ANTIBACTERIAL DRUG DEVELOPMENT TARGETING GUT PATHOGENS

by

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"O Allah, to You is praise as befits the glory of Your Face and the greatness of Your Might"

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LIST OF ABBREVIATIONS

AGs	Aminoglycosides
ANOVA	Analysis of Variances
ATCC	American Type Culture Collection
BEI resources	Biodefense and Emerging Infections Research Resources Repository
BHI	Brain heart infusion
BHIS	Supplemented brain heart infusion
C. difficile	*Clostridioides difficile*
CDAD	*C. difficile* associated diarrhea
CDC	Centers for Disease Control and Prevention
CDT	*C. difficile* binary toxin
CLSI	Clinical and Laboratory Standards Institute
CP	Cysteine protease
CDI	*Clostridioides difficile* infection
DMSO	Dimethyl sulfoxide
E. faecium	*Enterococcus faecium*
E. faecalis	*Enterococcus faecalis*
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
GSH	Glutathione
GT	Glucosyl-transferase
GTDs	Glucosyl transferase domains
IDSA	Infectious diseases society of America
IL	Interleukin
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAP	North American pulsotype
PACUC	Purdue University Animal Care and Use Committee
P_{app}	Apparent permeability coefficient
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
R&D	Research and development
RBD	Receptor binding domain
REA	Restriction endonuclease analysis
ROS	Reactive oxygen species
SAR	Structure-activity relationship
SCFA	Short chain fatty acids

SHEA	Society for healthcare epidemiology of America
TcdA	*Clostridioides difficile* toxin A
TcdB	*Clostridioides difficile* toxin B
TD_{Lo}	Lowest published toxic dose
TSA	Tryptic soya agar
TSB	Tryptic soya broth
VRE	Vancomycin-resistant *Enterococcus*

ABSTRACT

Over three million infections were reported in the United States of America in 2019. These infections were caused by either antibiotic-resistant pathogens or *Clostridioides difficile* and resulted in more than 50,000 deaths. Unfortunately, antibacterial agents are rapidly losing their ability to treat infections and the process of discovering new antibiotics is too slow to cope up with bacterial evolution. Repurposing FDA-approved drugs of well-studied safety, pharmacology and pharmacokinetics represents a faster alternative method of antibacterial drug discovery. Repurposing is more successful and less depleting method of drug discovery than classical *de novo* method in regard to both cost and time. In the following studies, two major pathogens are targeted, vancomycin-resistant *Enterococcus* (VRE) and *C. difficile*. Both bacteria are more prevalent in healthcare settings were more vulnerable population of elderly and immunocompromised individuals reside. In addition, healthcare settings are usually associated with higher frequency of receiving antibiotics which in turn, compromises the integrity of normal microbiota responsible for protection against invading pathogens. Furthermore, hospital stays are associated with exposure to bacterial shedding from other patients. Our aim was to identify FDA-approved drugs with novel ability to eradicate these two bacterial pathogens in the gastrointestinal tract (GIT). Notably, the GIT is considered the actual site of infection in case of *C. difficile* while it is only a transition site for VRE where the bacteria colonize before causing true infections in other tissues. Studies against both bacteria started with an *in vitro* screening of FDA-approved drugs and clinical molecules to identify potential candidates for further investigation.

For VRE, two drugs where identified with potent inhibitory activity and favorable pharmacokinetic profiles, auranofin and ebselen. Auranofin was approved in the 1960s for the treatment of rheumatoid arthritis due to its anti-inflammatory activity. Auranofin was found to exert potent bacteriostatic activity against both vancomycin-sensitive and vancomycin-resistant *Enterococcus* strains (minimum inhibitory concentration against 90% of the strains, MIC90 = 1 µg/mL). In addition, bacteria could not develop resistant mutants against auranofin upon prolonged exposure. On the other hand, ebselen is an organoselenium compounds currently in clinical trials for several indications. Similarly, ebselen was found to be a potent inhibitor of VRE growth (MIC90 = 2 µg/mL). In addition, ebselen successfully inhibited bacterial biofilm formation and

eradicated mature biofilms. In a mouse model of VRE colonization, both drugs inhibited bacterial shedding and reduced bacterial counts in the GIT of the colonized animals.

For *C. difficile*, auranofin was also found to exert potent inhibitory activity against bacterial growth (MIC90 = 2 µg/mL), toxin production and spore formation. Additionally, it was beneficial in protecting colon cells against *C. difficile* toxin-induced inflammation. Further, auranofin was found to not promote growth of VRE as seen with the current anticlostridial agents. In addition to auranofin, two more antiprotozoal drugs were found to potently inhibit *C. difficile* growth, ronidazole and secnidazole. Both drugs are 5-nitroimidazoles approved for human (secnidazole) or veterinary (ronidazole) applications. Secnidazole and ronidazole halted *C. difficile* growth at very low concentrations (MIC90 = 0.5 and 0.125 µg/mL, respectively). Furthermore, both drugs were superior to metronidazole in bacterial killing and had favorable activities against protective gut microbiota. In addition, they demonstrated efficient protection to mice in a *C. difficile* infection model.

Overall, several drugs were presented to possess favorable activities against *C. difficile* or VRE. These drugs merit more evaluation as potential candidates for the treatment of infection caused by either bacteria.

CHAPTER 1. INTRODUCTION

1.1 Global Threat of Bacterial Infection

"The post-antibiotic era is already here". This was one of the first statements in the last report by the Centers for Disease Control and Prevention (CDC) released in 2019 about infections with antibiotic-resistant bacteria and *Clostridioides difficile*. This is because unfortunately, a lot of antibiotics that were once considered miraculous have completely or partially lost their activity in fighting against bacteria. Consequently, over 3 million infections are reported annually with almost 50.000 deaths in the United States due to antibiotic-resistant infections and *C. difficile* [1]. Interestingly, there was a significant reduction in the numbers of infection-related deaths from the last CDC report in 2013. However, there was a strong recommendation to adopt more aggressive strategies to fight against bacterial infection and not to rely solely on antibiotics. Indeed, this was not the only call for more effective measures to prevent and treat bacterial infections throughout the years. Nevertheless, bacteria keep evolving in a plethora of forms to overcome all the new contending strategies [2]. Although everyone is at risk of contracting bacterial infection, hospitalized patients are considered a higher risk group due to their underlying conditions. Usually hospitalized patients are from more vulnerable categories like seniors or infants. In addition, they are mostly undergoing a procedure or receiving medications, like chemotherapies, that compromise their natural immunity against infections. Further, there is a higher possibility for hospitalized patients to get exposed to bacteria being shed from other patient in the same facility. As a result, bacterial infections are more frequent during, or sometimes immediately after, admission to a healthcare facility. Regrettably, Patients acquiring infections during their hospital stay will remain in hospital 2.5 times longer and will incur 3 times higher cost than uninfected patients. Furthermore, they will also require increased utilization of physicians, nurses and hospital resources even after discharge [3]. In the following studies, two of the most dangerous hospital related infective bacteria were targeted, *C. difficile* and vancomycin-resistant *Enterococci* (VRE), which were listed by the CDC as urgent and serious threats, respectively. The strategy that was followed was to target these two bacteria in the gastrointestinal tract (GIT). The GIT can be the true infection site, as in case of *C. difficile*, or a transient colonization site before causing a true infection of other tissues, as in case of VRE. In both cases, protection conferred through the GIT

normal bacteria, known as colonization resistance, is usually compromised pre-infection. Colonization resistance and risk factors involved in breaching the integrity of GIT protective bacteria and causing infection will be discussed below.

