.

3.5 References

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CHAPTER 4. *IN VIVO* EFFICACY OF AURANOFIN IN A HAMSTER MODEL OF *CLOSTRIDIOIDES DIFFICILE* INFECTION

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4.1 Abstract

Clostridioides difficile infections (CDIs) are an urgent public health threat worldwide and are a leading cause of morbidity and mortality in healthcare settings. The increasing incidence and severity of infections combined with the scarcity of effective anti-CDI agents has made treatment of CDI very challenging. Therefore, development of new, effective anticlostridial agents remains a high priority. The current study investigated the *in vivo* efficacy of auranofin in a CDI hamster model. All hamsters treated with auranofin (5 mg/kg) survived a lethal challenge with *C. difficile*. Furthermore, auranofin (5 mg/kg) was as effective as vancomycin, the drug of choice for treatment of CDIs, against relapsing CDI. Remarkably, auranofin (5 mg/kg) generated a 3.15-$\log_{10}$ reduction (99.97%) in *C. difficile* count in the cecal contents of hamsters, which surpassed vancomycin (20 mg/kg). These results indicate that auranofin warrants further investigation as a new agent to replenish the pipeline of anti-CDI therapeutics.

Key words: *Clostridioides difficile* infection (CDI), *C. difficile* ileocecitis, recurrence, auranofin, repurposing

4.2 Introduction

Clostridioides difficile is the most common cause of healthcare-associated infections and antibiotic-associated diarrhea; *C. difficile* imposes a heavy burden on most healthcare systems [1, 2]. *C. difficile* infection (CDI) is considered a significant source of morbidity and mortality in healthcare settings [3]. According to the U.S. Centers for Disease Control and Prevention (CDC), about 223,900 patients were hospitalized with a CDI in the United States in 2017, which resulted in around 12,800 deaths and over $1 billion in healthcare costs [2]. CDI is considered an urgent

threat by the CDC, and controlling CDI is a top priority for healthcare systems to mitigate both clinical and financial outcomes [2].

In spite of numerous calls for development of new anti-CDI therapeutics, only one new drug, fidaxomicin, has been developed for the treatment of CDIs during the past 30 years [4]. Currently, vancomycin and fidaxomicin are the only drugs approved by the U.S. Food and Drug Administration (FDA) for treatment of CDI. Although previously recommended as a first-line treatment for CDI in adults, metronidazole is no longer recommended for severe CDI cases and is restricted to patients who are unable to obtain or be treated with vancomycin or fidaxomicin [5]. Treatment of CDI currently relies heavily on vancomycin or metronidazole. However, both drugs have drawbacks including high treatment failure and frequent recurrence of disease (in 25-30% of cases) [6, 7]. Moreover, both antibiotics induce dysbiosis (disrupting gut microbiota diversity), which enhances susceptibility to CDI [8]. Furthermore, treatment of CDI with vancomycin and metronidazole has been shown to promote the overgrowth of vancomycin-resistant enterococci (VRE) [9]. Fidaxomicin is less damaging to gut microbiota compared to both vancomycin and metronidazole [10]. However, the clinical outcome of fidaxomicin is still unsatisfactory as it pertains to treatment failure, especially in cases of relapsing CDI [11]. Additionally, treatment with fidaxomicin is restricted by its high cost, which is almost 150 times more expensive than metronidazole [12, 13]. Emerging resistance or reduced susceptibility to currently available anti-CDI antibiotics has further compounded the challenge to treat CDIs [14, 15]. Consequently, there is a critical need to identify and develop new, effective anticlostridial drugs.

De novo drug discovery is a time-consuming and highly expensive process that can take up to 15 years and can cost more than $2 billion to develop one new drug [16]. Drug repurposing is an attractive approach that can lessen the time and cost to develop new therapeutics compared to *de novo* drug discovery [17-25]. Utilizing a drug repurposing strategy, we identified auranofin, an FDA-approved antirheumaic drug[20, 26], as a potent anticlostridial agent capable of inhibiting production of both toxins and spores *in vitro* [23]. When investigated in an *in vivo* mouse model, auranofin (at clinically achievable doses) significantly protected mice from *C. difficile* challenge[24]. Additionally, auranofin significantly prevented CDI recurrence in mice when compared with vancomycin [24]. Building upon our previous studies, the main objectives of the study herein were to investigate the *in vivo* efficacy of auranofin in a CDI hamster model, establish

a dose-response relationship for auranofin in the hamster model of CDI, and to evaluate auranofin's ability to prevent CDI recurrence.

4.3 Materials and Methods

4.3.1 Media and reagents

Media and reagents were purchased commercially: reinforced clostridial medium (Becton, Dickinson and Company, Cockeysville, MD, USA), phosphate buffered saline (PBS) (Corning, Manassas, VA, USA), vancomycin hydrochloride (Hospira, Lake Forest, IL, USA), oxyrase (Oxyrase Inc, West Mansfield, OH, USA) and clindamycin hydrochloride (TCI America, Portland, OR, USA).

4.3.2 Preparation of *C. difficile* inoculum for hamsters infection

C. difficile VA11 (UNT103-1) was used to infect hamsters. This bacterial strain is a toxigenic clinical isolate responsible for outbreaks of CDI in North America [43, 44]. The *C. difficile* inoculum used for infection was prepared as described previously [43]. Briefly, resuspended bacteria from overnight plates (in reinforced clostridial medium + 1% oxyrase) were diluted to 1×10^7 CFU/mL. The bacterial inoculum was diluted, plated, and counted on reinforced clostridial medium before being used to infect hamsters.

4.3.3 *In vivo* efficacy of auranofin in a CDI ileocecitis hamster model

The two studies were performed as a service provided by the University of North Texas Health Science Center (Fort Worth, TX, USA). Male Golden Syrian hamsters (weighing 80 – 100 g) were housed in individually ventilated cages (2 per cage) and received food and water *ad libitum*. The CDI hamster model was performed as described previously [30, 33, 34, 45]. On day -1, all animals received a single subcutaneous injection of clindamycin (10 mg/kg). Twenty-four hours after clindamycin pre-treatment, hamsters were infected via oral gavage with 0.75 mL of the previously prepared *C. difficile* inoculum (~7.5×10^6 CFU/hamster), using a ball tip metal feeder. The bacterial inoculum used was re-counted after infection to confirm the infective dose.

Following infection, hamsters were randomly allocated into groups (n=10) for treatment. Twenty-four hours post-infection, two groups were treated with low doses of auranofin (0.125 mg/kg and 0.25 mg/kg), one group was treated with vancomycin (20 mg/kg), and one group was treated with the vehicle (10% DMSO in PBS). In a follow-up study, two groups of infected hamsters were treated with higher doses of auranofin (1 mg/kg and 5 mg/kg), one group was treated with vancomycin (20 mg/kg), and one group was treated with the vehicle (10% DMSO in PBS). Treatments were administered orally via oral gavage and were continued once daily for five days. Hamsters were observed throughout the duration of each experiment for signs of mortality and morbidity, the presence of diarrhea (wet tail), and overall appearance (activity, general response to handling, touch, ruffled fur). Hamsters were weighed every other day. Animals judged to be in a moribund state were euthanized. Criteria used to assign a moribund state included extended periods of weight loss, progression to an emaciated state, prolonged lethargy (more than 3 days), signs of paralysis, skin erosions or trauma, hunched posture, and a distended abdomen.

4.3.4 *In vivo* efficacy of auranofin in a relapsing CDI hamster model

Hamsters in each experiment were infected, as described above. In the first study, two groups were treated with low doses of auranofin (0.125 mg/kg and 0.25 mg/kg), one group was treated with vancomycin (20 mg/kg), and one group was treated with the vehicle (10% DMSO in PBS) for 5 days. In the second study, two groups were treated with higher doses of auranofin (1 mg/kg and 5 mg/kg), one group was treated with vancomycin (20 mg/kg), and one group was treated with the vehicle (10% DMSO in PBS) for 5 days. Thereafter, treatments were discontinued and hamsters were observed for disease symptoms, recurrence of CDI and signs of mortality and morbidity (described above) until the 21st day. Animals judged to be in a moribund state were euthanized. Animals that died during the observation period in each experiment were necropsied. Additionally, the contents of deceased hamsters' cecal tissues were diluted in PBS and plated anaerobically onto modified reinforced clostridial agar to obtain *C. difficile* CFU counts. After the end of each experiment, surviving hamsters from each experiment were humanely euthanized using CO_2 asphyxiation. The contents from each hamster's cecal tissues were diluted in PBS and plated onto modified reinforced clostridial agar to obtain CFU counts.

4.3.5 Statistical analyses

Kaplan-Meir survival data were analyzed using the logrank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test, utilizing GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, CA, USA). The cecal *C. difficile* CFU counts were analyzed via a one-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P<0.05$).

4.4 Results and Discussion

4.4.1 *In vivo* efficacy of auranofin in a *C. difficile* ileocecitis hamster model

Auranofin was previously reported to exhibit potent antibacterial and antivirulence activities against *C. difficile in vitro* [23]. Additionally, auranofin, at clinically achievable concentrations, was able to protect mice against *C. difficile* challenge [24]. These results encouraged us to investigate auranofin's efficacy in a *C. difficile* ileocecitis hamster model and auranofin's potential to protect hamsters from CDI recurrence. The hamster model is routinely used to evaluate therapeutics for treatment of CDI. CDI in hamsters exhibits key morphological features similar to CDI in humans such as colon enlargement, fluid accumulation and pseudomembrane formation. Additionally, dysbiosis induced by clindamycin treatment, which leads to proliferation of *C. difficile*, is observed both in hamsters and in humans. [27, 28]. In contrast, CDI in hamsters is rapidly fatal if left untreated, a pattern that is not characteristic of human CDI. Thus, the CDI hamster model can be considered as a prevention of death model [29].

The Golden Syrian hamster model was used to evaluate auranofin's ability to prevent ileocecitis induced by *C. difficile*, compared to vancomycin. The initial study investigated the activity of low doses of auranofin. Two groups of infected hamsters (n=10) were treated with 0.125 mg/kg or 0.25 mg/kg of auranofin. One group was treated with vancomycin (positive control) and the last group received the vehicle alone (negative control). Treatments were continued for 5 days during which hamsters were observed for disease symptoms. As shown in Fig. 4.1A, vehicle-treated hamsters exhibited 100% mortality by day 5 of the study, in agreement with previous reports [30-32]. Vancomycin (20 mg/kg) protected 100% of the infected hamsters up to 5 days, in coincidence with previous reports [30, 33, 34]. Hamsters administered 0.125 mg/kg of auranofin exhibited 40% survival by day 5. Auranofin, at 0.25 mg/kg, was more efficacious resulting in 60%

survival of infected hamsters on day 5, which was statistically significant compared to the vehicle-treated group.

We next tested the effect of higher doses of auranofin (1 mg/kg and 5 mg/kg) (Fig. 4.1B). After 5 days of auranofin (1 mg/kg) treatment, 60% of hamsters infected with *C. difficile* survived. On the other hand, administration of 5 mg/kg auranofin resulted in 100% survival of infected hamsters during the 5-day treatment period. It is worth mentioning that the results obtained in the *in vivo C. difficile* ileocecitis hamster model were slightly different from the results previously reported in the *in vivo* CDI mouse model [24]. This effect could be attributed to a difference in auranofin's pharmacokinetic profile between hamsters and mice. The rates of metabolism and excretion for auranofin may differ between hamsters and mice, which could lead to a difference in the drug's concentration at the infection site. This factor would need to be further explored in future studies. Another factor that might have contributed to the difference in results obtained between the hamster and mice studies is the overall surface area of the infection site. The site of infection is expected to be larger in hamsters compared to mice. Thus, we suspect that higher doses of auranofin were needed in hamsters to achieve a similar protective effect observed in mice.

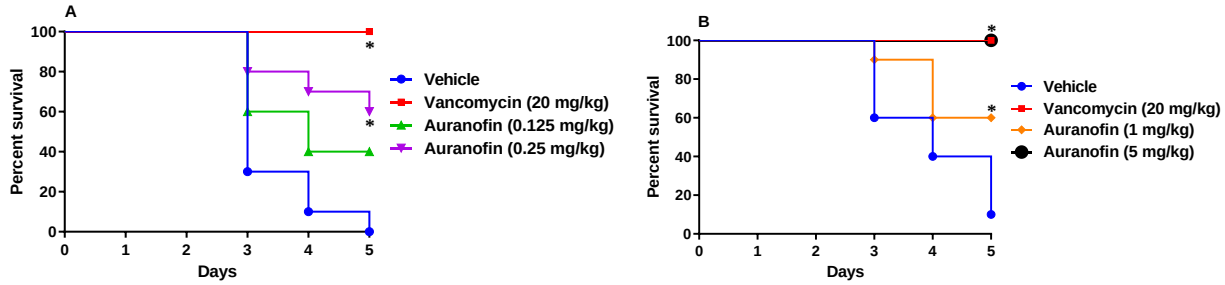

Figure 4.1 Efficacy of auranofin in treatment of CDI in hamsters: **A)** low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) and **B)** high doses of auranofin (1 mg/kg and 5 mg/kg). Hamsters were treated with auranofin, vancomycin (20 mg/kg), or the vehicle for 5 days after infection with *C. difficile* UNT103-1. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) and Gehan-Breslow-Wilcoxon tests ($P < 0.05$). An asterisk (*) denotes a statistically significant difference between hamsters treated with either auranofin or vancomycin in comparison with the vehicle-treated hamsters.

During the experiment, the average weight of surviving hamsters in each treatment group was measured every other day (Fig. 4.2A and 4.2B). In the first experiment (Fig. 4.2A), hamsters

in the vehicle-treated group experienced slight weight loss by day 4, but the weight loss was not statistically significant. No decrease in weight was observed for hamsters treated either with auranofin (at 0.125 mg/kg and 0.25 mg/kg) or vancomycin (Fig. 4.2A). Similarly, in the second experiment, hamsters treated with either auranofin (1 mg/kg and 5 mg/kg) or vancomycin did not exhibit signs of weight loss (Fig. 4.2B).

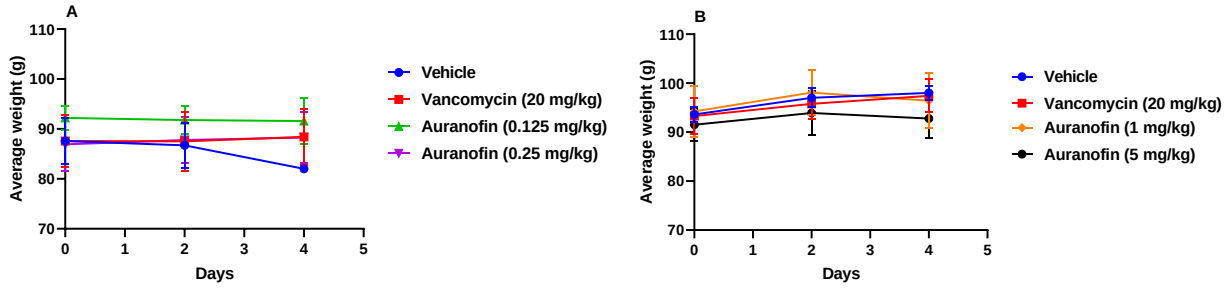

Figure 4.2 Average weight of surviving hamsters infected with *C. difficile* treated with: **A)** low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) and **B)** high doses of auranofin (1 mg/kg and 5 mg/kg).

Infected hamsters were treated with auranofin, vancomycin (20 mg/kg), or the vehicle for 5 days and weighed every other day. The data are presented as average weight (g) (mean ± standard deviation) for each group. A two-way ANOVA with post-hoc Dunnett's test for multiple comparisons ($P < 0.05$) found no significant difference between the average weight for each group after receiving treatment, as compared to before the start of treatment (day 0).

4.4.2 *In vivo* efficacy of auranofin in a relapsing CDI hamster model

One of the main problems associated with CDI is the high incidence of recurrence (in 15 to 50% of cases) following initial success with antibiotic treatment [35]. Relapsing CDI occurs due to the presence of *C. difficile* spores that germinate in the gut into vegetative cells that colonize the intestine and subsequently produce toxins [36, 37]. Recurrence of infection occurs in approximately 20% of patients [15, 38, 39]. Additionally, 20% of patients who experienced a relapsing episode of *C. difficile* reportedly died within 30 days of diagnosis [40]. Moreover, it was reported that up to 65% of patients successfully treated from CDI recurrence will relapse again in the future [41, 42]. Consequently, relapsing CDI represents a difficult and challenging problem facing healthcare systems that requires the discovery of new, more effective agents.

With this issue in mind, we sought to investigate the activity of auranofin in preventing *C. difficile* relapse in hamsters. We initially tested the activity of low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) in a relapsing CDI hamster model (Fig. 4.3A). This study was followed by another relapsing CDI hamster study investigating the activity of higher auranofin doses (1 mg/kg and 5 mg/kg) (Fig. 4.3B). In both studies, animals were infected with *C. difficile* and treatments were discontinued after 5 days. Hamsters were subsequently monitored for survival and possible CDI relapse.

As depicted in Fig. 4.3A, vehicle-treated hamsters became moribund following *C. difficile* challenge resulting in 100% mortality by day 5. This result is in agreement with previous studies [30-32]. Following discontinuation of treatment, vancomycin protected 100% of infected animals through day 12 (90% survival). By day 19, 80% of hamsters in the vancomycin treatment group were alive, which remained unchanged until the end of the experiment. On the other hand, animals administered auranofin (0.25 mg/kg) exhibited a recurrence rate of 50%. Three hamsters died during the post-treatment period resulting in 30% survival by the end of the experiment (statistically significant protection when compared to the vehicle-treated group). Auranofin (0.125 mg/kg) was slightly less efficacious with an overall survival of 20% (50% recurrence rate, as 2 out of 4 hamsters died after the discontinuation of treatment) (Fig. 4.3A).