1.2 Colonization Resistance and Risk Factors for Gut Infections

Human body serves as a scaffold for a multitude of bacteria that live with a human in a complex harmony along his life [4, 5]. The largest consortium of bacteria resides in the human intestine and is responsible for a variety of homeostatic functions. Along with their metabolic and immunologic functions, gut bacteria play an essential role in establishing colonization resistance to protect against numerous intestinal pathogens [6-8]. *C. difficile,* for instance, is one of the most virulent intestinal pathogens protected against by virtue of normal microbiota. *C. difficile* is naturally equipped with extremely resistant spores that mediate transmission. However, pathology of *C. difficile* infection, ranging from mild diarrhea to megacolon and shock, is attributed to toxin production [9, 10]. Intestinal microbiota protects against *C. difficile* colonization through production of short chain fatty acids (SCFAs). SCFAs stimulate the growth of gut epithelium, reduce inflammation through induction of regulatory T cells (Tregs), induce antimicrobial peptides like thuricin CD and augment the mucus barrier through increasing production of mucin. Furthermore, resident bacteria compete with the invading *C. difficile*, or other pathological bacteria, for intestinal niches and nutrients. In addition, microbiota is involved in the transformation of primary bile acids, *C. difficile* spore germinant, into secondary bile acids, *C. difficile* growth inhibitors, resulting in reduction of spore germination and inhibition of the vegetative growth [10, 11]. Overall, an intact intestinal microbiota is crucial for protection against *C. difficile* infection (CDI).

The use of antibiotics is the main risk factor for deteriorating the integrity of normal gut microflora. Antibiotics, specifically broad spectrum anti-anaerobic ones, exert profound and long-lasting effect on both the structure and function of intestinal microbiota [10, 12]. Several studies have been conducted to evaluate the effect of different antibiotics on the relative population and diversity of gut microbiota in humans and animals. Despite the disparity between different studies, there was a general theme of diminished bacterial diversity and species abundance after antibiotic administration [13-16]. In addition, the populations of *Bacteroidetes* and *Firmicutes*, the dominant phyla among microbiota, were dramatically decreased while the population of *Proteobacteria* was

relatively increased [17-22]. The antibiotic-induced dysbiosis of microbiota is accompanied with a reduction of its metabolic activity and eventually a decline in colonization resistance. Reduction of the metabolic function of the microbiota lowers the intestinal SCFAs levels, with a resultant loss of their protective and immunologic functions. Also, a defective bile acid metabolism results in elevated concentration of primary bile acids and reduced concentration of secondary bile acids, which in turn supports the germination of *C. difficile* spores and allows for vegetative outgrowth and toxin production. Additionally, the lack of niche exclusion and ecological competition between microbiota and invading bacteria increases host susceptibility for infection [8, 10, 23-26]. Notably, recovery of the microbiota can take several months after antibiotic cessation, the recovery period varies based on the host and the spectrum of the antibiotic used [8, 13]. Even after full recovery, the structure of the microbiota does not necessarily return to the pre-antibiotic state. Additionally, repeated use of antibiotics has a cumulative effect on the microbiota composition [10, 12].

Although individuals have different microbiota composition, the use of similar antibiotics will select for similar resistant organisms. Therefore, the use of different antibiotic classes will result in distinct microbiota profiles with variable susceptibility [10, 27]. Several retrospective meta-analyses were performed to classify antibiotics based on their risk to develop hospital or community acquired CDI. Clindamycin and cephalosporins were associated with the greatest risk of CDI followed by fluoroquinolones and carbapenems. Penicillins, macrolides and sulfonamides/trimethoprim were moderately associated with CDI, while tetracycline was not associated with any risk of the infection [28-32]. Susceptibility of the infecting strain can also play a role in infecting or colonizing the GIT. Resistance to clindamycin, cephalosporins and fluoroquinolones is very common in *C. difficile* and several outbreaks of clindamycin- or fluoroquinolone-resistant strains have been reported [30, 33]. Also, duration of antibiotic-induced disruption of microflora affects susceptibility for infection. Clindamycin, for instance, is excreted in bile which prolongs its intestinal activity leading to longer period of disruption of gut flora [34]. This is also observed with anticlostridial drugs, vancomycin has a more powerful and prolonged effect on the gut flora than metronidazole leading to longer vulnerability to CDI recurrence or developing VRE infection [35, 36]. Fidaxomicin, on the other hand, has a weaker influence on microbiota than both vancomycin and metronidazole, as a result, less recurrence of CDI is observed with fidaxomicin use [37].

The second main risk factor for developing GIT infections is aging. Advanced age is associated with several changes that increase the incidence of CDI and VRE colonization [38-40]. Colonization resistance declines with age due to reduction of the overall diversity of microbiota and loss of protective species from *Bacteroidetes* and *Firmicutes*, resembling the post-antibiotic exposure state. In addition, aging is associated with immunosenescence which results in lack of antibodies against *C. difficile* [9, 38]. Additionally, aging results in more frequent interactions with healthcare systems and more antibiotic exposure which raise the frequency of contact with *C. difficile* spores and VRE and increase susceptibility of infection [38]. Prevention of infections in elderly can be achieved primarily through avoidance of unnecessary use of antibiotics, which can reach up to 75% in nursing homes [38, 41, 42]. Whenever possible, the use of antibiotics with lower odds of developing CDI should be favored. In addition, reducing exposure to *C. difficile* spores and VRE through proper cleaning of surfaces with chlorine-based disinfectants and training the healthcare personnel on hand hygiene practice can reduce the incidence of infection [43, 44]. Several other promising preventative approaches like microbiota restoration, intestinal antibiotics inactivators, non-toxigenic *C. difficile*, and immunization are still under investigation and warrant further research [45]. Other risk factors for infection with VRE specifically include alcoholism, dementia and McCabe score of 2 [46]. While for *C. difficile* gastrointestinal procedures, anti-ulcer treatments, nasogastric tubing and malignant blood disorders are associated with higher risk of infection [31].

1.3 Vancomycin-resistant *Enterococcus*

Enterococci are Gram-positive facultative anaerobes that are ubiquitous in nature. They inhabit plants, water, water sediments, soil and sand. They are also considered as a part of the normal flora in humans, animals, birds and insects [47]. In human, *enterococci* reside in the oral cavity, genital tract and biliary system, however, they are most abundant in the gastrointestinal tract [48, 49]. In susceptible patients, resident *enterococci* can act as opportunistic pathogens and infect their host. Nonetheless, true infections can occur due to nosocomial transmission of virulent strains between patients in healthcare settings. As a result, *enterococci* are the second leading causes of hospital-acquired infections. [49, 50]. The most medically relevant strains are *E. faecium* and *E. faecalis*. They cause a variety of infections including; urinary tract infection, soft tissues infection, bacteremia, meningitis and endocarditis [51]. The medical importance of *enterococci* is primarily

attributed to their antibiotic resistance. *Enterococci* are intrinsically resistant and tolerant to some antibacterial agents, further, they can acquire resistance to all known antibiotics classes through either external transfer of genetic material or genetic mutation [52]. The molecular mechanisms of resistance to conventional antibiotics are explained below:

1. β-lactam antibiotics exert their antibacterial activity through binding to penicillin binding protein (PBP) and subsequent inhibition of peptidoglycan assembly. This results in cell wall synthesis inhibition and induction of apoptosis through production of reactive oxygen species. *Enterococci* are intrinsically resistant to β-lactams due to the production of low affinity PBP (PBP5). In addition, *enterococci* are tolerant to β-lactams, meaning they can only be killed at very high, clinically unachievable concentrations of the antibiotics [51, 53]. Tolerance is mediated via superoxide dismutase which alleviates oxidative stress and halts apoptosis [54]. Furthermore, *enterococci* can attain β-lactam resistance through acquisition of transferable plasmids encoding for β-lactamase, an enzyme that hydrolyze β-lactam antibiotics, from *staphylococcus aureus* [51].