We next tested the ability of auranofin at higher doses to prevent CDI recurrence. Two doses of auranofin (1 mg/kg and 5 mg/kg) were evaluated in addition to the vehicle (negative control) and vancomycin (standard-of-care antibiotic). As shown in Fig. 4.3B, CDI resulted in the mortality of vehicle-treated hamsters with 40%, 60%, 90% and 100% mortality observed on days 3, 4, 5 and 6, respectively. During the post-treatment stage, vancomycin protected all infected animals through day 13. Starting on day 14, a stepwise pattern of mortality was observed ultimately resulting in 60% survival (40% relapse) at the end of the experiment. This pattern is typically observed with vancomycin treatment [30, 42]. Auranofin (5 mg/kg) protected all infected hamsters after the discontinuation of treatment through day 8, at which point one hamster died. By day 10, 80% of hamsters treated with auranofin (5 mg/kg) were alive. By day 12, 60% of hamsters were alive, which was maintained until the end of day 21. On the other hand, auranofin (1 mg/kg) was less efficacious with 40% mortality observed during the treatment stage (until day 5). Following the discontinuation of treatment, 3 hamsters succumbed to relapsing CDI, which resulted in 30%

survival by the end of the experiment (statistically significant protection as compared to the vehicle-treated group) (Fig. 4.3B).

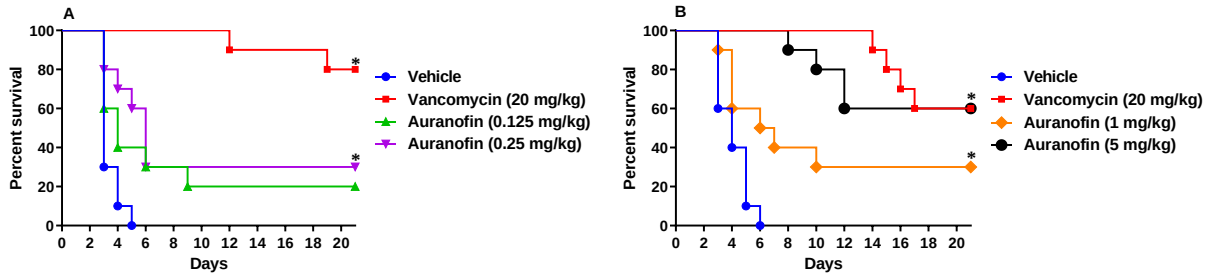

Figure 4.3 Efficacy of auranofin against CDI recurrence in hamsters: **A)** low doses (0.125 mg/kg and 0.25 mg/kg) and **B)** high doses (1 mg/kg and 5 mg/kg).

Hamsters were treated with auranofin, vancomycin (20 mg/kg), or the vehicle after infection with *C. difficile* UNT103-1. Treatments were discontinued after 5 days. Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) and Gehan-Breslow-Wilcoxon tests ($P < 0.05$). An asterisk (*) denotes a statistically significant difference between hamsters treated with either auranofin or vancomycin in comparison to the vehicle-treated hamsters.

Additionally, the average body weight results of the two experiments evaluating low and high doses of auranofin (Fig. 4.4A and .44B) found that hamsters treated with auranofin exhibited a slight loss in their average body weight through days 8-10. This was followed by an increase in body weight of hamsters until the end of the study. The initial weight loss was attributed to animals that died later in the study whereas hamsters that survived exhibited an overall increase in average body weight.

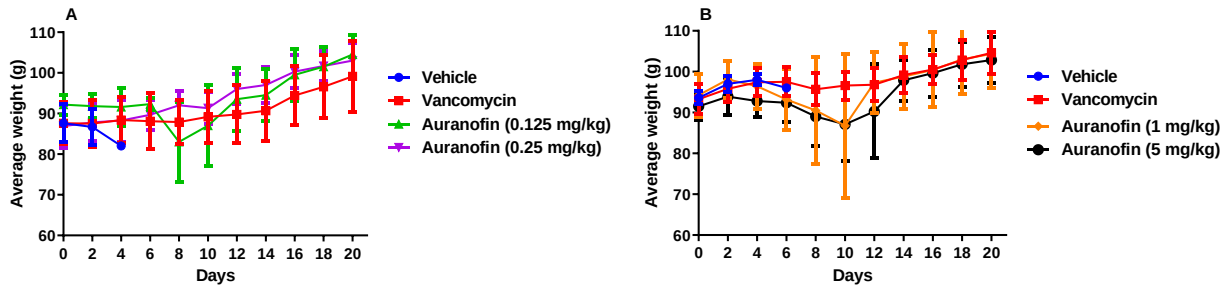

Figure 4.4 Average weight of all surviving hamsters in the CDI recurrence experiments: **A)** low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) and **B)** high doses of auranofin (1 mg/kg and 5 mg/kg).

Infected hamsters were treated with auranofin, vancomycin (20 mg/kg), or the vehicle for 5 days and treatments were discontinued thereafter. Hamsters were weighed every other day until the end of each experiment. The data are presented as average weight (g) (mean ± standard deviation) for each group.

After the conclusion of each experiment, hamsters were humanely euthanized and the cecal tissues were aseptically removed, homogenized, diluted and plated to determine the *C. difficile* CFU count inside each hamster's cecum.

Low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) were less effective in reducing the *C. difficile* counts inside the cecal tissues generating a 0.48-$\log_{10}$ reduction (with 0.125 mg/kg dose) and 1.2-$\log_{10}$ reduction (with 0.25 mg/kg dose), respectively (Fig. 4.5A). Statistical analysis of the data for auranofin (both at 0.125 mg/kg and 0.25 mg/kg) determined that this reduction in bacterial burden was not significant. Notably, two-thirds of hamsters in the auranofin (0.25 mg/kg) group that survived until the end of the study exhibited bacterial CFU counts in the ceca that were below the limit of detection (2.80 $\log_{10}$ (CFU/mL)). One hamster in the auranofin (0.125 mg/kg) group also exhibited a CFU count that was below the limit of detection. In contrast, vancomycin significantly reduced the bacterial CFU count by 3.1-$\log_{10}$, with 7 hamsters exhibiting bacterial CFU counts in the ceca that were below the limit of detection (Fig. 4.5A).

In the second experiment, evaluating the activity of higher doses, auranofin (5 mg/kg) was slightly superior to vancomycin in decreasing the burden of *C. difficile* in the cecal tissues of infected hamsters (Fig. 4.5B). Auranofin (5 mg/kg) significantly reduced the *C. difficile* CFU count generating a 3.15-$\log_{10}$ reduction. On the other hand, vancomycin (20 mg/kg) generated a 2.65-

log_{10} reduction. It is worth noting that 7 hamsters in the auranofin (5 mg/kg) group and 6 hamsters in the vancomycin group presented with *C. difficile* CFU counts that were below the limit of detection (2.80 log_{10} (CFU/mL)). Additionally, auranofin (1 mg/kg) significantly reduced the *C. difficile* CFU count by 1.75-log_{10}; 2 hamsters in this group exhibited bacterial CFU counts that were below the limit of detection.

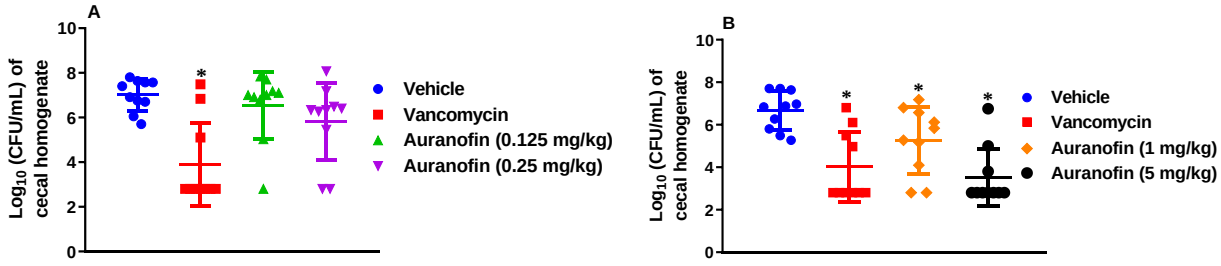

Figure 4.5 *C. difficile* UNT103-1 CFU counts in the cecal tissues of infected hamsters: **A)** low doses of auranofin (0.125 mg/kg and 0.25 mg/kg) and **B)** high doses of auranofin (1 mg/kg and 5 mg/kg).

Infected hamsters were treated with auranofin, vancomycin (20 mg/kg), or the vehicle for 5 days and treatments were discontinued thereafter. Bacteria were recovered from the cecal tissues of hamsters under anaerobic aseptic conditions, serially diluted, and plated. The data were analyzed via a one-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P<0.05$). An asterisk (*) denotes a statistically significant difference between hamsters treated with either auranofin or vancomycin in comparison to the vehicle-treated hamsters.

In conclusion, this study investigated the efficacy of auranofin *in vivo* in a CDI hamster model. Auranofin significantly protected hamsters against lethal CDI when administered at the doses of 1 mg/kg or 5 mg/kg. Furthermore, auranofin (5 mg/kg) was as effective as vancomycin in preventing CDI recurrence in hamsters. Interestingly, auranofin (5 mg/kg) was superior to vancomycin in reducing *C. difficile* counts present in the cecum of infected hamsters. These results indicate that auranofin merits further investigation as a supplement to the dry pipeline of anti-CDI therapeutics. Follow-up studies are warranted to investigate the efficacy of higher doses of auranofin and to evaluate the *in vivo* activity of auranofin in combination with other anti-CDI drugs.

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CHAPTER 5. ANTIVIRULENCE ACTIVITY OF AURANOFIN AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI: *IN VITRO* AND *IN VIVO* STUDIES

A version of this chapter has been reprinted with permission. Abutaleb, N. S. & Seleem, M. N. (2019). Antivirulence activity of auranofin against vancomycin-resistant enterococci: *in vitro* and *in vivo* studies. *Int J Antimicrob Agents,* 55(3), 105828. doi: 10.1016/j.ijantimicag.2019.10.009

5.1 Abstract

Vancomycin-resistant enterococci (VRE) are a leading cause of nosocomial infections due to the limited number of effective therapeutic options. In an effort to repurpose FDA-approved drugs against antibiotic-resistant bacteria, we identified auranofin as a potent drug against VRE. The present study determined that auranofin's antibacterial activity was not affected when evaluated against a higher inoculum size of VRE (~10^7 CFU/mL), and auranofin successfully reduced the burden of stationary phase VRE cells via a time-kill assay. In addition, auranofin reduced VRE production of key virulence factors including proteases, lipase and hemagglutinin. The promising features of auranofin prompted us to evaluate its *in vivo* efficacy in a lethal mouse model of VRE septicemia. All mice receiving auranofin at 0.125 mg/kg orally, 0.125 mg/kg subcutaneously (S.C.), or 0.0625 mg/kg S.C. survived the lethal VRE challenge. Additionally, auranofin was superior to linezolid, the current drug of choice, in reducing VRE burden in the liver, kidneys and spleen of mice. Remarkably, auranofin successfully reduced VRE below the limit of detection in murine internal organs after only four days of oral or subcutaneous treatment. These results indicate that auranofin warrants further investigation as a new treatment for systemic VRE infections.

Keywords: auranofin, septicemia, antivirulence, protease, lipase

5.2 Introduction

Multidrug-resistant enterococci, especially *Enterococcus faecalis* and *Enterococcus faecium*, have emerged as the leading cause of hospital-acquired infections since the 1980s [1]. Both species are associated with life-threatening infections including septicemia, endocarditis,

surgical site infections and urinary tract infections [2]. Both *E. faecalis* and *E. faecium* pose a major challenge in healthcare settings due to their ability to acquire or develop resistance to multiple antibiotics [3]. Two main mechanisms are involved in enterococcal resistance to antibiotics: a) intrinsic resistance to several antibiotics such as β-lactams and aminoglycosides, and b) ability to acquire resistance to glycopeptides, fluoroquinolones, tetracyclines, macrolides and streptogramins via horizontal gene transfer through transposons and plasmids [4].

Vancomycin has been a mainstay of treatment for enterococcal infections; however, vancomycin-resistant enterococci (VRE) isolates have emerged causing various infections that are difficult to be treated. As a direct consequence, VRE, in particular vancomycin-resistant *E. faecium*, are listed as a high priority pathogen by the World Health Organization for which new antibiotics are urgently needed [5].

Repurposing FDA-approved drugs is a novel way to reduce both the time and cost associated with antimicrobial innovation [6-8]. Using this approach, we previously identified auranofin as a potent antimicrobial agent against VRE *in vitro* and in a mouse model of VRE decolonization [9]. Auranofin was initially approved as a treatment for rheumatoid arthritis and has a well-studied safety profile with rare, mild and self-limiting adverse effects reported. Currently auranofin is undergoing Phase II clinical trials for the treatment of amoebic dysentery, giardiasis (ClinicalTrials.gov Identifier: NCT02736968) and tuberculosis (ClinicalTrials.gov Identifier: NCT02968927) demonstrating its potential to be repurposed for other diseases.

The goal of this study was to evaluate the antimicrobial activity of auranofin against standard and high inocula of VRE, assess auranofin's ability to inhibit production of proteases, lipase, and hemagglutination, and to investigate the efficacy of auranofin to enhance survival of mice in a VRE septicemia model. Results obtained further support the potential of auranofin to be repurposed as a novel antibacterial agent to treat VRE infections.

5.3 Materials and Methods

5.3.1 Bacterial strains and reagents

Auranofin, linezolid (Chem-Impex International, Wood Dale, IL, USA) and vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO, USA) were purchased from commercial vendors. Skim milk powder was purchased from Oxoid (Basingstoke, Hants, UK). Brain heart

infusion (BHI), tryptic soy broth (TSB), Tryptic soy agar (TSA) and enterococcosel agar were purchased from Becton, Dickinson and Company (Cockeysville, MD, USA) and phosphate-buffered saline (PBS) was purchased from Corning (Manassas, VA, USA). Egg yolk emulsion was purchased from HIMEDIA Laboratories (Thane, MH, India). Defibrinated horse blood was obtained from Hemostat Laboratories (Dixon, CA, USA). Clinical isolates of VRE (Table 5.2S) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) and the American Type Culture Collection (ATCC).

5.3.2 Effect of VRE inoculum size on the minimum inhibitory concentration of auranofin

The impact of VRE inoculum size on the minimum inhibitory concentration (MIC) of auranofin was determined using the broth microdilution assay following the guidelines of the Clinical and Laboratory Standards Institute [10]. Briefly, standard inoculum (SI: 5×10^5 CFU/mL) and high inoculum (HI: 5×10^7 CFU/mL) of each strain of VRE were prepared and tested against auranofin and linezolid.

5.3.3 Time-kill assay of auranofin against vancomycin-resistant *E. faecium* stationary phase cells

A time-kill assay was conducted for auranofin and linezolid against *E. faecium* NR-31909 stationary phase cells, as described previously [11]. Briefly, an overnight culture of *E. faecium* grown in TSB (~ 5×10^9 CFU/mL) was subsequently incubated with drugs (in triplicates) at 10 × MIC for 24 hours. After 12 and 24 hours, aliquots were diluted in PBS and plated onto BHI agar plates. Plates were incubated at 37°C for 18 hours before counting colonies to determine viable CFU/mL. Data are presented as average $\log_{10}$ (CFU/mL) of VRE stationary phase cells at the indicated time points. Data were analyzed via a two-way ANOVA with post-hoc Dunnett's test ($P < 0.05$) for multiple comparisons.

5.3.4 Protease inhibition assay

The modified skimmed milk method was utilized to investigate the protease inhibitory activities of auranofin and linezolid, as described previously [12]. Initially, the ability of strain *E. faecium* NR-31909 to produce proteases was confirmed by inoculation onto TSA containing 3%

skimmed milk and incubating at 37°C for 24 hours. The presence of a transparent zone around the colonies indicated the strain produced proteases [13]. In brief, an overnight culture of *E. faecium* NR-31909 incubated in TSB containing 0.25 × MIC of auranofin, linezolid or DMSO (negative control) (in triplicates) was centrifuged at 10,000 × *g* for 10 minutes. A total volume of 500 µL of culture supernatant was incubated with 1 mL skim milk (1.25%) at 37°C for 30 minutes. Afterwards, the OD_{600} was measured to indicate the degree of clearance of skim milk. Data are presented as percent protease production in the presence of each drug. DMSO (the solvent of the drugs) served as a control to determine the total protease production by the bacteria. TSB containing skim milk was used as a negative control to determine the OD values in the absence of protease production). Data were analyzed via unpaired Student t test ($p<0.05$). Auranofin was compared to untreated (*) and to linezolid (#).

5.3.5 Lipase inhibition activity

The ability if auranofin to inhibit *E. faecium* lipase production was detected as described previously [14], with some modifications. First, *E. faecium* NR-31909 was confirmed for the ability to produce lipase by culturing on TSA supplemented with egg yolk emulsion (10%) and incubated at 37°C for 24 to 48 hours. The formation of an opaque zone around colonies indicated lipolytic activity. An overnight culture of *E. faecium* NR-31909 grown in TSB containing 0.25 × MIC of auranofin, linezolid or DMSO (negative control) (in triplicates) was centrifuged at 10,000 × *g* for 10 minutes. An aliquot (500 µL) of supernatant was taken (in duplicates) and incubated with 1 mL of 10% egg yolk emulsion at 37°C for 30 minutes. The OD_{600} was measured to detect the lipolytic activity of *E. faecium* in the presence of drugs. The data are presented as percent lipase production relative to DMSO. DMSO served as a negative control to determine the total bacterial lipase production. Data were analyzed via unpaired Student t test ($p<0.05$). Auranofin was compared to untreated (*) and to linezolid (#).

5.3.6 Hemagglutination inhibition assay

To study the effects of auranofin and linezolid on the adhesive properties of hemagglutinins (involved in bacterial invasion) produced by enterococci, a hemagglutination inhibition assay was employed, as described before [15, 16]. *E. faecium* NR-31909 was first screened for the

hemagglutination assay using horse blood. Briefly, red blood cells (RBCs) were collected by washing 5 mL of horse blood with 10 mL of PBS four times with subsequent centrifugation at 2,000 × *g* for 3 minutes. The supernatant was subsequently removed without disrupting the RBCs. Afterward, a 10% RBC suspension was prepared in PBS, diluted 10-fold, and a total volume of 50 µL was added to a round bottom 96-well plate. A total volume of 50 µL of the overnight bacterial supernatant (prepared as described in the protease assay above) was added to the RBCs suspension. Plates were then incubated at room temperature for one hour. Negative hemagglutination results will appear as dots in the center of the well. Positive hemagglutination results will form a uniform reddish color across the well.

After confirming *E. faecium* NR-31909 produced hemagglutinins, the ability of auranofin and linezolid to interfere with production of hemagglutinins was investigated. Briefly, an overnight culture of *E. faecium* NR-31909 grown in TSB containing subinhibitory concentrations of auranofin, linezolid or DMSO (negative control) (in triplicate) was centrifuged at 4700 RPM for 10 minutes. A total volume of 50 µL of culture supernatant was incubated with 50 µL of 1% RBCs suspension in PBS at room temperature for one hour. DMSO served as a negative control (i.e. positive for hemagglutination).

5.3.7 *In vivo* mouse peritonitis animal model

The study was reviewed, approved and performed under the guidelines of the Purdue University Animal Care and Use Committee and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Each group of mice was housed in an individually ventilated cage and received food and water ad libitum throughout the duration of the experiment. The VRE mouse peritonitis lethal infection model was used, as described previously [17, 18], with some modifications. Eight-week-old female BALB/c mice (Jackson, ME, USA) were used for this study. *E. faecium* NR-31909 (a strain isolated from a human patient with bacteremia) was chosen for this study. Each group of mice (n = 5) was infected intraperitoneally with (LD_{50} ~2.5×10^7 CFU/mL) of *E. faecium* NR-31909 premixed with 20% sterile rat fecal extract (SRFE) in the ratio of 2:1. One hour post-infection, three groups were treated orally with auranofin (0.125 mg/kg, 0.25 mg/kg and 0.5 mg/kg), and three groups were treated subcutaneously (S.C.) with auranofin (0.0625 mg/kg, 0.125 mg/kg and 0.25 mg/kg). One group was treated orally with linezolid (20 mg/kg). Two groups were

treated with the vehicle (10% DMSO in PBS orally and S.C.). Treatments were continued once daily for four days before the mice were humanely euthanized five days post-infection. Bacteria were recovered from the liver, kidneys and spleen of mice under aseptic conditions, serially diluted in PBS, and plated on enterococcosel agar supplemented with vancomycin (8 μg/mL). Plates were then incubated for 48 hours at 37°C to determine VRE burden in each organ. The data were analyzed via one way ANOVA with post hoc Dunnett's test for multiple comparisons. (*) denotes statistical significant difference between the results obtained for auranofin or linezolid in comparison to the untreated group ($P < 0.05$). (#) denotes statistical significant difference between the results obtained for auranofin in comparison to linezolid ($P < 0.05$).

5.3.8 Statistical analyses

GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla CA) was used to conduct the statistical analyses presented in this study.

5.4 Results

5.4.1 The effect of inoculum size on the MIC of auranofin against VRE

VRE are known to colonize the intestinal tract in large populations. Consequently, the antibacterial activity of auranofin and linezolid against two different inoculum sizes of VRE: a standard inoculum (SI) (5×10^5) and high inoculum (HI) (5×10^7 CFU/mL) was investigated. As depicted in Table 5.1, the MIC_{90} of auranofin did not change after increasing the VRE inoculum size from 5×10^5 CFU/mL to 5×10^7 CFU/mL. In contrast, the MIC_{90} of linezolid against VRE increased by one-fold in agreement with a previous report [19].

Table 5.1 Minimum inhibitory concentration (MIC, µg/mL) of auranofin and linezolid against clinical isolates of vancomycin-resistant *E. faecium* and *E. faecalis* at standard and high inocula

Strains	**MIC (µg/mL)**			
	Auranofin		**Linezolid**	
	SI	**HI**	**SI**	**HI**
***E. faecium* NR-31916**	0.5	0.5	1	1
***E. faecium* ATCC 700221**	0.5	0.5	0.5	1
***E. faecium* NR-32054**	0.5	0.5	1	2
***E. faecalis* NR-31971**	0.5	0.5	1	2
***E. faecium* HM-952**	1	1	1	2
***E. faecium* NR-32065**	0.5	0.5	1	2
***E. faecium* NR-32094**	1	1	1	2
***E. faecalis* NR-31887**	1	1	1	2
***E. faecalis* HM-201**	1	1	1	1
***E. faecalis* HM-934**	1	1	1	2
***E. faecalis* NR-31970**	1	1	1	2
***E. faecium* HM-968**	1	1	1	2
***E. faecalis* HM-335**	1	1	1	2
***E. faecium* HM-965**	1	1	1	2
***E. faecium* NR-31909**	1	1	1	1
MIC_{90}	**1**	**1**	**1**	**2**

SI, standard inoculum (~5×10^5 CFU/mL); HI, high inoculum (~5×10^7 CFU/mL); MIC_{90}, the concentration of the test agent that inhibited the growth of 90% of the tested strains

5.4.2 Evaluation of the antibacterial activity of auranofin against vancomycin-resistant *E. faecium* stationary phase cells via a time-kill assay

After confirming the antibacterial activity of auranofin remained consistent against a high inoculum size of VRE, the drug's activity against stationary phase VRE cells was examined via a time-kill assay. These cells are highly resistant to most antibiotics, in part because most antibiotics target metabolic processes expressed by cells actively dividing (i.e. in logarithmic stage of growth). Auranofin significantly reduced stationary phase VRE cells by 0.64-log_{10} after 12 hours and by 1-log_{10} after 24 hours (Fig. 5.1). In contrast, linezolid was ineffective against stationary

phase cells as the bacterial CFU increased by 0.92-$\log_{10}$ after 12 hours and increased by 1.14-$\log_{10}$ after 24 hours. The result for linezolid is similar to what has been previously reported [20]. These results indicate that auranofin is superior to linezolid at reducing the burden of VRE stationary phase cells *in vitro*.

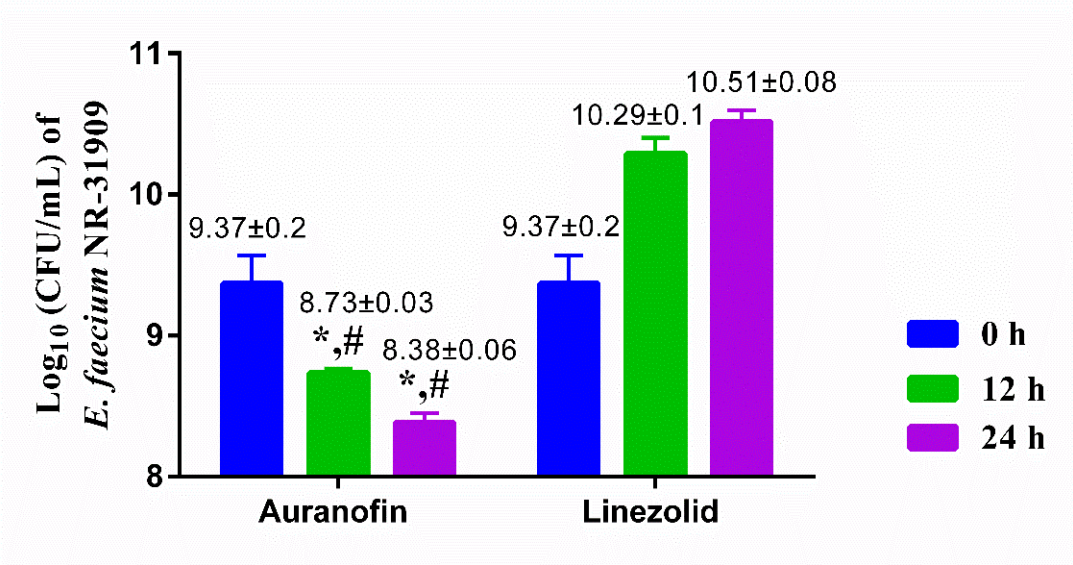

Figure 5.1 Time-kill kinetics assay of auranofin and linezolid against stationary phase vancomycin-resistant *Enterococcus faecium* NR-31909.

Bacteria were incubated with test agents, and samples were collected at 0, 12- and 24-h incubation period. The error bars represent standard deviation values obtained from triplicate samples used for each agent studied. (*) represents significant difference from 0 time. # represents significant difference from linezolid (*, $^{\#}$ $P < 0.05$). Data were analyzed with two way ANOVA with post hoc Dunnett's test.

5.4.3 Protease inhibition assay

Bacteria secrete proteases to invade and damage host epithelial cells. The ability of auranofin and linezolid to inhibit protease production in *E. faecium* NR-31909 was studied. Auranofin, at 0.25 × MIC, significantly outperformed linezolid in inhibiting total protease production ($P < 0.0001$) (Fig. 5.2). Auranofin inhibited the total protease production of *E. faecium* NR-31909 by 69.7%. Linezolid (which is known to inhibit protein synthesis) [21] inhibited 55.3% of the total proteases produced by the bacterial strain.

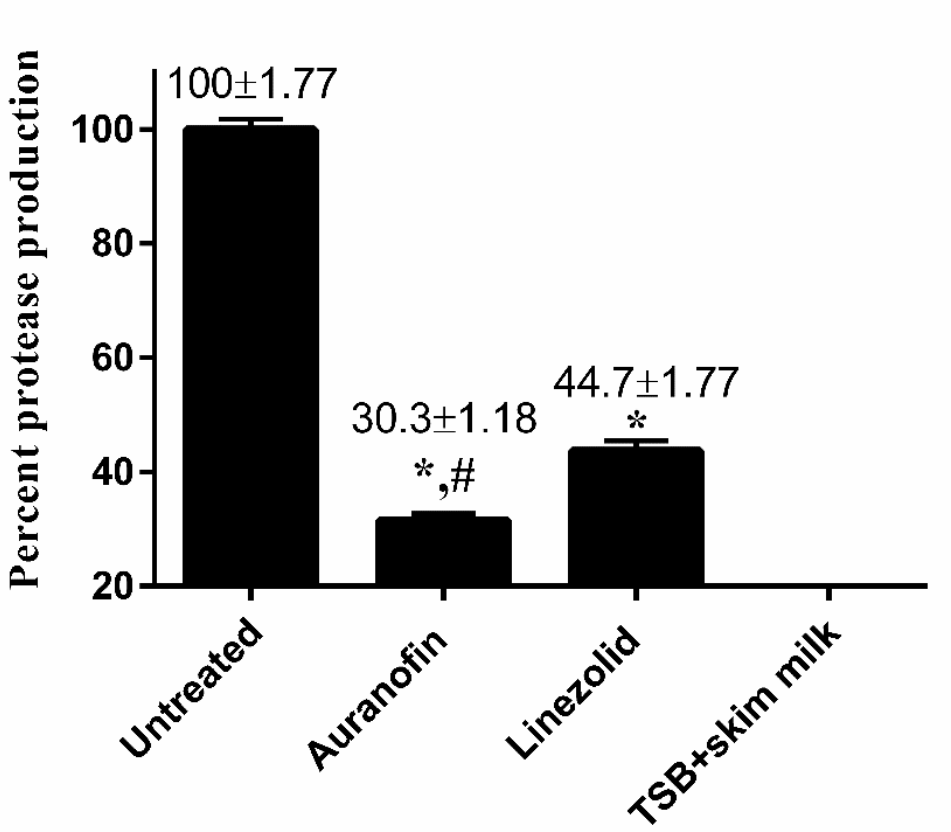

Figure 5.2 Total protease inhibition activity of auranofin and linezolid against vancomycin-resistant *E. faecium* NR-31909.

Data are presented as percent protease production of each drug (tested in sixruplicate). TSB with skim milk served as a negative control. Data were analyzed via unpaired Student t test ($p<0.05$). Auranofin was compared to untreated (*) and to linezolid (#).

5.4.4 Lipase inhibition activity

In addition to the secretion of proteases, VRE produce lipase which damages the host's epithelium and permits tissue invasion [13]. Given auranofin inhibited VRE protease production, the drug's ability to inhibit lipase produced by VRE was also investigated. Auranofin was once again significantly superior to linezolid in reducing VRE lipase production (*P* value of 0.0006) (Fig. 5.3). At 0.25 × MIC, auranofin reduced lipase production by 41.5%, while linezolid inhibited 35.4% of lipase production by *E. faecium* NR-31909.

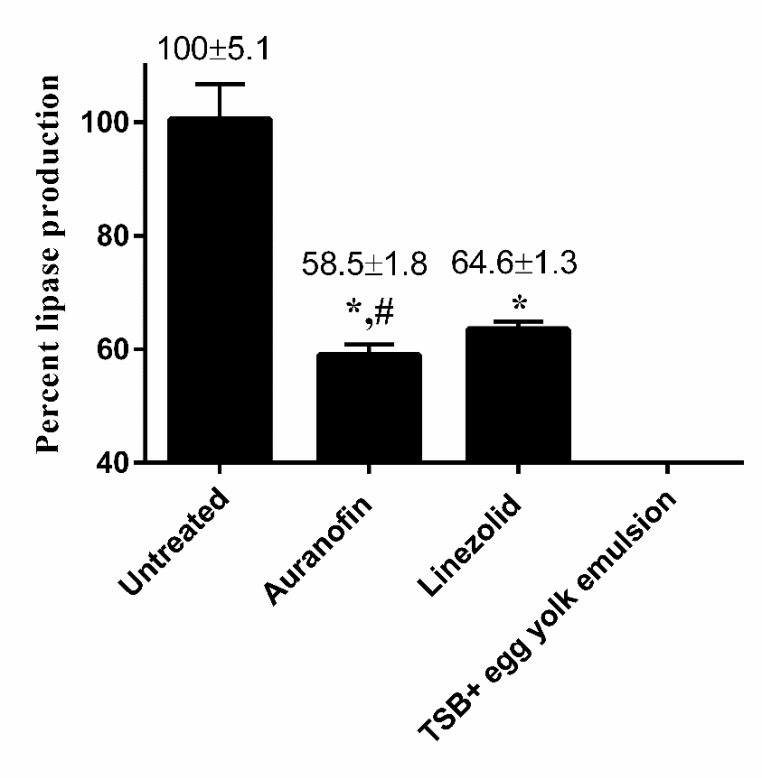

Figure 5.3 Lipase inhibition activity of auranofin and linezolid against vancomycin-resistant *E. faecium* NR-31909.

Data are presented as percent lipase production in presence of each drug (tested in sixruplicate). TSB with egg yolk emulsion served as a negative control. Data were analyzed via unpaired Student t test ($p<0.05$). Auranofin was compared to untreated (*) and to linezolid (#).

5.4.5 Hemagglutination inhibition assay

Hemagglutination properties are highly common in different enterococcal species, especially VRE [22]. Due to their adhesive properties, hemagglutinins aid in bacterial invasion leading to damage of host epithelial tissue [13]. To evaluate auranofin's ability to inhibit hemagglutination induced by VRE, a hemagglutination inhibition assay was performed. As depicted in Fig. 5.4, auranofin, at subinhibitory concentrations, was able to inhibit hemagglutination induced by *E. faecium* NR-31909. Linezolid was unable to achieve the same effect. Different subinhibitory concentrations of auranofin were evaluated to determine the lowest concentration capable of inhibiting hemagglutination. Auranofin successfully inhibited VRE hemagglutination at a concentration as low as 0.0625 µg/mL.

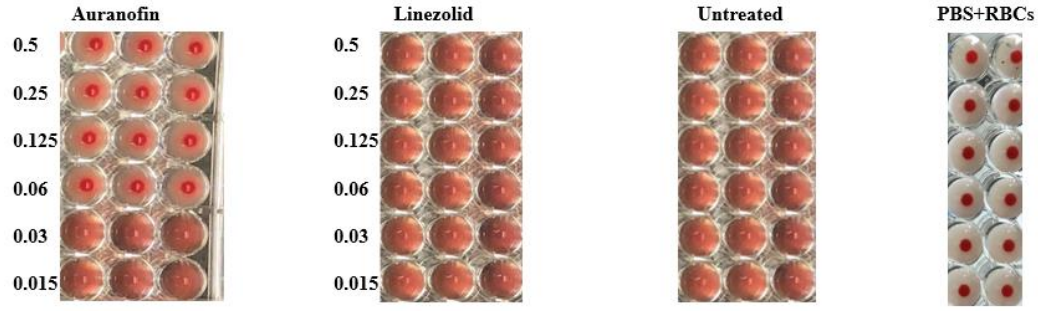

Figure 5.4 Hemagglutination inhibitory activities of auranofin and linezolid against *E. faecalis* NR-31909.

Sub-inhibitory concentrations (starting from 0.5 µg/mL to 0.015 µg/mL) of the drugs were added with bacterial supernatant and RBCs, and then incubated for 1 hour at 37 °C. Positive hemagglutination inhibition results appear as dots in the center of round-bottomed plates. Negative hemagglutination inhibition results appear as a uniform reddish color across the well.

5.4.6 *In vivo* murine peritonitis model

The promising *in vitro* antibacterial and antivirulence results led us to evaluate the ability of auranofin to treat septicemia induced by vancomycin-resistant *E. faecium* in a murine peritonitis model [17, 18]. As presented in Fig. 5.5, auranofin, at the same dose administered to human patients for treatment of rheumatoid arthritis (0.125 mg/kg orally), significantly protected 100% of the mice from a lethal challenge of VRE. Interestingly, higher oral doses of auranofin (0.25 mg/kg and 0.5 mg/kg) significantly protected only 80% of the mice. The same pattern was also observed with auranofin groups treated S.C. Auranofin administered at 0.25 mg/kg S.C. protected only 80% of the mice, while lower doses (0.0625 mg/kg and 0.125 mg/kg) of auranofin protected 100% of the mice. Linezolid (20 mg/kg) successfully protected 100% of the mice. Auranofin was significantly superior to linezolid in decreasing the burden of VRE in internal organs (liver, kidneys and spleen) of infected mice (Fig. 5.6). Remarkably, auranofin reduced VRE burden below the limit of detection (100 CFU/gm) from the liver, kidneys, and spleen of all mice after only four days of oral or S.C. treatment. Linezolid, in contrast, reduced VRE burden by approximately 1.64-$\log_{10}$, 1.38-$\log_{10}$ and 1.72-$\log_{10}$ in the liver, kidneys and spleen of mice, respectively.