2. Aminoglycosides (AGs) bind the 16S rRNA of the 30S ribosomal subunit and block bacterial protein production. *Enterococci* naturally have an impaired uptake of AGs which provides low-level resistance to these antibiotics. Moreover, 16S rRNA can be methylated by EfmM, a chromosomally encoded methyltransferase, to be sterically inaccessible by AGs [52, 54]. Intrinsically inactivating enzymes (e.g. 6'-acetyltransferase) bestow only low-level resistance to AGs. However, acquired enzymes (e.g. 2"-phosphotransferase-6'-acetlytransferase) provide high-level resistance to AGs and totally abolish their clinical efficacy [55].

3. Resistance to the **antifolate** combination sulfamethoxazole/trimethoprim is mediated through the ability of *enterococci* to utilize exogenous folate [51]. Additionally, the expression of *lsa* gene encoding for efflux pumps provides *E. faecalis* with natural resistance to **clindamycin** and **streptogramins** (quinupristin/dalfopristin, Q/D). In *E. faecium*, Q/D resistance involves inactivation of the drugs by acetyltransferase or lactonase. Another mechanism involved in Q/D resistance is target alteration [54]. Methylation of the 23S rRNA of the 50S ribosomal subunit, encoded by *ermB* gene, results in a phenotype referred to as MLS_B that is resistant to Q/D as well as **macrolides** and **lincosamides** [52]. Similarly, enzymatic methylation of the 23S rRNA results

in **oxazolidinone** resistance, however, it is mediated by a plasmid-carried gene, *cfr*. Other alterations in the 23S rRNA can result in resistance to linezolid such as mutation in the genes encoding 23S rRNA or ribosomal proteins [55]. Several other mechanisms are involved in the resistance development against less clinically important antibiotics. However, the most clinically important resistance associated with *enterococci* is resistance against vancomycin, explained below.

4. Glycopeptides, namely vancomycin and teicoplanin, wield their bactericidal action through complexing with the D-alanine-D-alanine terminal of peptidoglycan precursors leading to cell wall biosynthesis inhibition. Resistance to glycopeptides is mediated via modification of the terminal dipeptide into D-alanine-D-lactate or D-alanine-D-serine. Nine resistance operons have been identified (VanA, B, C, D, E, L, M and N), out of which VanA is the most prevalent. VanA can be transferred with a transposable element carried on pheromone-sensitive plasmid to confer glycopeptide resistance to recipient cells. VanA cassette encodes for seven enzymes that perform three distinct function; VanA and H assemble the modified dipeptide, VanX and Y hydrolyze the original dipeptide, VanR and S have two-component system regulatory function, while VanZ supports resistance through a yet unclear mechanism. Notably, VanA cluster can be mobilized on a wide host range plasmid to transfer glycopeptide resistance to *staphylococcus aureus* [52, 54].

Development of a resistance-proof antibiotic has been a practically unmet goal for antibacterial drug developers and researchers. Several approaches have been proposed to minimize the ability of bacteria to develop resistance; examples include using multi-drug combinations, antibacterial peptides, antivirulence agents, resistance suppressing drugs, iron quenching drugs and agents that prevent bacterial adhesion or quorum sensing [56-59]. Hitting several targets, or several pathways, with one drug is another tactic to curb antibiotic resistance. Raison d'être is that the probability for developing mutation in two or more targets will be much lower than in one target. Even if the bacteria could develop resistance to such agents, the fitness cost will limit the spread of the resistant phenotype [56, 60]. Interestingly, most of the successful antibiotics were found to have more than one bacterial target. As a result, multi-target antibiotics are favored over single-target ones (the multi-target hypothesis). A multi-target drug is also preferred to combination therapy due to less drug-drug interactions, toxicities and overall cost [60-62]. Several

methods are utilized to develop antibacterial polypharmacology starting from the serendipity screening approach up to structure optimization of a known antibiotic, hybridization of pharmacophores from distinct classes of antibiotics and novel computational fragment combination approach [63-65]. In following studies against VRE, two drugs were identified, ebselen and auranofin. Both drugs are thought to hit more than one target in the VRE cells, which is suggested to be the reason why it was found to be impractical to develop resistance strains against both drugs [66, 67].

1.4 *Clostridioides difficile* Infection (CDI)

Clostridioides difficile infection (CDI) is listed by the CDC as one of the most dangerous threats currently facing the nation. In 2017 CDI caused 223,900 people to require hospital care and was a direct cause of death of at least 12,800 patients. This means that a human being loses his life every 40 minutes due to CDI [68]. The CDC classified *C. difficile* as one of the urgent threats that calls for immediate and rigorous action [69]. There has been an increased in the severity and recurrence of CDI over the last two decades. The increased morbidity of CDI has led to more frequent need for colectomies and increased the mortality rate [70]. In addition, CDIs are not restricted only to the hospital settings, which represents only 50% of the cases, the community acquired infections represent about 41% of all CDIs while the remaining 9% occurred amongst the residents of retirement homes and long-term care services [71].

Clostridioides difficile is an anaerobic Gram-positive bacterium naturally armed with extremely resistance spores that mediate persistence, transmission and recurrence [72]. After ingestion, *C. difficile* spores germinate in the colon to yield the vegetative state. In the absence of a balanced gut microflora, due to broad spectrum antibiotic use for instance, *C. difficile* can colonize the colon and produce toxins [8]. *C. difficile* toxins, particularly toxin A and toxin B (TcdA and TcdB), are potent cytotoxic agents against intestinal epithelium. They are the main causative agent of CDI symptoms including diarrhea, pseudomembranous colitis, megacolon and could cause up to shock and death [73]. Both TcdA and TcdB are large proteins with glucosyl transferase domains (GTDs) that inactivate Rho GTPases in the colonic epithelial cells leading to loss of tight junctions, compromised epithelial integrity and promotion of mucosal inflammation [74]. CDI has suddenly become one of the most violent nosocomial infections due to the

emergence of hypervirulent strains with enhanced infectivity and virulence. An example of the hypervirulent strains of *C. difficile* is the North American pulsotype 1 (NAP1), PCR-ribotype 027, restriction endonuclease analysis (REA) group BI 8. This epidemic strain was first encountered in Quebec province, Canada in 2002 [75]. The main characteristic about hypervirulent strains is the secretion of increased levels of *C. difficile* toxins. The promotion in toxin production is attributed to a defect in the expression of a regulatory sigma factor responsible for TcdA and TcdB downregulation [76]. The hypervirulent strain of *C. difficile* are also equipped with a third toxin, binary toxin (CDT), which is thought to be associated with enhanced adhesion and pathogenicity [75, 77]. Below are the latest treatment options for CDI and some under-investigation methods.

1. Antibacterial treatment: Despite the numerous calls for effective anticlostridial agents, only three medications are used as anticlostridial agents, vancomycin, metronidazole and fidaxomicin. Vancomycin and metronidazole share the hitch of possessing activity against gut normal flora, added that metronidazole is completely absorbed form the intestine leaving a very minute concentration at the site of infection. As a result, high percentage of treatment failure and relapse are observed with metronidazole or vancomycin treatment [78, 79]. Fidaxomicin has a better profile than both drugs regarding the oral bioavailability and specificity [80]. Regrettably, the clinical outcome is still insufficient regarding treatment failure and relapse especially against the hypervirulent strains of *C. difficile*. Moreover, other antibacterials and antitoxins are under investigation for CDI treatment. Unfortunately, as for today none of them has been proved effective enough to be approved by the Food and Drug Administration (FDA) [70].