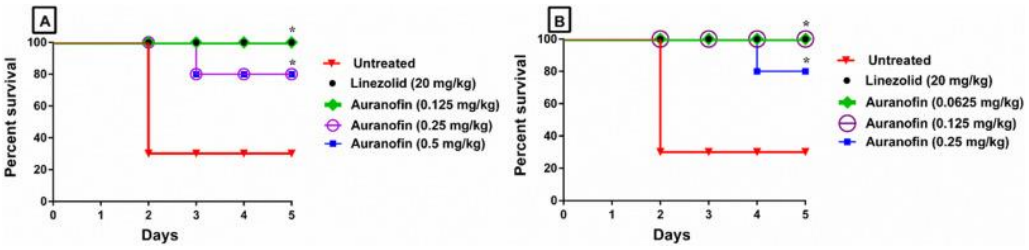

Figure 5.5 *In vivo* antibacterial activity of auranofin against *E. faecium* NR-31909 in the murine septicemia model when administered (A) Orally at 0.125 mg/kg, 0.25 mg/kg and 0.5 mg/kg; and (B) Subcutaneously at 0.0625 mg/kg, 0.125 mg/kg and 0.25 mg/kg compared to the vehicle control and the standard antibiotic linezolid given orally at 20 mg/kg.

Mice survival was monitored for 5 days. Results were analyzed for statistical difference utilizing graphpad prism. (*) Denotes significant difference between each treated group and the untreated group ($P < 0.05$). minimum inhibitory concentration (MIC, µg/mL) of auranofin and control drugs against vancomycin-resistant *Enterococcus faecium* isolates.

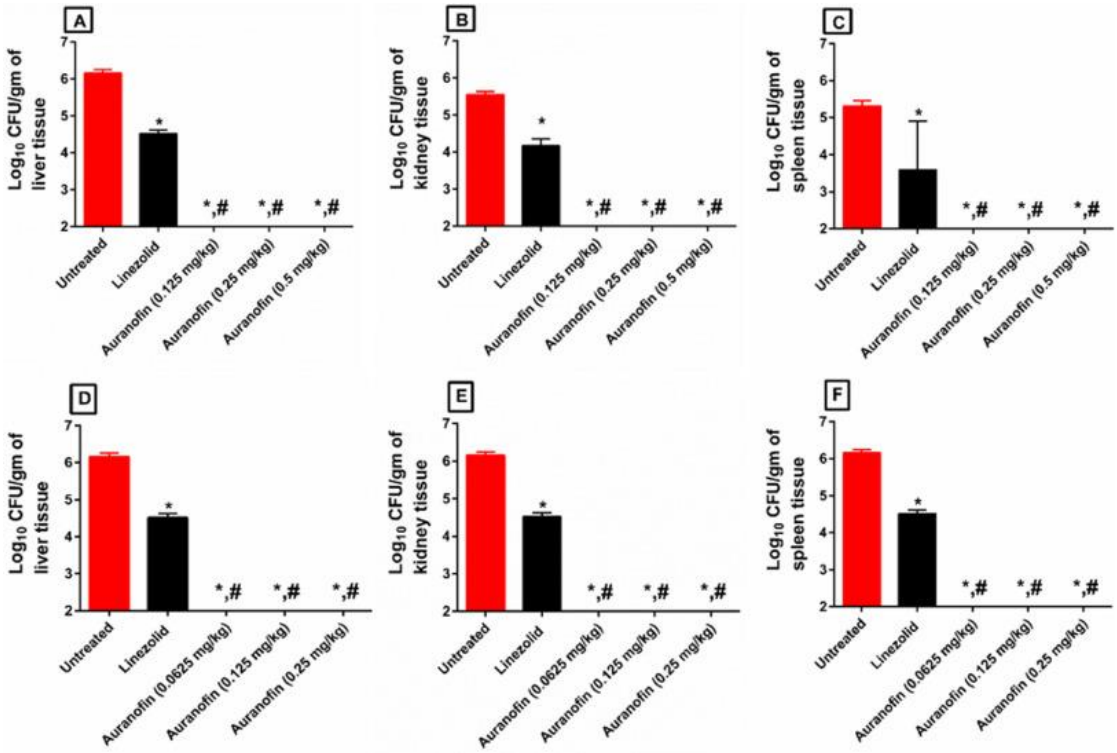

Figure 5.6 VRE counts in (A) liver, (B) kidney, and (C) spleen of the infected mice (5/group) orally treated with auranofin, and (D) liver, (E) kidney, and (F) spleen of the infected mice (5/group) subcutaneously treated after 4 days.

Auranofin was compared to untreated (*) and to linezolid (#). ($P < 0.05$).

5.5 Discussion

Enterococci are normal inhabitants of the gastrointestinal tract, and VRE colonization is often followed by translocation across human epithelial cells leading to systemic infections including sepsis, urinary tract infections, endocarditis and surgical site infections [9, 23]. The U.S. Centers for Disease Control and Prevention (CDC) has identified VRE as a serious public health threat because it is responsible for more than 20,000 infections annually leading to more than 5% of all deaths attributed to an antibiotic-resistant infection in the U.S. [24].VRE are a leading cause of nosocomial infections and limited therapeutic options are available [1].

Unlike for most Gram-positive pathogens, cell-wall inhibiting antibiotics, such as ampicillin or vancomycin, exhibit bacteriostatic activity against VRE. Bactericidal activity requires a combination of a cell wall inhibitor and an aminoglycoside. However, most enterococcal strains (especially VRE) are resistant to β-lactams and aminoglycosides [25]. Furthermore, enterococci, in general, and VRE specifically, have an uncanny ability to acquire or develop resistance to multiple antibiotics [3]. Most seriously, VRE strains have been isolated that exhibit resistance to last-resort antibiotics, such as linezolid, quinupristin-dalfopristin, daptomycin, tigecycline and oritavancin [20]. Given the dearth of effective therapeutic options, there is an urgent need to develop novel antimicrobial agents to treat VRE infections.

In an intensive search for new drugs to combat VRE infections, our group previously discovered niclosamide, auranofin and ebselen as effective VRE decolonizing agents [9, 26, 27]. Building upon our previous work, this study investigated auranofin's ability to inhibit production of key virulence factors utilized by VRE to promote infection. Furthermore, auranofin's ability to protect mice *in vivo* in a lethal VRE septicemia model was evaluated.

One of the major challenges associated with VRE infection is that they overgrow and colonize the human intestine in large numbers upon the disturbance of the normal microbiota [26, 27]. VRE typically colonize the gastrointestinal tract of humans in a larger population than the inoculum size used for standard antibacterial susceptibility assays. This higher inoculum size has a negative effect on the antibacterial activity of certain antibiotics. For example, increasing the inoculum size of enterococci resulted in increased MIC values for several antibiotics, in previous reports, such as piperacillin and piperacillin-tazobactam [28], vancomycin and teicoplanin [29] and daptomycin, linezolid, gentamicin and rifampin [19]. Consequently, higher doses of these antibiotics are needed to successfully suppress VRE growth. Thus, we first confirmed auranofin is

capable of inhibiting a high inoculum of VRE. Inoculum effect testing is typically performed using an inoculum 100-fold greater than the CLSI-recommended inoculum, as discussed previously [30].

In our study, increasing the inoculum size of VRE from 10^5 CFU/mL to 10^7 CFU/mL did not affect the MIC values of auranofin. In contrast, the MIC values for linezolid increased when evaluated against a higher inoculum size of VRE. This characteristic of auranofin is highly advantageous for treatment of severe VRE infections such as sepsis.

Next, a time-kill assay was utilized to study the killing kinetics of auranofin against stationary phase enterococcal cells. Stationary phase cells are well-known to be highly resistant to most antibacterial drugs. These cells contain a high percentage of persisters that are considered as the reason behind recurring infections and a major cause of drug resistance [31, 32]. Against stationary phase cells, auranofin was superior to linezolid in reducing the burden of VRE in the time-kill assay after 12 and 24 hours. Linezolid, in agreement with previous report [20], was ineffective at reducing VRE burden.

After confirming that auranofin's antibacterial activity was not affected by a high inoculum of VRE or VRE in stationary phase, auranofin's ability to inhibit key virulence factors that promote infections was investigated. VRE produces multiple virulence factors, such as proteases, gelatinase, lipase, hemolysin, hemagglutinin, and DNase, which lead to cell damage and progression of infection [13]. Proteases are considered one of the most important virulence factors produced by bacteria. Proteases have been shown to damage immunoglobulins, which protect mucous membranes, as well as disrupt tight junctions between host epithelial cells, leading to tissue invasion and damage [33]. Proteases can also cleave secreted toxins to regulate the abundance of virulence factors depending on the specific niche encountered within the host [34]. Additionally, it has been reported that gelatinase (a protease produced by enterococci) played an essential role in the progression and severity of infection in an *E. faecalis* endocarditis animal model [35]. Another study found a strong correlation between protease secretion and virulence in a murine model of enterococcal catheter-associated urinary tract infection [36]. In addition to proteases, lipases play an essential role in microbial nutrient acquisition through digesting lipids, especially when grown in carbohydrate-restricted environments [37]. In addition, lipases promote adhesion by degrading host surface molecules; the released free fatty acids are postulated to increase unspecific hydrophobic interactions with the host receptors [38].

Auranofin was previously reported to inhibit protein synthesis and virulence factors (namely toxin production) expressed by *Staphylococcus aureus* [39]. Consequently, we hypothesized that auranofin, at subinhibitory concentrations, would be able to inhibit virulence factors expressed by VRE. To test this hypothesis, the inhibitory activities of auranofin against VRE protease and lipase production was evaluated. Auranofin significantly outperformed linezolid in reducing the VRE protease and lipase production. These results collectively indicate that auranofin can reduce production of both proteases and lipase in VRE which may curb the pathogen's ability to attach and invade host tissues.

In addition to the secretion of lipase and proteases, hemagglutination of RBCs is another important virulence factor utilized by VRE. Bacterial binding to host tissues is dependent on microbial surface adhesins that recognize specific receptors on the host cell surface. Upon binding to the host, these bacterial adhesins cause agglutination of RBCs [40]. It has been reported that enterococci possess characteristic thermostable proteinaceous agglutinins capable of agglutinating rabbit, human and sheep RBCs, indicating the diversity in the surface structures involved in enterococcal adhesion [13]. The protein synthesis-inhibiting activities of auranofin and its adherence-inhibition activity (protease and lipase inhibitions) prompted us to evaluate the drug's hemagglutination-inhibition activity. As expected, auranofin inhibited the hemagglutination induced by *E. faecium* NR-31909 at a concentration as low as 0.0625 µg/mL. These results indicate auranofin has potent antivirulence activity and may be capable of interfering with VRE attachment to and invasion of host tissues.

The potent antibacterial and antivirulence activity of auranofin against VRE *in vitro* prompted us to evaluate its effectiveness in a murine VRE septicemia model. VRE bloodstream infections, in most cases, lead to infective endocarditis that can be fatal in up to 46% of cases [41]. Different doses of auranofin administered either orally or S.C. were evaluated to determine the most effective dose and route of administration. Interestingly, lower doses of auranofin (0.0625 mg/kg S.C. and 0.125 mg/kg oral and S.C.) protected 100% of the mice, while higher doses (0.25 mg/kg S.C. and oral and 0.5 mg/kg oral) protected 80% of the mice (considered a non-significant difference relative to mice treated with linezolid). Most importantly, auranofin was superior to linezolid in decreasing the burden of VRE in the internal organs (liver, kidneys and spleen) of infected mice. Remarkably, auranofin reduced VRE below the limit of detection from all three internal organs evaluated, after only four days of oral or S.C. treatment. In contrast, linezolid (20

mg/kg) did not efficiently clear VRE in internal organs. This pattern is postulated to be due to its bacteriostatic activity that could be the reason behind its lower efficacy in reducing VRE burden in internal organs, in agreement with previous studies [9, 26, 27]. It is worth noting that the oral dose of auranofin that provided 100% protection to mice is clinically achievable in humans.

In conclusion, the current study highlights that auranofin has potent *in vitro* antivirulence activity and *in vivo* activity against VRE and warrants further investigation as a novel treatment option for systemic VRE infections.

5.6 References

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5.7 Supplemental material

Table 5.2S Description of clinical isolates of vancomycin-resistant enterococci (VRE) used in this study

VRE strain	ID number	Source/description
E. faecium	NR-31916	Isolated in 1996 from turkey faeces in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecium	ATCC 700221	Isolated from human faeces, Connecticut. Resistant to vancomycin and teicoplanin.
E. faecium	NR-32054	Isolated in 2008 from swine faeces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin.
E. faecalis	NR-31971	Urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin and vancomycin.
E. faecium	HM-952	Human isolate from the USA. Resistant to vancomycin.
E. faecium	NR-32065	Isolated in 1994 in Aix-en-Provence, France. Resistant to vancomycin.
E. faecium	NR-32094	Isolated in 1996 in New York, USA. Resistant to vancomycin.
E. faecalis	NR-31887	Isolated from human blood in 1987 in USA. Resistant to gentamicin.
E. faecalis	HM-201	Isolated in January 2002 from the blood of a patient with endocarditis at Stamford Hospital, Connecticut, USA. Resistant to vancomycin.
E. faecalis	HM-934	Isolated from human secretion in Bogota, Colombia (2006). Resistant to vancomycin.
E. faecalis	NR-31970	Isolated from a urine sample in Michigan, USA (2001). Resistant to erythromycin and gentamicin.
E. faecium	HM-968	Isolated from human sputum in Colombia (2006). Resistant to ampicillin and vancomycin, and displays high-level resistance to streptomycin.
E. faecalis	HM-335	Isolated from the blood of a 64-year-old female haemodialysis patient with fatal bacteraemia after treatment with daptomycin (2004). Resistant to daptomycin and vancomycin.
E. faecium	HM-965	Isolated from human blood in Ecuador (2006). Resistant to ampicillin and vancomycin and displays high-level resistance to gentamicin and streptomycin.
E. faecium	NR-31909	Isolated from the stool of a human patient prior to bacteraemia. Resistant to vancomycin.

CHAPTER 6. *IN VIVO* ANTIBACTERIAL ACTIVITY OF ACETAZOLAMIDE AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI

A version of this chapter has been reprinted with permission. Abutaleb, N. S., Ahmed Elkashif, Daniel P. Flaherty & Seleem, M. N. *In vivo* antibacterial activity of acetazolamide against vancomycin-resistant enterococci. *Antimicrob Agents Chemother,* under review, manuscript ID: AAC01715-20R1

6.1 Abstract

Vancomycin-resistant enterococci (VRE) represent a major public health threat that seriously require developing new therapeutics. In the present study, acetazolamide (AZM) was evaluated against enterococci. It inhibited different enterococcal strains tested, at clinically achievable concentrations. Moreover, AZM outperformed linezolid (LIN), the drug of choice for VRE infections, in two *in vivo* VRE mouse models; murine colonization-reduction and VRE septicemia. Collectively, these results indicate that AZM warrants consideration as a promising treatment option for VRE infections.

Keywords: carbonic anhydrase inhibitors, acetazolamide, vancomycin-resistant enterococci (VRE), VRE bloodstream infections, VRE decolonization.

6.2 Main article

Vancomycin-resistant enterococci (VRE) are listed as high priority pathogens which urgently require the development of new antibiotics [1]. The growing problem of VRE in the healthcare settings is further exacerbated by the lack of effective treatments. Currently, linezolid is the only FDA-approved drug for treating VRE infections. Yet, the treatment outcomes of linezolid are non-satisfactory, especially in bloodstream infections, with high mortality rate that can reach as high as 30% and it demonstrates little activity as a VRE decolonizing agent [2]. Additionally, linezolid's treatment is associated with bone marrow toxicity and neurotoxicity [3, 4]. Further compounding the VRE problem is the VRE's growing resistance to available treatment options such as linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline [5-8]. Furthermore, VRE can efficiently acquire or develop resistance to multiple antibiotics in addition

to their intrinsic resistance to several antibiotics such as β-lactams and aminoglycosides [9]. Consequently, there is a critical need for development of new anti-VRE therapeutics.

Repurposing FDA-approved drugs is an attractive strategy for drug discovery that reduces time, cost, and risk associated with antimicrobial drug innovation [10-18]. Utilizing this approach, we identified the FDA-approved carbonic anhydrase inhibitors (CAIs); acetazolamide (AZM), methazolamide (MZM) and ethoxzolamide (EZM) as a novel class of promising anti-VRE agents [11]. Building upon our previous work, the aim of the current study is to evaluate the antimicrobial activity of these CAIs against enterococci and evaluate AZM's *in vivo* efficacy in two VRE mouse models, VRE decolonization and VRE peritonitis.

Bacterial isolates, drugs, media and reagents used in the study and were purchased commercially. Susceptibility determinations were performed (3 independent replicates) by broth microdilution following the CLSI guidelines [19]. The *in vivo* activity of AZM was evaluated in a VRE decolonization murine model, as described previously [12-14]. Further, the activity of AZM was evaluated in the VRE mouse peritonitis model, as described earlier [15].

The antibacterial activity of the CAIs; AZM, EZM and MZM, was evaluated against a wide panel of clinical VRE strains. AZM and EZM exhibited the most potent activity against the tested isolates (MICs ranging from 1 to 4 μg/mL) (Table 6.1). They inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the tested isolates (MIC_{50}) at a concentration of 1 μg/mL and 2 μg/mL, respectively. MZM inhibited the tested VRE strains at concentrations ranged 1 to 8 μg/mL with MIC_{50} and MIC_{90} of 4 μg/mL and 8 μg/mL, respectively. Notably, the MICs of AZM are several folds lower than its clinically achievable blood concentration where AZM's serum concentration reaches up to 100 μg/mL after a single oral dose [20].

Table 6.1 Minimum inhibitory concentrations (MICs, in µg/mL) of CAIs and control drugs against clinical VRE isolates

VRE strains	**CAIs/** Control antibiotics				
	AZM	**EZM**	**MZM**	LIN	VAN
E. faecalis NR-31971	2	1	2	1	64
E. faecium NR-31914	1	1	4	1	>128
E. faecium HM-968	1	1	4	1	>128
E. faecalis NR-31972	2	1	2	1	>128
E. faecium NR-28978	1	2	8	1	>128
E. faecium NR-31903	2	1	8	16	>128
E. faecium NR- 31909	1	1	4	1	>128
E. faecium NR-31912	1	1	4	0.5	>128
E. faecium NR-31915	1	2	8	1	>128
E. faecium NR-31916	1	2	8	0.5	128
E. faecium NR-32052	2	1	8	0.5	>128
E. faecium NR-32053	1	1	8	0.5	>128
E. faecium NR-32054	1	1	8	0.5	128
E. faecium NR-32065	1	1	1	0.25	>128
E. faecium NR-32094	1	1	8	0.5	>128
E. faecium HM-952	1	1	8	1	>128
E. faecium HM-965	1	2	4	0.5	>128
E. faecium ATCC 700221	1	1	4	0.5	>128
E. faecalis ATCC 51299	1	1	1	1	64
E. faecalis HM-201	2	1	2	1	>128

Table 6.1 continued

VRE strains	**CAIs/** Control antibiotics				
	AZM	**EZM**	**MZM**	LIN	VAN
E. faecalis HM-334	1	1	1	1	>128
E. faecalis HM-335	2	1	1	0.5	>128
E. faecalis HM-934	4	4	4	1	>128
E. faecium HM-970	1	1	2	1	>128
MIC_{50}	1	1	4	1	>128
MIC_{90}	2	2	8	1	>128

CAIs, carbonic anhydrase inhibitors; AZM, acetazolamide; EZM, ethoxzolamide; MZM, methazolamide; LIN, linezolid; VAN, vancomycin

Next, we investigated the activity of AZM and EZM against vancomycin-sensitive enterococci. Similarly, they maintained potent activity against vancomycin-sensitive enterococci (MICs = 1-4 μg/mL). Moreover, both were tested against other non-*faecalis* non-*faecium* enterococcal species. Diseases due to these bacterial strains are significantly increasing worldwide. *E. gallinarum* and *E. casseliflavus* infections are of special interest because of their intrinsic resistance to vancomycin [21, 22]. These strains can also, cause hospital-acquired infections, particularly bloodstream, urinary tract, and surgical wound infections [23]. Moreover, it was found that translocation of *E. gallinarum*, to the liver and other systemic tissues promotes autoimmune disorders in mice and humans [24]. Interestingly, AZM and EZM maintained the same potency (or even better) against these strains (Table 6.2). Advantageously, they were two-fold more potent than LIN against *E. saccharolyticus* and *E. durans.* In addition, they were as effective as LIN against *E. hirae* and *E. casseliflavus* (with the exception of EZM, which was 4 times more potent than LIN, (MIC = 0.25 μg/mL)).

Table 6.2 MICs (μg/mL) of AZM and EZM and control drugs against clinical vancomycin-sensitive *Enterococcus faecium* and *Enterococcus faecalis* isolates as well as other clinically important enterococcal species

Enterococcal strains	**CAIs**/Control antibiotics			
	AZM	**EZM**	LIN	VAN
E. faecium NR-31933	4	4	2	4
E. faecium NR-31935	2	2	0.5	1
E. faecium NR-31937	2	2	1	2
E. faecium NR-31954	4	4	0.5	2
E. faecalis NR-31975	2	1	1	1
E. faecalis NR-31970	2	2	1	1
E. gallinarum ATCC 49573	4	2	0.5	16
E. saccharolyticus ATCC 43076	0.5	0.5	1	0.5
E. casseliflavus ATCC 700327	1	0.25	1	16
E. hirae ATCC 10541	2	2	1	1
E. durans ATCC 11576	0.25	0.25	0.5	1

Dysbiosis caused by patients exposure to broad-spectrum antibiotics can lead to VRE colonization, which serves as the origination point for VRE to spread in the body leading to life-threatening infections such as endocarditis, bloodstream infections and urinary tract infections [25]. Besides, dysbiosis and colonization by VRE were found to contribute to, and exacerbate irritable bowel disorders such as Crohn's disease [26-28]. Thus, VRE decolonization from the GIT is an important strategy to curb VRE infections, particularly in immunocompromised, organ transplant and intensive care units patients [12, 29]. Yet, there is unfortunately, no effective drug currently approved for enterococcal decolonization [25]. Therefore, we evaluated AZM's activity as a VRE decolonizer. AZM was superior to LIN in decreasing the VRE burden in the intestinal organs of the colonized mice (Fig. 6.1 and 6.2). After 5 days of treatment, AZM (10 mg/kg)

significantly reduced the VRE burden in mice fecal samples by 1.43-$\log_{10}$ (96.3% reduction), while AZM (20 mg/kg) significantly reduced the burden by 1.88-$\log_{10}$ (98.7% reduction). LIN, in contrast, generated a 0.9-$\log_{10}$ reduction (87.4%) in VRE count. The VRE burden continued to significantly decrease with AZM (20 mg/kg) treatment, resulting in a 2.75-$\log_{10}$ (99.8%) reduction of VRE in fecal samples after 7 days of treatment. On the other hand, AZM (10 mg/kg) generated a similar reduction to that produced after 5 days of treatment. Conversely, LIN only generated a 1.14-$\log_{10}$ (92.6%) reduction in VRE CFU after 7 days of treatment (Fig. 6.1).

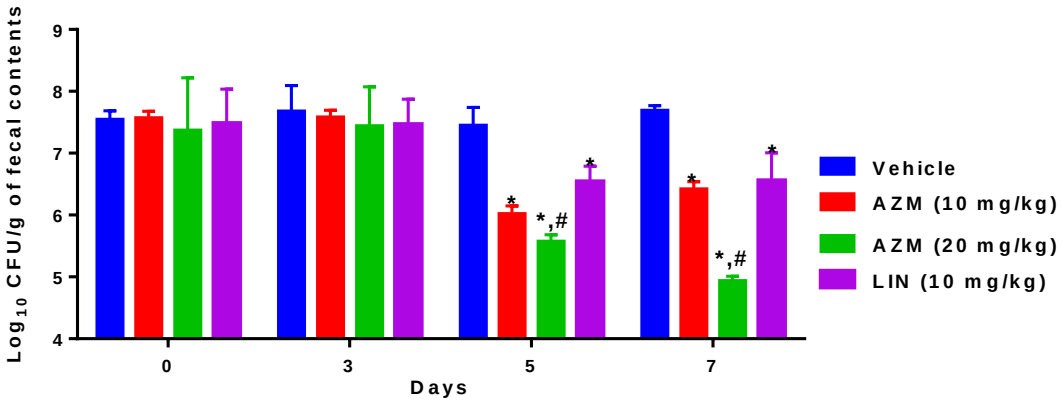

Figure 6.1 Burden of vancomycin-resistant *E. faecium* HM-952 in the fecal contents of colonized mice.

The CFU data were analyzed via a two-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a significant difference (P <0.05) between mice treated with AZM or LIN compared with untreated mice. A pound (#) indicates a significant difference (P <0.05) between mice treated with AZM compared to LIN-treated mice.

Similar to the results of the fecal samples, AZM significantly reduced the VRE count in the mice cecal and ileal contents (Fig. 6.2). In the cecal contents, AZM (10 mg/kg) significantly decreased the VRE burden by 1.06-$\log_{10}$ (91.2% reduction). This was similar to the reduction obtained with LIN, that decreased the VRE count in the cecal contents by 1.1-$\log_{10}$ (93.2% reduction). On the other hand, AZM (20 mg/kg) significantly outperformed LIN, generating a 2.71-$\log_{10}$ (99.8%) reduction in the cecal VRE burden. In the ileal contents, AZM (10 mg/kg) and (20 mg/kg) significantly surpassed LIN in reducing the VRE burden (0.82-$\log_{10}$ (84.6%) reduction, and a 2.54-$\log_{10}$ (99.7%) reduction, respectively). LIN did not reduce the VRE count in the ileal

contents (Fig. 2), in accordance with previous reports [12, 13]. The lower activity of LIN in reducing the bacterial burden in the GIT could be attributed to its rapid absorption from the GIT [30], its low concentration in the stool [31], or its limited activity against a high bacterial inoculum (~10^8 CFU) as in the case of VRE colonization [32].

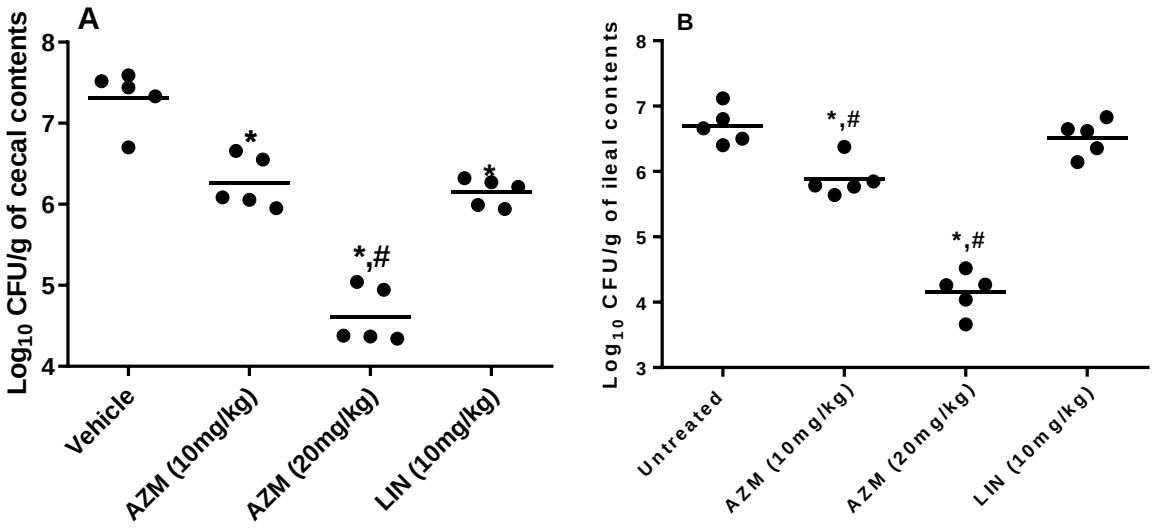

Figure 6.2 Burden of vancomycin-resistant *E. faecium* HM-952 in: **A)** the cecal contents of colonized mice, and **B)** the ileal contents of colonized mice.

The CFU data were analyzed via a one-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a significant difference (P <0.05) between mice treated with AZM or LIN compared with untreated mice (vehicle). A pound (#) indicates a significant difference (P <0.05) between mice treated with AZM compared to LIN-treated mice.

Finally, we aimed to investigate the activity of AZM in the murine VRE septicemic peritonitis model. Enterococci, mainly VRE, are a common cause of nosocomial bloodstream infections and their incidence is continually rising. VRE bloodstream infections often lead to infective endocarditis that can be fatal in up to 46% of cases [33]. Management of VRE bloodstream infections is compromised by the enterococcal resistance to several antibiotics, especially cell wall inhibitors and aminoglycosides that are commonly used in combination for treatment of such infections [9]. As a result, there is a critical need for new agents effective against systemic VRE infections. Thus, the efficacy of AZM in an *in vivo* VRE peritonitis murine model was evaluated. As presented in Fig. 6.3, AZM (20 mg/kg) and LIN (20 mg/kg) significantly

protected 100% of the mice from a lethal dose of VRE. However, AZM was significantly superior to LIN in decreasing the burden of VRE in the internal organs of the infected mice (Fig. 6.4). AZM reduced the VRE burden in mice liver, kidney and spleen tissues by 1.9-$\log_{10}$ (98.7%), 2.43-$\log_{10}$ (99.6%), and 2.13-$\log_{10}$ (99.3%) reduction, respectively. The highest reduction observed with AZM was in the kidney tissue, which could be attributed to the fact that AZM is excreted unmetabolized through the kidneys and urine [34]. LIN generated 1.42-$\log_{10}$ (96.1%), 1.14-$\log_{10}$ (92.8%) and 1.1-$\log_{10}$ (91.9%) reduction of the VRE in the livers, kidneys and spleens of mice, respectively (Fig. 6.4), in agreement with a previous report [15].

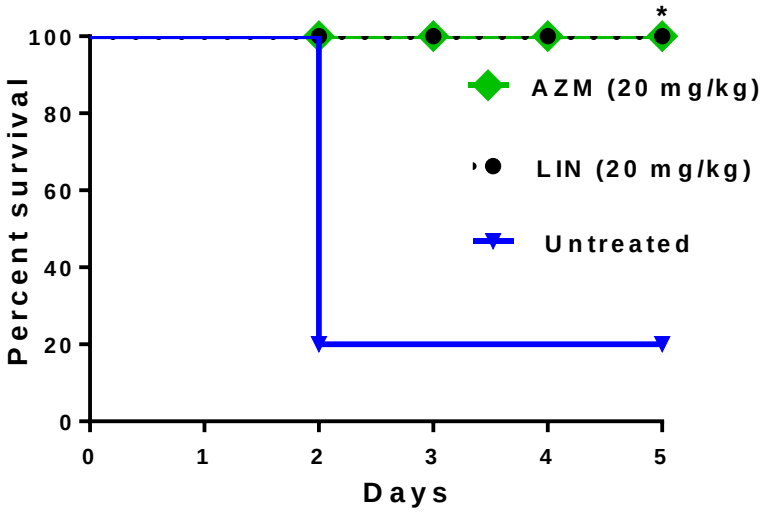

Figure 6.3 *In vivo* activity of AZM in the murine VRE peritonitis model.

Kaplan–Meier survival curves were analyzed using a log-rank (Mantel–Cox) test. Asterisks (*) denote statistical significant difference ($P < 0.05$) between mice treated with either AZM or LIN in comparison with the vehicle-treated mice.

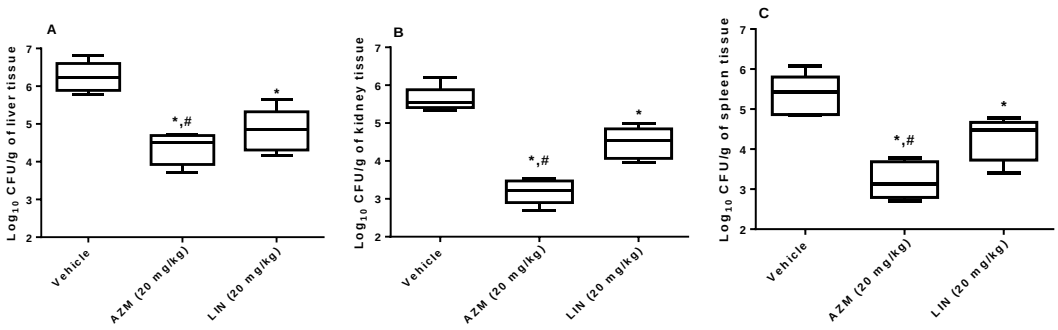

Figure 6.4 Burden of vancomycin-resistant *E. faecium* NR-31909 in: **A)** liver, **B)** kidneys and **C)** spleen of the infected mice.

The CFU data were analyzed via a one-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a significant difference (P <0.05) between mice treated with AZM or LIN compared with untreated mice (vehicle). A pound (#) indicates a significant difference (P <0.05) between mice treated with AZM compared to LIN-treated mice.

The current study focused on AZM as a representative CAI for several reasons: 1) AZM is listed on the WHO list of essential medicines due to its safety profile, low cost, high efficacy, and high availability [35], 2) it is highly safe and can be administered in dosages up to 1 g/day with no toxicity to humans [36], and 3) it possesses highly acceptable pharmacokinetic properties [37].

In conclusion, the current study highlights CAIs (for example AZM) as a new class of potent anti-enterococcal agents. AZM exhibited potent *in vitro* activity against enterococci. Moreover, AZM was superior to LIN *in vivo*, in two VRE mouse models. Thus, AZM represents a promising, novel treatment option for VRE infections.

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6.4 Supplemental material

6.4.1 Materials and Methods

6.4.1.1 Bacterial strains, reagents and media

Clinical enterococcal isolates (Table 6.3S) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) (Manassas, VA, USA), and the American Type Culture Collection (ATCC) (Manassas, VA, USA). Drugs used in the study were purchased commercially: AZM (Alfa Aesar, Ward Hill, MA, USA), EZM (Sigma-Aldrich, Saint Louis, MO, USA), MZM, LIN and vancomycin (VAN) (Chem-Impex International, Wood Dale, IL, USA), and ampicillin (IBI Scientific, Peosta, IA, USA). Media and reagents were purchased from commercial vendors: tryptic soy broth (TSB), tryptic soy agar (TSA), enterococcosel agar (Becton, Dickinson and Company, Cockeysville, MD, USA), and phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA).

Table 6.3S. Description of enterococcal isolates utilized in this study.

Enterococcal strains	**Source/Description**
E. faecalis NR-31971	Isolated from urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin and vancomycin.
E. faecium NR-31914	Isolated in 1995 from ascites fluid of a hospitalized patient in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecium HM-968	Isolated from human oral sputum in Colombia (2006). Resistant to ampicillin and vancomycin, and displays high-level resistance to streptomycin.
E. faecalis NR-31972	Isolated in 2003 from a human urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin and vancomycin.
E. faecium NR-28978	Collected from a hospitalized person free of enterococcal infection in the Netherlands in 2000 during a surveillance program.
E. faecium NR-31903	Isolated from the stool of a human patient prior to bacteraemia. Resistant to linezolid and vancomycin.
E. faecium NR- 31909	Isolated from the stool of a human patient prior to bacteraemia. Resistant to vancomycin.
E. faecium NR-31912	Isolated from the stool of a human patient having dominance of VRE in the stool but no bacteraemia.
E. faecium NR-31915	Isolated in 1996 from turkey faeces in the Netherlands. Resistant to gentamicin and vancomycin.