2. Monoclonal antibodies: Toxin neutralizing antibodies against TcdA and TcdB have been recently demonstrated to enhance the outcome of antibiotic treatments in regard to recurrence and mortality. Although the controversy about the outcome of antitoxin B, bezlotoxumab, it was recently approved by the FDA for the treatment of severe cases of CDI especially in elderly patients at high risk of recurrence [81]. Several other studies, at different phases of clinical trials, are aiming to develop and test the activity of monoclonal antibodies against *C. difficile* toxins.

3. Restoration of gut flora: Intestinal microbiome is the natural host defense against CDI. Additionally, CDI is known to be a direct outcome of impaired gut microbiome integrity due to

several reasons including antibiotic administration, aging and stress. Several approaches have been visited employing restoration of gut normal bacteria in order to fight against CDI. Transplantation of microbiota harvested from healthy human fecal matter have been shown to cure CDI and prevent recurrence. Other less laborious and more acceptable methods are being evaluated. Several microbiota preparations (suspensions, capsules and lyophilized powders) are at different levels of clinical trial trials [82, 83].

4. Active immunization: Although no vaccine is approved yet against *C. difficile*. Long term immunization against *C. difficile* sounds promising. Three candidate toxoid vaccine are currently undergoing clinical trials. Various vaccine strategies have been adapted to immunize against CDI. *C. difficile* surface proteins and carbohydrates have been used to elicit humoral immunity against *C. difficile*. Unlike toxoid immunization, they can inhibit *C. difficile* colonization. Formalin inactivated toxoids A and B have been classically used as vaccines. The effectiveness of mucosal versus systemic immunity of these vaccines is debatable, induction of both have been reported to increase survival in hamsters [84]. Unfortunately, toxoid vaccines are associated with safety issues arising from large scale production of toxins and spores [85]. Recombinant toxin-peptide antigens can also confer protection against CDI with the hazardous production of large-scale toxins. A question to investigate is which domain of the *C. difficile* toxin is more antigenic. *C. difficile* toxins include 3 functional domains: An N-terminal enzymatic domain, glucosyl-transferase (GT), a cysteine protease (CP) moiety and a C-terminal receptor binding domain (RBD). RBD was reported to induce protection against CDI without causing cytoskeletal damage to the host cell. DNA vaccines represent a safe and easy to manufacture alternative. Recently, a DNA vaccine utilized RBD of TcdA inserted in human adenovirus was found to produce a high humoral and cellular response [86].

1.5 Challenges Facing Antibacterial Drug Discovery

Discovery of antibacterial agents was a revolution in human history. Antibiotics enabled humans to fight against an invisible killer that afflicted and ended their lives for centuries without having tools to fight back. Unfortunately, the fight did not end at that point as bacteria evolved throughout the years under the selective pressure of antibiotics to be able to escape the inhibitory

effect of these medicines. Today, bacteria have developed resistances to all known antibiotic classes, moreover, some bacterial species combine them all at the same time being pan-resistant to all antibiotics used in treatment [1, 62, 87]. The best way to tackle this problem is through development of new antibacterial agents that either inhibit new targets in the bacterial cells or have improved activity over old ones. However, the discovery and approval of a new antibacterial small chemical molecules is a very slow and arduous process. Failure rate for a novel antibiotic development is estimated to be 95% with a cost that exceeds hundreds of millions of Dollars [88]. Surprisingly, the huge cost and risk for developing a new medicine is acceptable with classes of drugs that are not antibiotics. This is because pharmaceutical companies expect to financially profit from selling these non-antibiotic medicines. Nonetheless, this is not the case with antibacterial agents as the primary purpose of these new drugs is to be used only with cases that are resistant to other older antibiotics. In addition, new successful antibiotics are stewarded and kept only as last resort medications due to the fear of development of resistance against them. Further, raising the unit price of these new antibiotics is not feasible due to the design of their clinical trials and the hospital incentive system for using cheaper antibiotics. Also, developing resistance against any agent after being used in the open market remains a huge fear that can drastically affect the profitability of this agent. Consequently, most of the big pharmaceutical companies have aborted their antibacterial development programs leaving it for small and medium size companies. However, the great cost associated with antibacterial development is not affordable by these medium size companies and there are examples of entities that declared bankruptcy after introducing new antibiotics. Out of the 42 antibiotics currently undergoing clinical trials, only 11 of them are targeting pathogens on the World Health Organization's threat list. Thus, more financial support is required to guarantee the completion of the clinical trials for these drugs. The case is even worse with pediatric treatment due to the lack of evidence-based treatment for pediatric infections. As a result, only a handful of pediatric-directed drug development are currently in place [88, 89].

Besides increasing the funding for antibacterial drug discovery of small-molecules, other alternative approaches have been proposed to address the problem of dwindling pipeline of antibacterials. Classically, a large number of compounds, either natural, semisynthetic or fully synthetic, are screened for their inhibitory activity against actively growing bacteria. This approach is still widely used in identifying potential antibacterial leads which can be further optimized. In

addition, researchers are trying to synthesize derivative of antibiotics to which resistance arose with the hope that they might overcome this resistance. Therefore, families of active antibiotics are produced, e.g. penicillins, cephalosporins, macrolides, etc. The major drawback of this method is the likelihood of cross resistance between the parent molecule and its analog due to structural similarity. Genome hunting is another alternative approach to develop target-based antibiotics. Based on the revealed genome sequence of many bacterial special, it is possible to identify targetable structures against which no antibiotic has been previously developed. Therefore, already existing antibacterial resistances will be avoided through seeking new targets. One of the promising methods for antibacterial drug discovery is drug repurposing which is discussed in more details in the following section [90, 91].

1.6 Repurposing, the Shortcut for Antibacterial Development

Repurposing is the utilization of an FDA-approved drug outside the scope of its indicated activity to treat a new ailment, bacterial infection in this case. The definition also includes clinical molecules which failed in the clinical trials, but we still have valuable information known about them. FDA-approved drugs and clinical molecules have a well-characterized profile of safety and biological activity. Uncovering the cryptic antibacterial properties of these drugs and proposing them as candidates for antibacterial development is becoming more attractive recently. Both pharmaceutical companies and research institutions are leveraging repurposing approach for antibacterial drug discovery and for drug development in general [92, 93]. The promise in repurposing drugs is based on the hidden antimicrobial activity of some drugs and the commonality of pathways between various disorders. Drug repurposing is advantageous to *de novo* drug development in having lesser rate of failure. Repurposed drugs have at least acceptable safety profile in human so it is less likely that these drugs will fail in safety assessment. Also, less time and money are invested for drug development of a repurposed drug relative to classical drug discovery. The time frame for a *de novo* drugs development is 10-17 years compared to 3-12 years in case of a repurposed drug. Also, repurposing is estimated to save about $120 million of the overall cost associated with classical drug discovery, estimated to be $800 million. Although phase III cost will remain the same, the overall cost is significantly reduced in case of repurposed drugs through bypassing several preclinical steps. Several drugs have been repurposed from their initial use and are now being marketed for their new indication. For example, celecoxib was initially used

as non-steroidal anti-inflammatory drug and was found to be useful in the management of familial adenomatous polyps in 2000. The new indication resulted in a profit of $2.69 billion by the end of 2014. Another famous example is sildenafil that was initially indicated for the treatment of angina pectoris and was later repurposed for the management of erectile dysfunction [92-94]. Although there is a lot of success stories of repurposed drugs, no repurposed antibacterial agent has been approved by the FDA so far. However, several drugs are currently being investigated for their potential us as antibacterial agent [95-100]. In the following studies, several FDA-approved drugs were identified and investigated for their activity against VRE or *C. difficile*. In both bacteria we started by screening libraries of FDA-approved drugs and clinical molecules. Afterwards, potential hits were confirmed and their *in vitro* and *in vivo* activities were evaluated. Details about each study will be discussed in the following chapters.