Table 6.3S continued

E. faecium NR-31916	Isolated in 1996 from turkey faeces in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecium NR-32052	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin
E. faecium NR-32053	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin
E. faecium NR-32054	Isolated in 2008 from swine faeces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin.
E. faecium NR-32065	Isolated in 1994 in Aix-en-Provence, France. Resistant to vancomycin.
E. faecium NR-32094	Isolated in 1996 in New York, USA. Resistant to vancomycin.
E. faecium HM-952	Human isolate from the USA. Resistant to vancomycin.
E. faecium HM-965	Isolated from human blood in Ecuador (2006). Resistant to ampicillin and vancomycin, and displays high-level resistance to gentamicin and streptomycin.
E. faecium ATCC 700221	Isolated from Human feces, Connecticut. Resistant to vancomycin and teicoplanin.
E. faecalis ATCC 51299	Isolated from peritoneal fluid, St. Louis, MO. Quality control strain for susceptibility testing.
E. faecalis HM-201	Isolated in January 2002 from the blood of a patient with endocarditis at Stamford Hospital in Connecticut, USA. Resistant to vancomycin.
E. faecalis HM-334	Isolated in 2004 from the blood of a 64 year-old female haemodialysis patient with fatal bacteraemia. Resistant to vancomycin.
E. faecalis HM-335	Isolated in 2004 from the blood of a 64-year-old female haemodialysis patient with fatal bacteraemia. Resistant to daptomycin and vancomycin.
E. faecalis HM-934	Isolated from human secretion in Bogota, Colombia (2006). Resistant to vancomycin.
E. faecium HM-970	Isolated from human faeces collected in Colombia (2008). Resistant to vancomycin.
E. faecium NR-31933	Isolated in 2001 from the faeces of a miniature pig in Germany. Resistant to vancomycin.
E. faecium NR-31935	Isolated in 1956 from cheese in Norway.
E. faecium NR-31937	Isolated in 1957 from the blood of a hospitalized patient in the Netherlands.
E. faecium NR-31954	Isolated in 2006 from the blood of a hospitalized patient in the Netherlands.
E. faecalis NR-31975	Isolated in 1985 from the blood of a patient with bacteremia in Wisconsin, USA. Resistant to erythromycin and gentamicin

Table 6.3S continued

E. faecalis NR-31970	Isolated in 2001 from a urine sample in Michigan, USA. Resistant to erythromycin and gentamicin
E. gallinarum ATCC 49573	Isolated from chicken intestine. Presence of *vanC-1* gene confirmed by PCR.
E. sacchrolyticus ATCC 43076	Isolated from Straw bedding, England. Quality control strain.
E. casseliflavus ATCC 700327	Quality control strain.
E. hirae ATCC 10541	Quality control strain.
E. durans ATCC 11576	Isolated from dairy products. Quality control strain

6.4.1.2 Antibacterial activity of CAIs against enterococcal strains

The minimum inhibitory concentrations (MICs) of the tested CAIs; AZM, EZM and MZM were determined against a panel of enterococcal strains, using the broth microdilution assay following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [1]. Briefly, 0.5 McFarland bacterial solution was prepared and diluted to an inoculum size of about 5 x 10^5 CFU/mL, and incubated with serial dilutions of the tested drugs aerobically at 37°C for 18-20 hours. MICs reported are the lowest drug concentrations that completely inhibited the bacterial growth, as observed visually. MIC_{50} and MIC_{90} are the lowest concentration of each drug that inhibited the growth of 50% and 90% of the tested isolates, respectively.

6.4.1.3 *In vivo* VRE colonization-reduction mouse model

Each group of mice was housed in an individually ventilated cage, received food and water ad libitum. In order to evaluate the ability of AZM in decreasing the burden of VRE present in the gastrointestinal tract (GIT) of mice, the VRE decolonization murine model described previously [2-4], was utilized. Briefly, 8-week-old female C57BL/6 mice (Jackson laboratories, ME, USA) were exposed to ampicillin (0.5 g/l in drinking water) for 7 days before they were infected via oral gavage with 1.3×10^8 CFU/mL of *E. faecium* HM-952. Seven days post infection, mice were randomly allocated into groups (n=5) for treatment. Two groups were treated with AZM (10 mg/kg and 20 mg/kg), one group was treated with LIN (10 mg/kg) and one group was treated with the vehicle (10% DMSO in PBS) (negative control). Treatments were administered via oral

gavage once daily for eight consecutive days. Fecal pellets were aseptically collected from mice on days 0, 3, 5 and 7. Afterwards, mice were humanely euthanized via carbon dioxide asphyxiation. Following euthanasia, the ceca and ilea were aseptically collected. Bacteria were recovered from fecal pellets, and cecal and ileal contents, diluted in PBS and plated on enterococcosel agar plates (supplemented with 8 μg/mL vancomycin to select for VRE). Plates were incubated at 37 °C for 48 hours to determine the bacterial count present in each sample. The data of CFU count in fecal contents were analyzed via two-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P < 0.05$). The cecal and ileal contents data were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P < 0.05$). Asterisk (*) denotes statistical significant difference between the results obtained for AZM or LIN in comparison to the untreated group (vehicle). Pound (#) denotes statistical significant difference between the results obtained for AZM in comparison to LIN.

6.4.1.4 *In vivo* VRE peritonitis mouse model

The activity of AZM was evaluated in the VRE mouse peritonitis lethal infection model, as described previously [5]. Eight-week-old female BALB/c mice (Jackson, ME, USA) were used for this study and were infected intraperitoneally with 3×10^7 CFU/mL of *E. faecium* NR-31909 premixed with 20% sterile rat fecal extract (SRFE) (2:1). One-hour post-infection, groups of mice (n=5) were treated orally with either AZM (20 mg/kg), LIN (20 mg/kg), or the vehicle (10% DMSO in PBS). Treatments were continued once daily via oral gavage for four days before the mice were humanely euthanized. Afterwards, mice internal organs (livers, kidneys and spleens) were aseptically removed. Bacteria were recovered from the internal organs, serially diluted, and plated on enterococcosel agar supplemented with vancomycin (8 μg/mL). Plates were then, incubated for 48 hours at 37°C. The data were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P < 0.05$). Asterisks (*) denote statistical significant difference between the results obtained for AZM or LIN in comparison to the untreated group ($P < 0.05$). Pounds (#) denote statistical significant difference between the results obtained for AZM in comparison to LIN.

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CHAPTER 7. *IN VITRO* AND *IN VIVO* ACTIVITIES OF THE CARBONIC ANHYDRASE INHIBITOR, DORZOLAMIDE, AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI

A version of this chapter has been reprinted with permission. Abutaleb, N. S., Elhassanny, A.E.M, Flaherty, D.P & Seleem, M. N. *In vitro* and *in vivo* activities of the carbonic anhydrase inhibitor, dorzolamide, against vancomycin-resistant enterococci. *PeerJ*, under review, manuscript ID: 55340.

7.1 Abstract

Vancomycin-resistant enterococci (VRE) are a serious public health threat and a leading cause of healthcare-associated infections. Bacterial resistance to antibiotics recommended for the treatment of enterococcal infections complicates the management of these infections. Hence, there is a critical need for the discovery of new anti-VRE agents. We previously reported carbonic anhydrase inhibitors (CAIs) as new potent VRE inhibitors. In the present study, the activity of the CAI, dorzolamide was evaluated against VRE both *in vitro* and *in vivo*. Dorzolamide exhibited potent activity against a panel of clinical VRE isolates, with minimum inhibitory concentration (MIC) values ranging from 1 µg/mL to 8 µg/mL. A killing kinetics experiment determined that dorzolamide exhibited a bacteriostatic effect against VRE, which was similar to the drug of choice (linezolid). Dorzolamide interacted synergistically with gentamicin against 4 strains of VRE, and exhibited an additive interaction with gentamicin against 6 VRE strains, reducing gentamicin's MIC by several folds. Moreover, dorzolamide outperformed linezolid in an *in vivo* VRE colonization reduction mouse model. Dorzolamide significantly reduced the VRE burden in fecal samples of mice by 2.9-$\log_{10}$ (99.9%) and 3.86-$\log_{10}$ (99.99%) after 3 and 5 days of treatment, respectively. Furthermore, dorzolamide reduced the VRE count in the cecal (1.74-$\log_{10}$ (98.2%) reduction) and ileal contents (1.5-$\log_{10}$ (96.3%)) of mice, which was superior to linezolid. Collectively, these results indicate that dorzolamide represents a promising treatment option that warrants consideration as a supplement to current therapeutics used for VRE infections.

Key words: carbonic anhydrase inhibitors, vancomycin-resistant enterococci (VRE), antibiotics resistance, VRE decolonizing agents.

7.2 Introduction

Vancomycin-resistant enterococci (VRE) infections are a major challenge globally and require the development of new therapeutics. Prolonged hospitalizations can lead to colonization of the gastrointestinal tract (GIT) by VRE, which in turn can result in life-threating infections, such as endocarditis, systemic infections, and urinary tract infections (UTI) [1]. In addition, VRE infections are associated with increased rates of mortality as well as high economic burden due to extended periods of hospitalizations [2]. The U.S. Centers for Disease Control and Prevention (CDC) recently reported that VRE infections contributed to nearly 55,000 hospitalizations in the United States in 2017, which resulted in a 10% mortality rate and cost nearly $540 million in healthcare costs [3].

The lack of effective treatment options for VRE infections has created a serious need for the development of new, effective anti-VRE therapeutics. Currently, linezolid is the only drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of VRE infections [4]. However, linezolid treatment is associated with several concerns. For example, linezolid treatment of VRE bloodstream infections has been linked with a mortality rate that can reach as high as 30%. Additionally, linezolid exhibits limited activity in decolonizing VRE from the GIT [5]. Furthermore, linezolid treatment is associated with serious side effects, including bone marrow toxicity and neurotoxicity [6, 7]. The combination of quinupristin/dalfopristin was previously approved by the FDA for treatment of VRE infections. However, this drug combination is rarely used due to concerns about toxicity [8]. Daptomycin is another antibiotic that is frequently used in clinical practice as an anti-VRE treatment option [9, 10]. However, daptomycin is not approved by the FDA to treat VRE infections, and the lack of standard dosing for VRE infections is a concern [11]. The serious threat of VRE is further compounded by the emergence of strains exhibiting resistance to linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline [12-15]. Furthermore, life-threatening infections caused by VRE, such as endocarditis and bloodstream infections, often require a β-lactam/aminoglycoside combination. However, most VRE strains are resistant to aminoglycosides and β-lactams, which compromises the treatment of these life-threatening infections [16]. Moreover, in addition to their intrinsic resistance to several antibiotics, VRE are able to develop resistance rapidly to multiple antibiotics via modification of the drug target or through horizontal gene transfer of transposons or plasmids carrying resistance elements

[17]. Consequently, the aforementioned reasons highlight the critical need to develop new, effective treatment options for VRE infections.

Drug repurposing is an efficient approach to drug discovery that saves both time and costs associated with drug innovation [18-25]. In an effort to meet the critical need for development of new, effective anti-VRE agents, we identified the FDA-approved carbonic anhydrase inhibitors (CAIs) acetazolamide, methazolamide and ethoxzolamide as promising anti-VRE agents [19]. Additionally, through structure-activity relationship modifications to acetazolamide, our team developed acetazolamide analogs that exhibited potent *in vitro* activity against clinical isolates of VRE [26]. CAIs are FDA-approved drugs that suppress the activity of carbonic anhydrase enzymes (CAs) and are clinically used as mild diuretics, anti-glaucoma medications, antiepileptics, and in the management of mountain sickness [27]. CAs act as catalysts hydrating carbon dioxide to bicarbonate and protons; this reaction constitutes the basis of regulation of pH in most living organisms [28]. Bacterial carbonic anhydrases (CAs) have recently garnered attention as bacterial targets for the development of novel antibacterial agents [29, 30].

The aim of the current study was to evaluate the antimicrobial activity of dorzolamide both *in vitro* and *in vivo* against VRE. Dorzolamide is a CAI used to treat glaucoma [31]. The antibacterial activity of dorzolamide was evaluated against a wide panel of clinical VRE strains. The *in vitro* killing kinetics of dorzolamide against VRE and the potential of dorzolamide to be combined with gentamicin were also investigated. Finally, the efficacy of dorzolamide in an *in vivo* VRE colonization reduction mouse model was evaluated.

7.3 Materials and Methods

7.3.1 Bacterial strains, media and chemicals

Enterococcal strains used in the study were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) (Manassas, VA, USA), and the American Type Culture Collection (ATCC) (Manassas, VA, USA). Media and reagents were purchased from commercial vendors: tryptic soy broth (TSB), tryptic soy agar (TSA), enterococcosel agar (Becton, Dickinson and Company, Cockeysville, MD, USA), and phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA). Drugs used in the study were purchased

commercially: dorzolamide (TCI America, Portland, OR, USA), linezolid and vancomycin (Chem-Impex International, Wood Dale, IL, USA), and ampicillin (IBI Scientific, Peosta, IA, USA).

7.3.2 Antibacterial activity of dorzolamide against enterococci

MICs determination was performed utilizing the broth microdilution assay, as described before [32]. The MICs experiments were repeated at least 3 times. MICs reported are the lowest drug concentrations that completely inhibited the bacterial growth, as observed visually. MIC_{50} and MIC_{90} are the lowest concentration of each drug that inhibited the growth of 50% and 90% of the tested isolates, respectively.

7.3.3 Killing kinetics of dorzolamide against VRE

A time-kill assay was performed for dorzolamide and linezolid against *E. faecium* HM-952, following a method described previously [22, 24].

7.3.4 Combination testing of dorzolamide with gentamicin against VRE

To evaluate the interactions between dorzolamide and gentamicin against VRE clinical isolates, a standard checkerboard assay was utilized [24, 33, 34]. The fractional inhibitory concentration indices (FICIs) were calculated. Interactions where the FICI was $\leq$ 0.5 were categorized as synergistic (SYN). An FICI value of > 0.5 – 1.25 was considered additive (ADD), an FICI value of >1.25 – 4 was considered indifferent, and FICI values of > 4 were considered antagonistic [35, 36].

7.3.5 *In vivo* VRE colonization-reduction mouse model

All animal housing and experiments were reviewed, approved and performed under the guidelines of the Purdue University Animal Care and Use Committee (protocol number 1905001908) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in individually ventilated cages (5 per cage, 12 hours light/dark cycle) with free access to food and water. All mice were acclimatized for seven days before any experimental procedure. The VRE colonization

reduction murine model, described previously [20, 21], was performed to evaluate the ability of dorzolamide to reduce the VRE burden present in the GIT of mice. Briefly, 8-week-old female C57BL/6 mice (Jackson laboratories, ME, USA) were sensitized with 0.5 g/l ampicillin in drinking water, for 7 days before being infected with 1.3×10^8 CFU/mL of *E. faecium* HM-952 via oral gavage. Seven-days post-infection, mice were randomly allocated into three groups (n=5/each) for treatment via oral gavage: one group for dorzolamide (10 mg/kg), one group for linezolid (10 mg/kg), and one group for the vehicle (10% DMSO:90% PBS) (negative control). Treatments were continued quaque die (q.d.) for eight consecutive days. The VRE colonization reduction model does not involve expected mice mortality throughout the experiment. However, certain criteria for exclusion and euthanizing the animals prior to the planned end of the experiment were established. Any animal that meets any two of the group I criteria (a. rough coat and unkempt, b. eyes are full or partially closed for 10 minutes, c. markedly diminished resistance to being handled (grimace response), d. markedly decreased movement/lethargy, e. hunched posture, and f. distended abdomen), will be excluded and euthanized. Any mouse having one of group II criteria (a. inability to eat or drink, b. moribund/unresponsive, c. failure to right itself when placed on its back, d. dyspnea, or e. 15% or more loss in the body weight) will be euthanized. Treatments were administered daily on the same arrangement and at the same time, and cages locations were kept at the same positions throughout the experiment to minimize confounders.

Mice fecal pellets were aseptically collected on days 0 (before treatment) and days 3, 5 and 7 (after the start of treatment). Thereafter, all mice were euthanized humanely, via carbon dioxide asphyxiation, and their cecal and ileal tissues were aseptically collected (all mice were included in the analysis). Fecal pellets and cecal and ileal contents were diluted in PBS and plated on enterococcosel agar plates containing 8 μg/mL vancomycin. Plates were incubated at 37 °C for 48 hours before determining the bacterial CFU present in each sample. The data of CFU counts in fecal contents were analyzed via two-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P < 0.05$), while that of CFU counts in cecal and ileal contents were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons ($P < 0.05$). Asterisks (*) denote statistically significant difference between the results obtained for dorzolamide or linezolid in comparison to the negative control group (vehicle). Pounds (#) denote statistically significant difference between the results obtained for dorzolamide in comparison to linezolid.

7.3.6 Statistical analysis

GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, CA, USA) was used to conduct the statistical analyses presented in this study. The time kill assay results and data obtained from fecal samples were analyzed via two-way ANOVA with Dunnett's test for multiple comparisons. The data obtained from cecal and ileal contents were analyzed via one-way ANOVA with post hoc Dunnett's test for multiple comparisons.

7.4 Results

7.4.1 Dorzolamide exhibits potent activity against strains of VRE

The antibacterial activity of dorzolamide was evaluated against a panel of 29 enterococcal strains that included 23 clinical VRE strains. As presented in Table 7.1, dorzolamide exhibited potent *in vitro* activity against all enterococcal strains tested. Dorzolamide inhibited growth of enterococcal isolates at concentrations that ranged from 1 μg/mL to 8 μg/mL. Dorzolamide, at 4 μg/mL, inhibited growth of both 50% (MIC_{50}) and 90% (MIC_{90}) of enterococcal isolates. Moreover, dorzolamide's MIC values were similar when tested against strains of VRE, vancomycin-sensitive *E. faecalis* and *E. faecium* strains, and the linezolid-resistant *E. faecium* NR-31903 strain. Linezolid, at 1 μg/mL, inhibited 50% and 90% of the enterococcal strains tested.

Table 7.1 MICs (μg/mL) of dorzolamide against clinical enterococcal isolates

Enterococcal strains	**Dorzolamide**	Linezolid	Vancomycin
E. faecium NR-28978	4	1	>128
E. faecium NR-31903	4	16	>128
E. faecium NR- 31909	4	1	>128
E. faecium NR-31912	4	0.5	>128
E. faecium NR-31914	4	1	>128
E. faecium NR-31915	4	1	>128

Table 7.1 continued

E. faecium NR-31916	4	0.5	128
E. faecalis NR-31971	4	1	64
E. faecalis NR-31972	2	1	>128
E. faecium NR-32052	4	0.5	>128
E. faecium NR-32053	4	0.5	>128
E. faecium NR-32054	4	0.5	128
E. faecium NR-32065	1	0.25	>128
E. faecium NR-32094	8	0.5	>128
E. faecalis HM-201	4	1	>128
E. faecalis HM-334	2	1	>128
E. faecalis HM-335	2	0.5	>128
E. faecalis HM-934	4	1	>128
E. faecium HM-952	4	1	>128
E. faecium HM-965	2	0.5	>128
E. faecium HM-968	4	1	>128
E. faecium HM-970	4	1	>128
E. faecium ATCC 700221	1	0.5	>128
E. faecium NR-31933	8	1	4
E. faecium NR-31935	4	1	1
E. faecium NR-31937	8	1	2
E. faecium NR-31954	4	1	2
E. faecalis NR-31970	4	1	1

Table 7.1 continued

E. faecalis NR-31975	4	1	1
MIC_{50}	**4**	**1**	**>128**
MIC_{90}	**4**	**1**	**>128**

MIC_{50}, the concentration of the test agent which inhibited 50% of the tested strains; MIC_{90}, the concentration of the test agent which inhibited 90% of the tested strains.