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CHAPTER 2. REPURPOSING AURANOFIN AS AN INTESTINAL DECOLONIZING AGENT FOR VANCOMYCIN-RESISTANT ENTEROCOCCI

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2.1 Abstract

Multidrug-resistant enterococcal pathogens, especially vancomycin-resistant enterococci (VRE), are among the pathogens that require new antibiotic innovation. The colonization of the gut represents a major pathway by which VRE can cause infection and spread to other patients. In the current study, auranofin (FDA-approved rheumatoid arthritis drug) is evaluated for its potential use as a decolonizing agent for VRE. Auranofin was found to exert potent antimicrobial activity against a wide range of enterococcal clinical isolates with a minimum inhibitory concentration of 1μg/mL. No resistant mutants could be developed against auranofin over the course of 14 passages. Auranofin was also found to exert potent anti-biofilm activity against VRE. Auranofin was superior to linezolid, the drug of choice for VRE infection treatment, in the *in vivo* mouse model. Auranofin significantly reduced the VRE burden in feces, cecum, and ileum contents after 8 days of treatment. Accordingly, this study provides valuable evidence that auranofin has significant promise as a novel gastrointestinal decolonizing agent for VRE.

2.2 Introduction

Enterococcal species are one of the major pathogens of healthcare settings [1]. Two strains, *Enterococcus faecium* and *Enterococcus faecalis*, are of major concern. Both strains can lead to bloodstream infection, endocarditis, meningitis, urinary tract infection, and other infections [2]. *Enterococci*, especially *E. faecium*, exhibits intrinsic resistance against several classes of antibiotics and can also develop resistance via mutation or exogenous gene transfer, which resulted in the emergence of multi-drug resistant enterococcal strains. The most pronounced resistance is against vancomycin. Vancomycin-resistant enterococcal (VRE) infections comprise most

Enterococcus faecium infections as well as a significant proportion of *Enterococcus faecalis* infections [1].

The widespread use of broad-spectrum antibacterial agents has contributed to the elevated prevalence of these opportunistic pathogens and the reduction of their antibiotic susceptibility [2]. *Enterococci* are normal inhabitants of several tissues of the human body, particularly the gastrointestinal tract (GIT). In the GIT *Enterococci* remain under the control of other intestinal commensals and gut cell receptors. The administration of high antibiotic concentrations, like in hospital settings, leads to reduced populations of susceptible gut commensals, which allows *Enterococci* to overgrow [3,4]. The VRE domination of the gut's microbial consortium can persist even after the cessation of antibiotic treatment and is usually followed by the translocation of the antibiotic-resistant bacteria across the mucosal barrier, which causes systemic infections [4].

An effective decolonizing agent is required to prevent systemic VRE infections and limit VRE endemicity in healthcare settings [5]. Unfortunately, no drugs are approved by the Food and Drug Administration (FDA) to decolonize VRE from the intestine. The clinical molecule ramoplanin is in Phase II of clinical studies for the VRE decolonization from the gut; however, the recurrence rate is high [6]. Accordingly, there is an urgent need for an effective decolonizing agent for VRE. Repurposing FDA-approved drugs for which human safety, bioavailability, and efficacy have already been proven is an efficient approach to drug discovery. Auranofin is an FDA-approved drug for the treatment of rheumatoid arthritis, it has a well-studied safety profile. Adverse effects associated with auranofin administration are rare and mostly associated with long-term use. These adverse reactions include diarrhea (2 – 5% of the patients), skin rash and extremely rare thrombocytopenia. In most cases, these adverse effects are self-limited when the dug is reduced or stopped [7]. We recently showed that auranofin exerts broad-spectrum antibacterial and antifungal activities [8,9]. Auranofin was also shown to possess antiprotozoal activity and is currently in Phase II studies for the treatment of amoebic dysentery and giardiasis [10,11]. In addition to exhibiting potent antibacterial activity against VRE, auranofin can only be absorbed at a low rate [12]. These two characteristics make auranofin a potential candidate to decolonize VRE in the GI tract. In the current study, auranofin is being evaluated for its ability to decolonize VRE in the GI tract.

2.3 Materials and Methods

2.3.1 Bacterial strains and chemicals.

Vancomycin-resistant *Enterococci* (VRE) strains (Table 2.1) were obtained from the American type culture collection (ATCC) and Biodefense and Emerging Infections Research Resources Repository (BEI Resources). All experiments were carried out in accordance with relevant guidelines and regulations and were approved by the Institutional Biosafety Committee of Purdue University. All chemicals and reagents were purchased from commercial vendors. Auranofin, linezolid (Chem-impex International, Wood Dale, IL), ampicillin (Peosta, IA), vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO), gentamicin sulfate (Fisher Bioreagents, Fairlawn, NJ), and ramoplanin (Sigma-Aldrich, St. Louis, MO) were purchased commercially. Brain heart infusion (BHI), tryptic soya broth (TSB), tryptic soya agar (TSA) and enterococcosel broth were purchased from BD (Becton, Dickinson and Company, Cockeysville, MD) and Phosphate buffered saline (PBS) was purchased from corning (Corning, NY).

2.3.2 *In vitro* antibacterial assay

The standard broth microdilution assay was utilized to assess the minimum inhibitory concentration (MIC) of auranofin and the control antibiotics following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [38]. The minimum inhibitory concentrations (MICs) reported are the lowest concentration of each drug that could inhibit the visual turbidity due to bacterial growth. MIC_{90} is the drug concentration that can inhibit the growth of 90% of the tested isolates.

2.3.3 Killing kinetics of auranofin and control drugs

A time-kill assay was performed as described previously [15,39]. Briefly, an overnight culture of *E. faecium* HM-952 was diluted to approximately 10^6 CFU/ml. Three- and six-fold MIC of auranofin and the control drugs, in triplicates, were incubated with the bacterial suspension for 24 hours. At the indicated time intervals, samples were taken, diluted, and cultured on BHI agar to detect the bacterial counts at each time point.

2.3.4 *In vitro* development of resistant mutants

To assess the propensity of VRE to develop resistance against auranofin, *E. faecium* strain HM-952 was serially passaged with sub inhibitory concentrations of auranofin and control drugs (linezolid, ramoplanin, and gentamicin) in 14 passages over a period of two weeks. Resistance was defined as a 4-fold shift in the MIC [15,16].

2.3.5 Auranofin activity against VRE biofilm

A Biofilm Inhibition assay was utilized to examine the effect of sub-inhibitory concentrations of auranofin on the ability of VRE to form biofilm as described before [40]. Briefly, an overnight culture of *E. faecalis* NR 31972 was diluted 1:100 in TSB + 1% glucose and seeded in 96-well plates. Different sub-inhibitory concentrations of auranofin and control antibiotics (linezolid and ramoplanin) were added, and the plates were incubated for 24 hours at 37 ˚C. After incubation, the medium containing drugs and planktonic bacteria was discarded, and the adherent biofilms were washed twice with Phosphate buffered saline (PBS). The plates were then stained with 0.1% crystal violet for 30 minutes and washed again to remove the non-adherent stain. The remaining stain was solubilized using 95% ethanol for 45 minutes. Then the OD595 was measured using a kinetic microplate reader (SpectraMax i3x, Molecular Devices LLC, Sunnyvale, CA).
The auranofin activity was assessed against the mature biofilms using the aforementioned protocol. The drugs were added at concentrations higher than the MIC to allow for the effective eradication of the mature biofilm. All experiments were carried out in quadruplicates and repeated at least twice.