7.4.2 Dorzolamide exhibits a bacteriostatic effect against VRE

To determine if dorzolamide exhibits a bactericidal or bacteriostatic effect *in vitro* against VRE, a time-kill assay was conducted. As presented in Fig. 7.1, in the presence of dorzolamide (at 10 × MIC), the bacterial count of *E. faecium* HM-952 remained almost constant over 24 hours but was significantly reduced as compared to the negative control (DMSO). This indicates that dorzolamide exhibited a bacteriostatic effect *in vitro* against VRE. Similarly, linezolid demonstrated a bacteriostatic effect against VRE, which is in agreement with previous reports [20, 21].

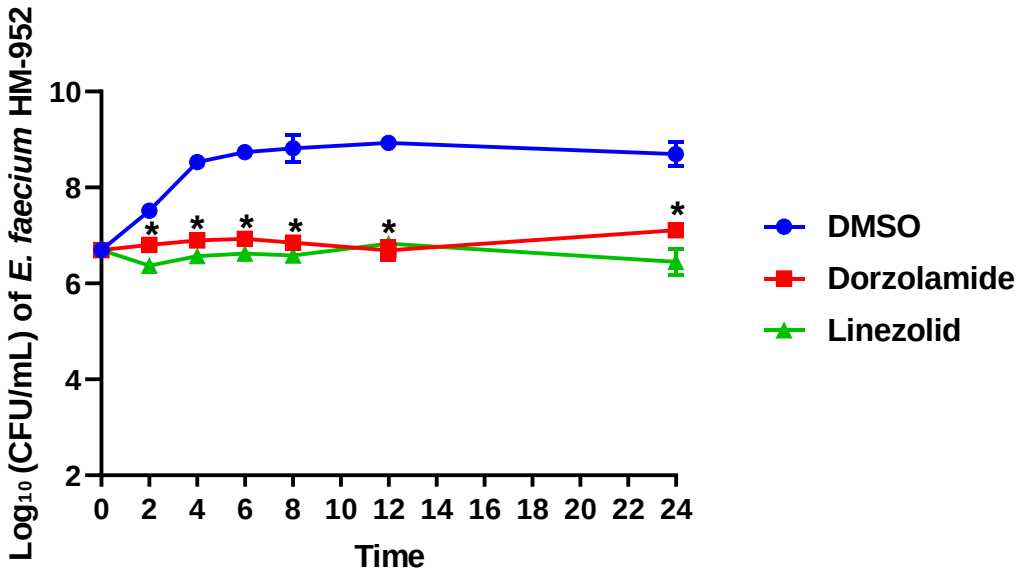

Figure 7.1 Time-kill assay of dorzolamide and linezolid (tested in triplicates, at 10 × MIC) against *E. faecium* HM-952.

DMSO (vehicle) served as a negative control. The error bars represent standard deviation values for each drug studied. The data were analyzed via a two-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a statistically significant difference (P <0.05) between dorzolamide or linezolid treatment compared to DMSO treatment (negative control).

7.4.3 Dorzolamide exhibits a synergistic interaction with gentamicin against VRE

Enterococci are relatively impermeable to aminoglycosides. As a consequence, the concentration of aminoglycosides necessary to kill VRE could be higher than their clinically achievable concentration [37, 38]. Therefore, using the standard checkerboard assay, we investigated whether the combination of an aminoglycoside (gentamicin) with dorzolamide could enhance the activity of gentamicin against VRE. As presented in Table 7.2, dorzolamide exhibited a synergistic interaction with gentamicin against 4 out of 10 tested strains, with a fractional inhibitory concentration index (FICI) that ranged from 0.31 to 0.50. The dorzolamide/gentamicin combination demonstrated an additive relationship against 6 of the tested strains. Remarkably, in the presence of 0.5 × MIC of dorzolamide, the MIC values of gentamicin were reduced significantly in 4 of these strains. The MIC of gentamicin improved from 32 µg/mL to 4 µg/mL in two strains, from 64 µg/mL to 4 µg/mL in one strain, and from 128 µg/mL to 2 µg/mL in one strain.

Table 7.2 MICs (µg/mL) of dorzolamide and gentamicin tested alone and in combination against VRE clinical isolates.

VRE strain	MIC (µg/mL)				FICI[1]	Interaction*
	Dorzolamide		Gentamicin			
	Alone	Combined with gentamicin	Alone	Combined with dorzolamide		
E. faecium NR-31912	4	1	64	16	0.50	SYN
E. faecium NR-31915	4	1	16	4	0.50	SYN
E. faecium NR-31916	4	2	32	4	0.63	ADD
E. faecalis NR-31971	4	1	256	32	0.38	SYN
E. faecalis NR-31972	2	1	512	64	0.63	ADD
E. faecalis HM-934	4	1	64	4	0.31	SYN
E. faecalis HM-201	4	2	512	32	0.56	ADD
E. faecalis HM-335	2	1	512	16	0.53	ADD
E. faecium HM-968	4	2	128	2	0.52	ADD
E. faecium HM-970	4	2	32	4	0.63	ADD

[1]FICI; fractional inhibitory concentration index

*FICI $\leq$ 0.5 is considered synergistic (SYN); FICI > 0.5 – 1.25 is considered additive (ADD); FICI >1.25 – 4 is considered indifference; FICI > 4 is considered antagonistic.

7.4.4 Dorzolamide significantly reduced the burden of VRE in the GIT in a colonization reduction murine model

Next, we evaluated dorzolamide's ability to decrease the burden of VRE in mice intestinal tissues in a VRE decolonization mouse model. Dorzolamide was found to be superior to linezolid in the mouse study (Fig. 7.2 and Fig. 7.3). After only 3 days of treatment, dorzolamide (10 mg/kg) significantly reduced the burden of VRE in mice fecal samples by 2.9-$\log_{10}$ (99.9% reduction). In contrast, linezolid (10 mg/kg) did not reduce the burden of VRE in mice fecal samples (Fig. 7.2). The burden of VRE continued to decrease with dorzolamide treatment, resulting in a 3.86-$\log_{10}$

(99.99%) reduction after 5 days. Dorzolamide significantly outperformed linezolid (0.91-$\log_{10}$ reduction) in reducing the burden of VRE in fecal samples after 5 days of treatment. After 7 days of dorzolamide treatment, the count of VRE slightly increased (compared to day 5) resulting in a 2.69-$\log_{10}$ (99.8%) reduction compared to vehicle-treated mice. Dorzolamide's reduction of VRE in fecal samples significantly surpassed the 1.1-$\log_{10}$ (92%) reduction in VRE CFU observed with linezolid after 7 days of treatment (Fig. 7.2). Notably, the VRE count in the fecal samples of the vehicle-treated group remained in the range of 10^7 CFU/mL during the experiment. This indicates that the decrease in VRE burden observed in the dorzolamide- or linezolid-treated mice was mainly due to the treatments received.

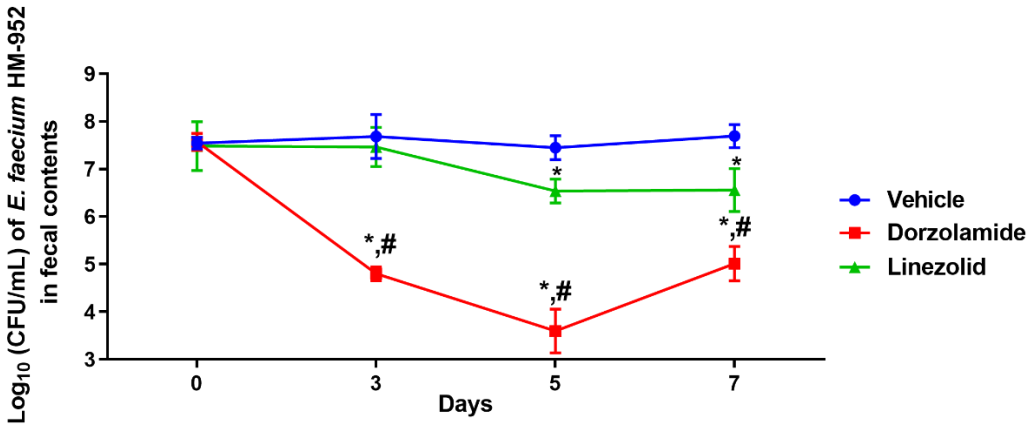

Figure 7.2 Log_{10} (CFU/mL) of vancomycin-resistant *E. faecium* HM-952 in the fecal contents of infected mice.

Mice were orally treated once daily for 8 days with each drug. Fecal samples were collected from each group of mice on day 0 (before the start of treatment) and on days 3, 5 and 7 (post-treatment) and VRE colonies were counted. The CFU data were analyzed via a two-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a statistically significant difference (P <0.05) between mice treated with dorzolamide or linezolid compared to the vehicle (negative control). A pound sign (#) indicates a statistically significant difference (P <0.05) between mice treated with dorzolamide compared to linezolid.

Furthermore, VRE burden in the cecal and ileal tissues of mice, after euthanasia, was determined. Dorzolamide significantly reduced the VRE count in the cecal and ileal contents of mice. In the cecal contents, dorzolamide decreased VRE burden by 1.74-$\log_{10}$ (98.2% reduction). Linezolid decreased the VRE burden in the cecal contents by 1.2-$\log_{10}$ (93.2% reduction) (Fig. 7.3). In the ileal contents of mice, dorzolamide significantly reduced VRE burden compared to

linezolid. Dorzolamide treatment resulted in a 1.5-$\log_{10}$ (96.3%) reduction in VRE compared to vehicle-treated mice. In contrast, linezolid did not reduce VRE burden in the ileal contents of mice (Fig. 7.3).

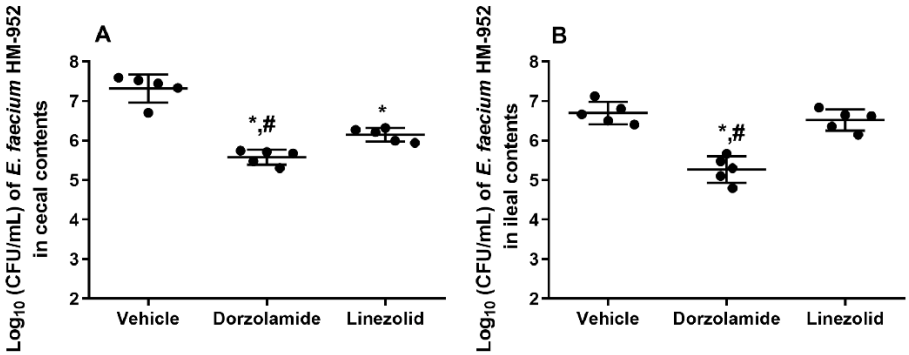

Figure 7.3 Log_{10} (CFU/mL) of vancomycin-resistant *E. faecium* HM-952 in: **A)** the cecal contents of infected mice, and **B)** the ileal contents of infected mice.

Mice were orally treated once daily for 8 days with each drug. Mice ceca and ilea were aseptically removed from each group after euthanasia, diluted, and counted. The data were analyzed via a one-way ANOVA with post-hoc Dunnett's test for multiple comparisons. An asterisk (*) indicates a statistically significant difference (P <0.05) between mice treated with dorzolamide or linezolid compared with vehicle-treated mice. A pound sign (#) indicates a statistically significant difference (P <0.05) between mice treated with dorzolamide compared to linezolid-treated mice.

7.5 Discussion

Vancomycin-resistant enterococci are a leading cause of nosocomial infections, but the therapeutic options available for treatment of these infections are limited [39]. VRE are responsible for more than one third of infections caused by all enterococci and over 5% of all deaths attributed to an antibiotic-resistant bacterial infection in the U. S. are due to VRE [20]. VRE are capable of overgrowing the body's normal flora in the gastrointestinal tract, particularly after the administration of broad-spectrum antibiotics. After colonizing the GIT, VRE can translocate across human epithelial cells, which leads to systemic infections such as septicemia, UTI, endocarditis, and surgical site infections [40]. Given the dearth of effective therapeutic options and

increasing resistance to the available treatment options, there is an urgent need to develop new therapeutics to treat VRE infections.

We recently identified carbonic anhydrase inhibitors, namely acetazolamide and its analogs, as potent inhibitors of VRE [19, 26]. This study aimed to investigate the activity of dorzolamide (an FDA-approved CAI) against VRE both *in vitro* and *in vivo*. Dorzolamide was tested against 23 clinical VRE strains. It exhibited potent *in vitro* inhibitory activity against all 23 strains tested (MIC values ranged from 1 µg/mL to 8 µg/mL). Moreover, dorzolamide effectively inhibited growth of both vancomycin-resistant *E. faecium* and *E. faecalis* strains, unlike the combination of quinupristin/dalfopristin, which is reported to be less efficacious against *E. faecalis* strains [41]. In addition, the MIC values of dorzolamide were consistent against both vancomycin-resistant and vancomycin-sensitive strains. Moreover, we determined that dorzolamide exhibits a bacteriostatic effect against VRE *in vitro*, which is similar to linezolid [20, 21].

One of the major challenges in treating enterococcal infections with a single agent is it often provides a bacteriostatic effect, even with drugs which are typically bactericidal, such as β-lactams [10, 38]. Accordingly, current guidelines recommend combination therapy with a β-lactam and an aminoglycoside (to exert bactericidal activity) to treat systemic infections caused by enterococci, particularly endocarditis [10]. However, many enterococcal strains are relatively impermeable to aminoglycosides, and enterococcal resistance to aminoglycosides is prevalent [42]. Consequently, we evaluated the combination of dorzolamide with the aminoglycoside gentamicin against 10 VRE strains. A checkerboard assay found synergistic interactions between dorzolamide and gentamicin against 4 strains of VRE and an additive effect against 6 strains of VRE. Interestingly, dorzolamide resensitized some tested VRE strains to gentamicin reducing its MIC by 8- to 64-fold. Therefore, using dorzolamide in combination with gentamicin could potentially decrease the dose of gentamicin administered to patients clinically. Using a lower treatment dose is highly desirable in the treatment of systemic VRE infections, especially in patients with comorbid conditions.

Finally, our study investigated dorzolamide's effect in an *in vivo* VRE colonization reduction murine model. Enterococci normally inhabit the human GIT and remain under the control of the normal flora present in the gut. Disturbance of the normal flora balance can lead to

VRE overgrowth and colonization of the gut. VRE can subsequently spread throughout the body causing serious infections including endocarditis, bloodstream infections, and UTIs [40]. In addition, dysbiosis and colonization by VRE was found to exacerbate irritable bowel disorders such as Crohn's disease [43-45]. Thus, suppressing VRE colonization of the GIT is considered an alternative strategy to curb VRE infections, particularly in highly-susceptible people such as immunocompromised patients, organ transplant recipients, and patients in intensive care units [20, 46]. Though enterococcal colonization of the GIT contributes to the development of systemic infections, there is no effective drug currently approved for enterococcal decolonization [40]. Linezolid, the only FDA-approved antibiotic to treat VRE infections, is ineffective as a VRE decolonizing agent, which could be attributed to its rapid absorption from the GIT. Consequently, the need to develop new agents that can successfully decolonize VRE from the GIT cannot be overemphasized. Although both dorzolamide and linezolid exhibited bacteriostatic activity against VRE *in vitro*, dorzolamide was superior to linezolid in reducing the burden of VRE in the GIT of infected mice in our mouse model. This result suggests that agents exhibiting bacteriostatic activity *in vitro* could be effective decolonizing agents and should not be excluded from consideration. Linezolid, in accordance with previous reports [20, 21], exhibited lower activity in reducing the burden of VRE in the GIT of infected mice. The limited effect of linezolid in reducing the burden of VRE in the GIT could be due to several reasons such as linezolid's 1.) rapid absorption from the GIT [47], 2.) low concentration in the stool [48], or 3.) limited activity against a high bacterial inoculum (~10^8 CFU), as is the case for VRE colonization of the GIT [49]. Although dorzolamide proved to be effective in the VRE colonization reduction mouse model, a future investigation will need to investigate whether dorzolamide has any deleterious impact on the gut microbiota.

In conclusion, the current study presents dorzolamide as a new drug for treatment of VRE infections. Dorzolamide exhibited a potent *in vitro* inhibitory activity against enterococci. Additionally, dorzolamide interacted synergistically with gentamicin, reducing its MIC values to low clinically achievable concentrations. Moreover, dorzolamide outperformed linezolid in an *in vivo* VRE colonization reduction mouse model. The results altogether suggest that, dorzolamide represents a promising novel therapeutic option for the treatment of VRE infections.

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7.7 Supplemental material

Table 7.3S Description of enterococcal isolates utilized in this study.

Enterococcal strains	**Source/Description**
E. faecium NR-28978	Collected from a hospitalized person free of enterococcal infection in the Netherlands in 2000 during a surveillance program.
E. faecium NR-31903	Isolated from the stool of a human patient prior to bacteremia. Resistant to linezolid and vancomycin.
E. faecium NR- 31909	Isolated from the stool of a human patient prior to bacteremia. Resistant to vancomycin.
E. faecium NR-31912	Isolated from the stool of a human patient having dominance of VRE in the stool but no bacteremia.
E. faecium NR-31914	Isolated in 1995 from ascites fluid of a hospitalized patient in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecium NR-31915	Isolated in 1996 from turkey faeces in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecium NR-31916	Isolated in 1996 from turkey faeces in the Netherlands. Resistant to gentamicin and vancomycin.
E. faecalis NR-31971	Isolated from urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin and vancomycin.
E. faecalis NR-31972	Isolated in 2003 from a human urine sample obtained in Michigan, USA. Resistant to erythromycin, gentamicin and vancomycin.
E. faecium NR-32052	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin
E. faecium NR-32053	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin
E. faecium NR-32054	Isolated in 2008 from swine faeces in Michigan, USA. Resistant to erythromycin, tetracycline and vancomycin.
E. faecium NR-32065	Isolated in 1994 in Aix-en-Provence, France. Resistant to vancomycin.
E. faecium NR-32094	Isolated in 1996 in New York, USA. Resistant to vancomycin.
E. faecalis HM-201	Isolated in January 2002 from the blood of a patient with endocarditis at Stamford Hospital in Connecticut, USA. Resistant to vancomycin.
E. faecalis HM-334	Isolated in 2004 from the blood of a 64 year-old female haemodialysis patient with fatal bacteraemia. Resistant to vancomycin.
E. faecalis HM-335	Isolated in 2004 from the blood of a 64-year-old female haemodialysis patient with fatal bacteraemia. Resistant to daptomycin and vancomycin.

Table 7.3S continued

E. faecalis HM-934	Isolated from human secretion in Bogota, Colombia (2006). Resistant to vancomycin.
E. faecium HM-952	Human isolate from the USA. Resistant to vancomycin.
E. faecium HM-965	Isolated from human blood in Ecuador (2006). Resistant to ampicillin and vancomycin, and displays high-level resistance to gentamicin and streptomycin.
E. faecium HM-968	Isolated from human oral sputum in Colombia (2006). Resistant to ampicillin and vancomycin, and displays high-level resistance to streptomycin.
E. faecium HM-970	Isolated from human faeces collected in Colombia (2008). Resistant to vancomycin.
E. faecium ATCC 700221	Isolated from Human feces, Connecticut. Resistant to vancomycin and teicoplanin.
E. faecium NR-31933	Isolated in 2001 from the faeces of a miniature pig in Germany. Resistant to vancomycin.
E. faecium NR-31935	Isolated in 1956 from cheese in Norway.
E. faecium NR-31937	Isolated in 1957 from the blood of a hospitalized patient in the Netherlands.
E. faecium NR-31954	Isolated in 2006 from the blood of a hospitalized patient in the Netherlands.
E. faecalis NR-31970	Isolated in 2001 from a urine sample in Michigan, USA. Resistant to erythromycin and gentamicin
E. faecalis NR-31975	Isolated in 1985 from the blood of a patient with bacteremia in Wisconsin, USA. Resistant to erythromycin and gentamicin

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28. Mohammad H, **Abutaleb NS**, Seleem MN. Auranofin Rapidly Eradicates Methicillin resistant Staphylococcus aureus (MRSA) in an Infected Pressure Ulcer Mouse Model. *Sci Rep* 2020, 10:7251. **IF= 4.25**.
29. Zhang M, Hong W, **Abutaleb NS**, Li J, Dong PT, Zong C, Wang P, Seleem MN, Cheng JX. Rapid Determination of Antimicrobial Susceptibility by Stimulated Raman Scattering Imaging of D2O Metabolic Incorporation in a Single Bacterium. *Adv Sci (Weinh)* 2020, 7:2001452. **IF= 15.84**.
30. **Abutaleb NS**, Seleem MN. Auranofin, at clinically achievable dose, protects mice and prevents recurrence from Clostridioides difficile infection. *Sci Rep* 2020, 10:1-8. **IF= 4.25**.
31. **Abutaleb NS**, Seleem MN. Repurposing the Antiamoebic Drug Diiodohydroxyquinoline for Treatment of Clostridioides difficile Infections. *Antimicrob Agents Chemother* 2020, 64. **IF= 4.715**.
32. Kaur J, Cao X, **Abutaleb NS**, Elkashif A, Graboski AL, Krabill AD, AbdelKhalek AH, An W, Bhardwaj A, Seleem MN. Optimization of acetazolamide-based scaffold as potent inhibitors of vancomycin-resistant Enterococcus. *J Med Chem* 2020, 63:9540-9562. **IF= 6.05**.
33. Hagras M*, **Abutaleb NS***, Elhosseiny NM, Abdelghany TM, Omara M, Elsebaei MM, Alhashimi M, Norvil AB, Gutay MI, Gowher H, et al. Development of Biphenylthiazoles Exhibiting Improved Pharmacokinetics and Potent Activity Against Intracellular Staphylococcus aureus. *ACS Infect Dis* 2020. **IF= 4.91 (* Equal contribution)**.
34. Shahin IG*, **Abutaleb NS***, Alhashimi M, Kassab AE, Mohamed KO, Taher AT, Seleem MN, Mayhoub AS. Evaluation of N-phenyl-2-aminothiazoles for treatment of multi-drug resistant and intracellular Staphylococcus aureus infections. *Eur J Med Chem* 2020, 202:112497. **IF= 4.83 (* Equal contribution)**.
35. Naclerio G*, **Abutaleb NS***, Li D, Seleem MN, Sintim HO. Ultrapotent inhibitor of Clostridioides difficile growth, which suppresses recurrence in vivo. *J Med Chem* 2020. **IF= 6.05 (* Equal contribution)**.
36. Morales-de-Echegaray AV, Lin L, Sivasubramaniam B, Yermembetova A, Wang Q, **Abutaleb NS**, Seleem MN, Wei A. Antimicrobial Photodynamic Activity of Gallium-Substituted Haemoglobin on Silver Nanoparticles. *Nanoscale* 2020. **IF= 6.89**.

37. Hamann HJ*, **Abutaleb NS***, Pal R, Seleem MN, Ramachandran PV. β, γ-Diaryl α-methylene-γ-butyrolactones as potent antibacterials against methicillin-resistant Staphylococcus aureus. *Bioorg Chem* 2020, 104:104183. **IF= 3.94 (* Equal contribution)**.

Accepted manuscripts

38. Song J, Malwal S, Baig N, Schurig-Briccio L, Gao Z, Vaidya G, Yang K, **Abutaleb NS**, Seleem MN, Gennis R, Pogorelov T, Oldfield E, Feng X. Discovery of prenyltransferase Inhibitors with in vitro and in vivo antibacterial activity. *ACS Infect Dis* 2020, Manuscript ID: id-2020-00472w. **IF= 4.91**

Manuscripts under review

39. **Abutaleb NS**, Elkashif A, Flaherty DP & Seleem MN. *In vivo* antibacterial activity of acetazolamide against vancomycin-resistant enterococci. *Antimicrob Agents Chemother*, under review, manuscript ID: AAC01715-20R1. **IF= 4.715**.
40. **Abutaleb NS**, Elhassanny AEM, Flaherty DP & Seleem MN. *In vitro* and *in vivo* activities of the carbonic anhydrase inhibitor, dorzolamide, against vancomycin-resistant enterococci. *PeerJ*, under review, manuscript ID: 55340. **IF= 2.34**.
41. **Abutaleb NS**, & Seleem MN. *In vivo* efficacy of auranofin in a hamster model of *Clostridioides difficile* infection. *Sci Rep*, under review, manuscript ID: 155f00ed-d4d0-4517-abb4-e4a81e19889c. **IF=3.99**.
42. Sayed A*, Hagras M*, **Abutaleb NS***, Salama E, Seleem MN, Mayhoub AS. Evaluation of Bisphenylthiazoles as a Promising Class for Combating Multidrug-resistant Fungal Infections. *Plos One*, under review, Manuscript ID: EMID:0f0b7baa002576c4. **IF= 2.74**. **(* Equal contribution)**.
43. Mohammad H, **Abutaleb NS**, Dieterly A, Lyle L, Seleem MN. Investigating Auranofin for the Treatment of Infected Diabetic Pressure Ulcers in Mice and Dermal Toxicity in Pigs. *Diabetes Metab Res Rev*, under review, Manuscript ID: DMRR-20-RES-515.R1. **IF= 3.314**.
44. Coscia E, **Abutaleb NS**, Hostetter B, Seleem MN, Breur G, McCain R, Crain C, Slaby O, Capoor M, Narayanan S, Coscia M. *Cutibacterium acnes* Intradiscal Infectious Disease: a Pilot Study in Sheep. *Sci Rep*, under review. **IF=3.99**.

45. Mohammad H, **Abutaleb NS**, Dieterly A, Lyle L, Seleem MN. Evaluation of ebselen in resolving a methicillin-resistant *Staphylococcus aureus* infection of pressure ulcers in obese and diabetic mice. *PloS One*, under review, Manuscript ID: PONE-D-20-26445. **IF= 2.74**.

Conference presentations:

1. **Abutaleb NS**, AbdelKhalek A, Seleem MN. Repurposing carbonic anhydrase inhibitors to decolonize and treat enterococcal infections. 34th Annual Herbert C. Brown Lectures, April, 14, 2017, West Lafayette, IN, USA. (Poster)
2. **Abutaleb NS**, Mohammad H, AbdelKhalek A, Seleem MN. Repurposing Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-Resistant Enterococci. Institute for Drug Discovery 4th Annual Symposium, October, 11, 2017, West Lafayette, IN, USA. (Poster)
3. **Abutaleb NS,** AbdelKhalek A, and Seleem MN. Auranofin combines the antibacterial, antitoxin and antiinflammatory activities in the treatment of Clostridium difficile infections. Sigma Xi Graduate Student Research Poster Award Competition, February 21, 2018, West Lafayette, IN, USA. (Poster)
4. **Abutaleb NS**, AbdelKhalek A and Seleem MN. Repurposing an FDA approved drug for the treatment of Clostridium difficile infections. Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 1st, 2018, West Lafayette, IN, USA. (Poster)
5. **Abutaleb NS**, Eldesouky HE, Li X and Seleem MN. Overcoming the azoles resistance in emerging multidrug resistant Candida auris. Indiana Branch of the American Society of Microbiology (IBASM). April 7th, 2018, Indianapolis, IN, USA. (Poster)
6. **Abutaleb NS**, Eldesouky HE, Li X and Seleem MN. Overcoming the azoles resistance in emerging multidrug resistant Candida auris. Indiana Branch of the American Society of Microbiology (IBASM). April 7th, 2018, Indianapolis, IN, USA. (oral presentation)
7. **Abutaleb NS**, Eldesouky HE, Li X and Seleem MN. Overcoming the azoles resistance in emerging multidrug resistant Candida auris. Annual Phi Zeta Research Day. April 9th,

2018, Purdue University, College of Veterinary Medicine. West Lafayette, IN, USA. (Poster)

8. **Abutaleb NS**, Elsebaei MM, Mohammad H, Abouf M, Hegazy Y, Ghiaty A, Mayhoub AS and Seleem MN. Phenylthiazoles: a new systemically active antibiotic series against methicillin-resistant staphylococcus aureus with unique mechanism. The thirty-fifth Herbert C. Brown Lectures in organicchemistry symposium, April 13th, 2018, West Lafayette, IN, USA. (Poster)
9. **Abutaleb NS**, Kotb A, Seleem MA, Mohammad H, Hagras M, Ghiaty A, Mayhoub AS and Seleem MN. Phenylthiazoles with tert-Butyl Side Chain: A new metabolically stable antibiotic series with anti-biofilm activity. Biomolecular Galaxy Sympoisum, May 9th, 2018, West Lafayette, IN, USA. (Poster)
10. **Abutaleb NS**, Hagras M, Elsebaei MM, Ali AO, Abdel-Aleem JA, Mayhoub AS and Seleem MN. A potential new antibiotic class against multi-drug resistant bacteria. 5th Annual Drug Discovery Symposium. September 28th, 2018, West Lafayette, IN, USA. (Poster) **1st place**
11. **Abutaleb NS**, Alhashimi M, Elkashif A and Seleem MN. After decades of a shared life with vancomycin-resistant enterococci, is it time to breakup? Sigma Xi Graduate Student Research Poster Award Competition, February 20, 2019, West Lafayette, IN, USA. (Poster) **1st place prize**
12. **Abutaleb NS**, Alhashimi M, Elkashif A and Seleem MN. After decades of a shared life with vancomycin-resistant enterococci, is it time to breakup? Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 1st, 2018, West Lafayette, IN, USA. (Poster) **1st place prize**
13. **Abutaleb NS**, Alhashimi M, Elkashif A and Seleem MN. Repositioning the Anti-Rheumatoid drug Auranofin for Treatment of Fatal Vancomycin-Resistant Enterococci Infections2019 PVM Research Day Phi Zeta Poster Awards: Basic Research, West Lafayette, IN. March 23rd, 2017.
14. **Abutaleb NS**, Alhashimi M, Elkashif A and Seleem MN. *In vivo* and *in vitro* activities of auranofin against vancomycin-resistant enterococci infections. Biomolecular Galaxy Sympoisum, May 8th, 2019, West Lafayette, IN, USA. (Poster) **1st place prize**

15. **Abutaleb NS**, Alhashimi M, Elkashif A, and Seleem MN. Auranofin, the gold drug, can it win the gold against Vancomycin-Resistant Enterococci fatal Infections? 6th Annual Drug Discovery Symposium. October 14th, 2019, West Lafayette, IN, USA. (Poster)
16. **Abutaleb NS**, Elkashif A, Flaherty D, and Seleem MN. Novel drugs for superbugs: acetazolamide analogs combating vancomycin-resistant enterococci lethal infections. Sigma Xi Graduate Student Research Poster Award Competition, February 12, 2020, West Lafayette, IN, USA. (Poster) **1st place prize**
17. Mohammad H, **Abutaleb NS**, and Seleem MN. Pressure's Up: Can auranofin treat infected diabetic pressure ulcers (PUs)? Sigma Xi Graduate Student Research Poster Award Competition, February 12, 2020, West Lafayette, IN, USA. (Poster) **1st place prize**.
18. **Abutaleb NS**, Elkashif A, Flaherty D, and Seleem MN. Teaching an old drug a new trick: Discovery of novel acetazolamide analogs for treatment of vancomycin-resistant enterococci infections. Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 3rd, 2020, West Lafayette, IN, USA. (Poster) **2nd place prize**.

Awards

1. **First place award:** poster session

   Title: Carbonic anhydrase inhibitors curb vancomycin-resistant enterococci gut colonization through a unique target.

   Symposium: Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 1, 2018, West Lafayette, IN, USA.

2. **First place award:** poster session

   Title: A potential new antibiotic class against multi-drug resistant bacteria.

   Symposium: 5th Annual Drug Discovery Symposium. September 28th, 2018, West Lafayette, IN, USA. (Poster) **1st place prize**

3. **First place award:** poster session

   Title: After decades of a shared life with vancomycin-resistant enterococci, is it time to breakup?

Symposium: Sigma Xi Graduate Student Research Poster Award Competition, February 20, 2019, West Lafayette, IN, USA. (Poster)

4. **First place award:** poster session

   Title: After decades of a shared life with vancomycin-resistant enterococci, is it time to breakup?

   Symposium: Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 1st, 2018, West Lafayette, IN, USA.

5. **First place award:** poster session

   Title: *In vivo* and *in vitro* activities of auranofin against vancomycin-resistant enterococci infections.

   Symposium: Biomolecular Galaxy Symposium, May 8th, 2019, West Lafayette, IN, USA.

6. **First place award:** poster session

   Title: Novel drugs for superbugs: acetazolamide analogs combating vancomycin-resistant enterococci lethal infections.

   Symposium: Sigma Xi Graduate Student Research Poster Award Competition, February 12, 2020, West Lafayette, IN, USA.

7. **First place award:** poster session

   Title: Pressure's Up: Can auranofin treat infected diabetic pressure ulcers (Pus)?

   Symposium: Sigma Xi Graduate Student Research Poster Award Competition, February 12, 2020, West Lafayette, IN, USA.

8. **Second place award:** poster session

   Title: Teaching an old drug a new trick: Discovery of novel acetazolamide analogs for treatment of vancomycin-resistant enterococci infections.

   Symposium: Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 3rd, 2020, West Lafayette, IN, USA.

9. Purdue Veterinary Medicine best Graduate Student Award, May 19th, 2020.

Disclosures

Diiodohydroxyquinoline for the treatment of Clostridium difficile infection (2018). M. N. Seleem, N. Abutaleb. App Number. 6274300

Teaching experience

Research mentor:

- Purdue University, USA: mentored 9 graduate students
- Zagazig University, Egypt: mentored 10 graduate students

Teaching Assistant:

- Teaching Assistant, Microbiology & Immunology, Faculty of Pharmacy, Zagazig University, Egypt (2009-2016).
- Taught the following undergraduate courses: General Microbiology, Immunology, General Microbiology, Immunology, Pharmaceutical Microbiology, Clinical microbiology, Pharmaceutical biotechnology, Parasitology and pathology, Public health

Courses

Undergraduate Courses:

Physics, Physical Chemistry, Organic Chemistry, Botany, Zoology, Computer, General Pharmacognosy & Medicinal Plants, Pharmaceutics, Analytical Chemistry, Anatomy, Histology, Physiology, Mathematics, Psychology, Pharmaceutical Microbiology, Pharmacology, Medicinal Chemistry, Applied Pharmacognosy, Industrial Pharmacy, Bioassay of Drugs & Biostatistics, Toxicology& Forensic Chemistry.

M.Sc. (Microbiology) Courses:

Physical Chemistry, Mathematics& Statistics and Instrumental Analysis.

PhD courses:

Statistical methods of biology, responsible conduct of research, biological and structural aspects of drug design and action, protein mass spectroscopy and proteomics, molecular bacterial pathogenesis, immunobiology, and general biochemistry I.

Professional Memberships and Community involvement

- Ad Hoc reviewer in Plos One, Scientific Reports, & Arabian journal of chemistry: 2020-present.
- Member of the American Association for the Advancement of Science (AAAS): 2020-present.
- Vice-president of the Egyptian student association at Purdue University (ESAP): 2019-2020.

- Member of Purdue Arab society organization (PAS)
- Purdue Graduate Student Government (PSGC)
- March 2019, The 67th Annual Lafayette regional science and engineering fair, poster judge.
- March 2020, The 68th Annual Lafayette regional science and engineering fair, poster judge.
- General Syndicate of Egyptian Pharmacists.
- Qualyobia Syndicate of Egyptian Pharmacists.
- Zagazig Scientific Pharmaceutical Students' Association.