2.3.6 Decolonization of VRE from the gastro-intestinal tract of mice

The study was reviewed, approved, and performed under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) 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. To assess the efficiency of auranofin in decolonizing VRE from the gut in an animal model, the VRE mice decolonization model was followed [3]. Briefly, 6-week-old female C57BL/6 mice (Envigo, Indianapolis, IN) were sensitized by ampicillin (0.5 gm/ml in drinking

water) for seven days. One day later mice were orally infected with 3 x 10^8 CFU/mL of VRE strain *E. faecium* HM-952. Seven days post infection, the mice were divided into groups and treated orally with either auranofin (0.5 mg/kg), linezolid (10 mg/kg), ramoplanin (10 mg/kg), or PBS. Treatments were continued for eight days before the mice were humanely euthanized on day 9 via CO_2 asphyxiation. Fecal samples were taken freshly from the mice on days 0, 3, 5 and 7 post treatment. The cecum and ileum contents were collected following euthanasia. The contents of the cecum and ileum as well as the fecal sample were weighed, diluted with PBS, plated on enterococcosel agar (supplemented with vancomycin, 8µg/mL), and incubated for 48 hours at 37° C to determine the bacterial count in each sample.

2.3.7 Statistical analysis

GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla CA) was utilized in performing the statistical analysis. Two-way ANOVA followed by Dunnett's multiple comparisons test was performed to analyze biofilm inhibition and eradication data and the data from fecal samples. For the data generated from cecum and ileum content, one-way ANOVA was used and was followed by t test for the post hoc pairwise comparison.

2.4 Results

2.4.1 Susceptibility of enterococcal isolates to auranofin

We tested the activity of auranofin against a wide panel of enterococcal isolates and compared auranofin to the control antibiotics. Using the standard microdilution assay, auranofin was found to exhibit potent activity against the 27 tested isolates with an MIC range of 0.5 to 1 µg/ml (Table 2.1). Linezolid showed an MIC range of 0.5 to 16 µg/ml, ramoplanin showed an MIC range of 0.25 to 4 µg/ml, and vancomycin showed an MIC range of 0.5 to > 128. The MIC90 (the minimum inhibitory concentration that inhabited 90% of the strains) of auranofin was found to be 1 µg/ml. The MIC90 for linezolid and ramoplanin was 2 and 4 µg/ml, respectively.

Table 2.1 The minimum inhibitory concentration (MIC, µg/mL) of auranofin and control antibiotics against VRE isolates used in the study

Strains	MIC µg/mL				Source and comments
	Auranofin	Linezolid	Ramoplanin	Vancomycin	
E. faecalis NR 31971	1	1	2	64	Urine sample obtained in Michigan, USA. Resistant to vancomycin.
E. faecium NR 31914	1	1	2	>128	Isolated in 1995 from ascites fluid of a hospitalized patient in the Netherlands.
E. faecium HM 968	1	1	1	>128	Isolated from human oral sputum collected in Colombia, 2006.
E. faecalis NR 31972	1	1	4	>128	Isolated in 2003 from a human urine sample obtained in Michigan, USA.
E. faecium NR 28978	1	1	2	>128	Hospitalized person free of enterococcal infection in the Netherlands in 2000 during a hospital surveillance.
E. faecium NR 31903	1	16	2	>128	Isolated from the stool of a human patient prior to bacteremia.
E. faecium NR 31909	1	1	4	>128	Isolated from the stool of a human patient prior to bacteremia.
E. faecium NR 31912	1	1	2	>128	Isolated from the stool of a human patient having dominance of VRE in the stool but no bacteremia.
E. faecium NR 31915	0.5	1	1	>128	Isolated in 1996 from turkey feces in the Netherlands. Resistant to gentamicin.
E. faecium NR31916	0.5	1	1	128	Isolated in 1996 from turkey feces in the Netherlands.
E. faecium NR 32052	1	1	0.5	>128	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin and tetracycline.
E. faecium NR 32053	1	1	0.25	>128	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin and tetracycline.
E. faecium NR 32054	0.5	1	0.25	128	Isolated in 2008 from swine feces in Michigan, USA. Resistant to erythromycin and tetracycline.
E. faecium NR 32065	0.5	1	2	>128	Isolated in 1994 in Aix-en-Provence, France.

Table 2.1 continued

E. faecium NR 32094	1	0.5	2	>128	Isolated in 1996 in New York, USA.
E. faecium HM 952	1	1	2	>128	Human isolate from the United States.
E. faecium HM 965	1	1	1	>128	Human blood in Ecuador, 2006. Resistant to ampicillin, gentamycin and streptomycin.
E. faecium HM 970	1	1	0.5	>128	Human feces collected in Colombia, in 2008. Resistant to vancomycin.
E. faecium ATCC 700221	1	1	4	>128	Human feces, Connecticut. Resistant to Vancomycin and Teicoplanin.
E. faecalis HM 201	1	1	2	>128	Isolated in 2002 from the blood of a patient with endocarditis at Stamford Hospital in Connecticut, USA.
E. faecalis HM 334	1	1	2	>128	Isolated in 2004 from the blood of a 64-year-old female hemodialysis patient with fatal bacteremia.
E. faecalis HM 335	1	1	2	>128	Isolated in 2004 from the blood of a 64-year-old female hemodialysis patient with fatal bacteremia.
E. faecalis HM 934	1	1	4	>128	Isolate from a human secretion in Bogota, Colombia, in 2006.
E. faecium NR 31933	1	2	2	4	Isolated in 2001 from the feces of a miniature pig in Germany.
E. faecium NR 31935	1	2	2	≤1	Isolated in 1956 from cheese in Norway.
E. faecium NR 31937	1	1	2	2	Isolated in 1957 from the blood of a hospitalized patient in the Netherlands.
E. faecium NR 31954	1	1	2	2	Isolated in 2006 from the blood of a hospitalized patient in the Netherlands.
MIC_{90}	**1**	**2**	**4**	**>128**	

2.4.2 Killing kinetics of auranofin

After confirming the potent activity of auranofin against VRE, we next assessed the growth kinetics of VRE strain *E. faecium* HM 952 when exposed to auranofin at two different concentrations: 3X and 6X the MIC. As depicted in Figure 2.1, auranofin at 3X and 6X reduced VRE by 2.57 and 2.72 after 72 hours, respectively. Linezolid, a known bacteriostatic against VRE,

as expected did not reduce CFU after 72 hours of exposure. Ramoplanin, a known rapid bactericidal [13,14], demonstrated rapid bactericidal activity against VRE and cleared the VRE within 4 hours.

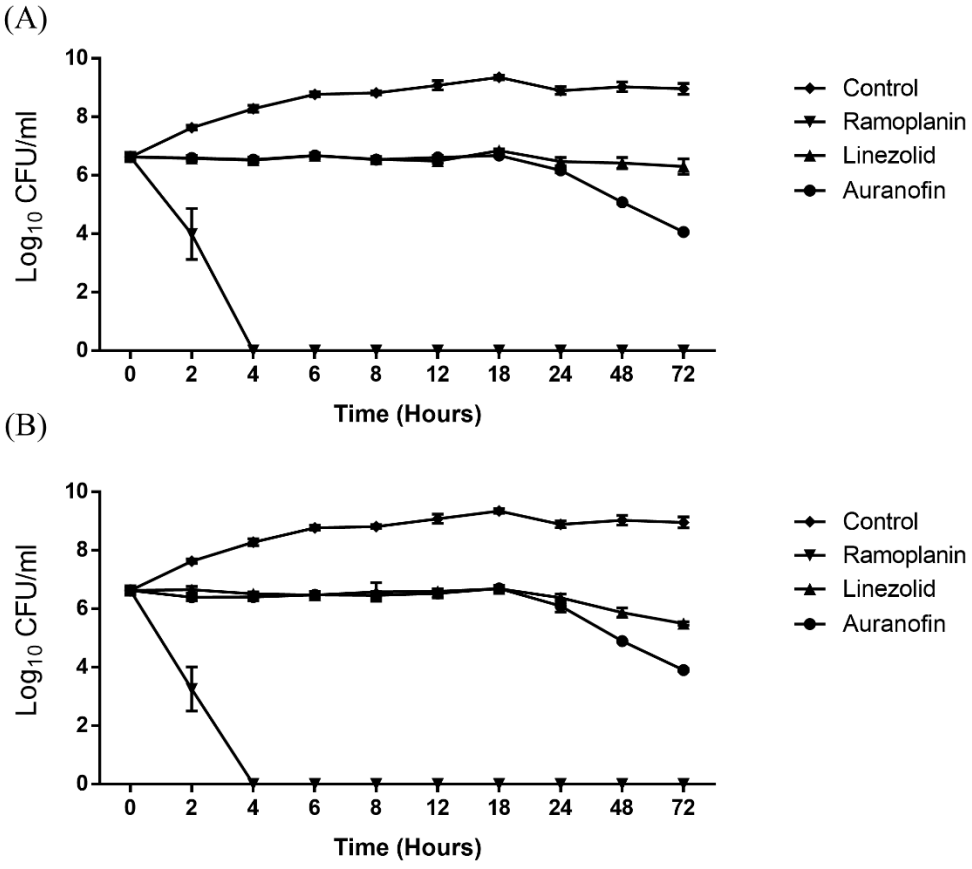

Figure 2.1 Time-kill assay for Auranofin linezolid and ramoplanin,

Compounds were tested at (A) 3 × MIC and (B) 6 × MIC. *E. faecium* HM 952 was aerobically incubated with the indicated concentrations of the drugs, in triplicates, for 72 hours at 37 ℃ and samples were counted at the indicated time points

2.4.3 *In vitro* multistep resistance development of VRE against auranofin

In order to test the likelihood of *enterococci* to develop resistance against auranofin, we serially passaged VRE isolate *E. faecium* HM 952 daily in the presence of a subinhibitory concentration of auranofin and control antibiotics for 14 days [15]. A four-fold shift in the MIC was considered resistance [16]. As presented in Figure 2.2, the MIC of auranofin did not change over 14 passages, which indicates that VRE did not develop resistance to auranofin. There was a one-fold increase in the linezolid's MIC observed over 14 passages. There was a rapid shift and

increase to a 7-fold MIC after the second passage for gentamicin. VRE developed a 32-fold increase in MIC to gentamicin after 14 passages with the drug.

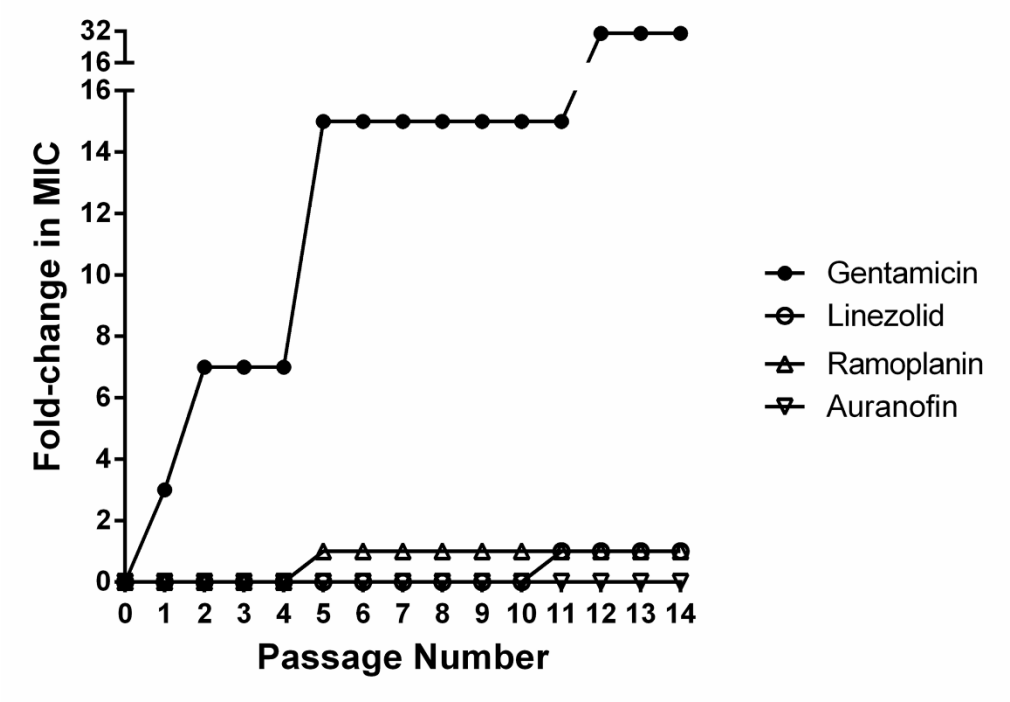

Figure 2.2 Multi-step resistance selection of auranofin, gentamicin, linezolid, and ramoplanin against VRE strain *E. faecium* HM 952.

VRE was serially passaged with drugs for 14 days and the broth microdilution assay was used to determine the minimum inhibitory concentration of each drug against VRE after each successive passage. A 4-fold increase in the MIC is considered resistance.

2.4.4 Activity of auranofin against VRE biofilm

To examine whether the potential therapeutic application of auranofin could be expanded beyond the inhibition of planktonic VRE, we tested the ability of auranofin to inhibit biofilm formation in VRE and remove established biofilm. As presented in Figure 2.3, auranofin at subinhibitory concentrations (0.0625X and 0.125X MIC) resulted in a significant reduction (~50%) of the VRE's biofilm-forming ability. Linezolid did not inhibit the biofilm formation in VRE, and ramoplanin exhibited a 30% VRE reduction at 0.125X MIC.

Next, we tested auranofin activity against established VRE biofilm. Auranofin at concentrations of 4, 8, and 16X MIC significantly reduced the established VRE biofilm by 50, 60 and 95%, respectively. Linezolid at 4, 8, and 16X MIC reduced the established VRE biofilm by 30, 40 and 50%, respectively. Ramoplanin concentrations of at 4, 8, and 16X MIC reduced the established VRE biofilm by 55, 55, and 65%, respectively.

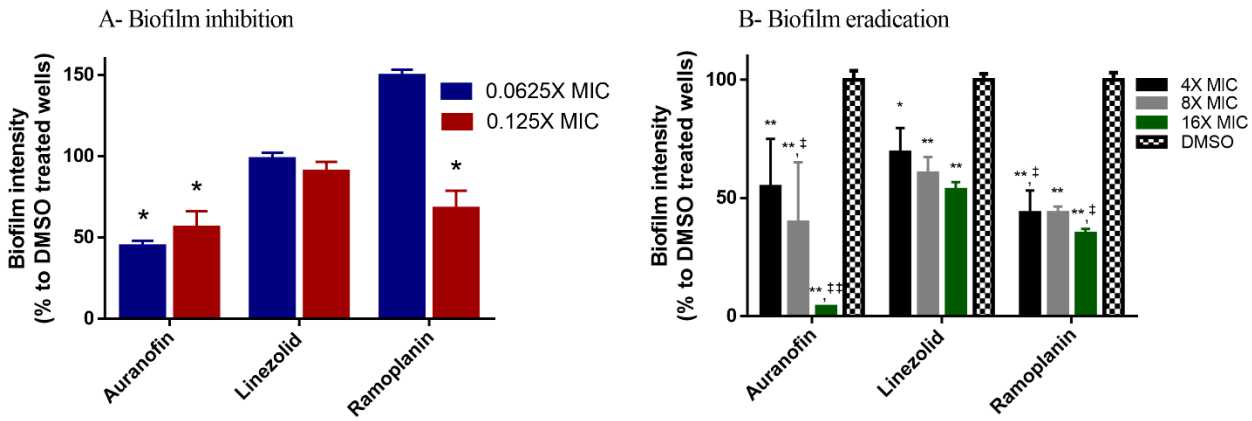

Figure 2.3 The anti-biofilm activity of auranofin against *E. faecalis* NR 31972

(A) Biofilm inhibition activity of auranofin; Sub-inhibitory concentrations of the drugs were added at the same time with the bacteria in TSB + 1% glucose and incubated for 24 hours at 37 ℃, then the biofilm density was measured using crystal violet. **(B)** Biofilm eradication activity of auranofin; The bacteria were incubated for 24 hours in TSB + 1% glucose to allow for the formation of mature biofilm. drugs were then added and incubated with the bacterial biofilm for additional 24 hours before the biofilm density was measured. (*) denotes significant difference from the DMSO treated control, while (‡) denotes a significant difference from the linezolid treated wells at equal concentration.

2.4.5 Activity of auranofin against enterococci in an *in vivo* model of intestinal VRE colonization

In order to validate our *in vitro* results, the VRE-colonization model [3,17] was utilized to assess the ability of auranofin to reduce the shedding and burden of VRE in the gastrointestinal tract of mice. As presented in Figure 2.4, auranofin and ramoplanin were superior to linezolid in decreasing the burden of VRE in fecal samples collected from mice. After three days of treatment, auranofin significantly reduced the burden of VRE in fecal samples by more than 97.79%, a rate

similar to ramoplanin (99.5%). Linezolid, in contrast, was unable to reduce the burden of VRE after three days of treatment. The presence of VRE in the fecal samples of mice treated with auranofin diminished to a remarkable 99.12% reduction after five days of treatment. Linezolid generated a 52.18% reduction in the VRE CFU count of fecal samples after five days of treatment. Mice treated with ramoplanin exhibited a 99.99% reduction of VRE in the fecal samples after five days for treatment. The burden of VRE (~10^7 CFU/gram feces) remained consistent in the control group (untreated mice) throughout the course of even days, which suggests that the decrease in VRE burden observed in mice receiving auranofin, ramoplanin, or linezolid was primarily due to the treatment received (rather than the excretion elimination of bacteria from the intestinal tract), data is shown in Figure 2.5.

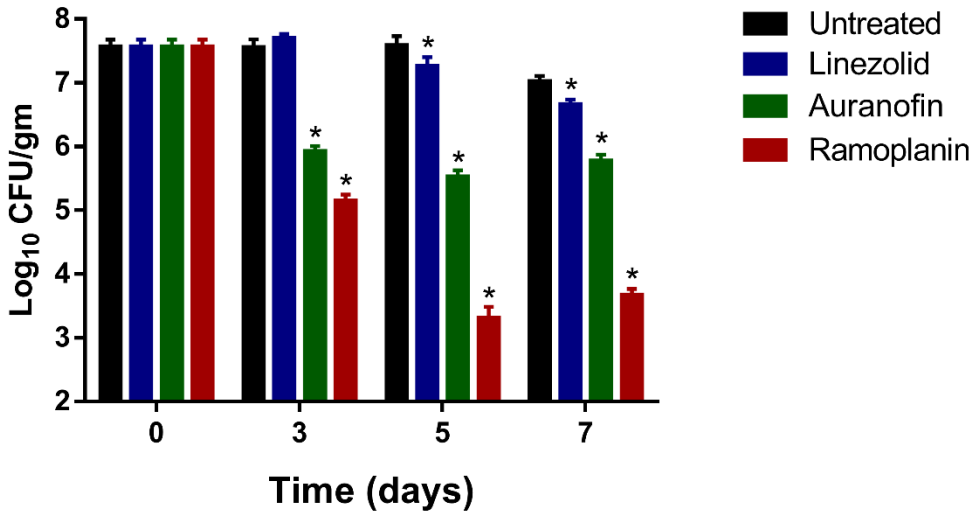

Figure 2.4 Bacterial counts of *E. faecium* HM-952 in the fecal samples of the mice. Infected mice were orally treated with auranofin (0.5 mg/kg), linezolid (10 mg/kg) and ramoplanin (10 mg/kg) daily for 8 days, one group was left untreated. Fecal samples were freshly collected from each group in days 0, 3, 5 and 7 post treatment. (*) denotes significant difference from the untreated group ($P < 0.05$).

A similar pattern was observed in the cecum and ileum contents. Linezolid did not reduce the cecum and ileum bacterial counts. Auranofin and ramoplanin significantly reduced the VRE load in the cecum and ileum. Auranofin decreased the burden of VRE in the cecal content by 1.4-log10 and in the ileal content by 1.3-log10, relative to the untreated mice. Ramoplanin decreased

the burden of VRE in the cecal content by 2.3-log10 and in the ileal content by 1.8-log10, relative to untreated mice.

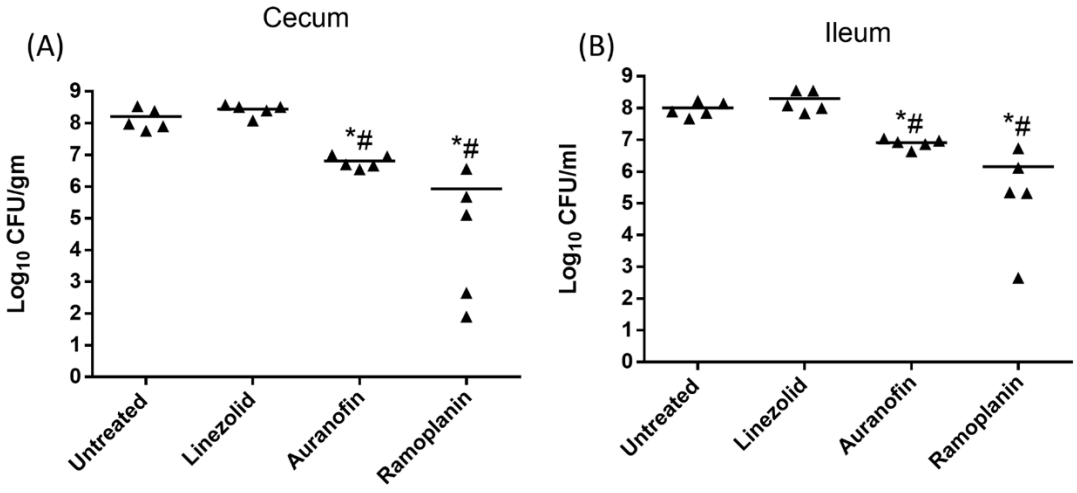

Figure 2.5 Bacterial counts of *E. faecium* HM 952 in (A) cecum and (B) ileum contents of the mice.

Infected mice (5/group) were orally treated with auranofin (0.5 mg/kg), linezolid (10 mg/kg) and ramoplanin (10 mg/kg) daily for 8 days, one group was left untreated. Cecum and ileum contents were collected on day 9. (*) denotes significant difference from the untreated group ($P < 0.05$) (#) denotes significant difference from the Linezolid-treated group ($P < 0.05$).

2.5 Discussion

Enterococci are normal inhabitants of several human body niches, especially the GIT. *Enterococci* have the capacity to overgrow other normal flora and colonize the gut, particularly after the administration of broad-spectrum antibiotics. Heavy VRE gut colonization usually precedes infections and is considered the initial source of bacteremia-induced endocarditis [2,3,18,19]. Moreover, the increased number of colonized patients increases the colonization pressure and the enterococcal acquisition by other patients [20]. *Enterococci* are naturally resistant to several antibiotics and can rapidly develop resistance to antibiotics via several mechanisms [21,22]. Vancomycin resistance is one of the most remarkable resistances developed by *enterococci*. More than one third of enterococcal infections in the U.S. are caused by VRE, and the percentage is higher for infections caused by *E. faecium* (almost 80%) [23]. Furthermore, VRE also displays resistance to the other clinically important antibiotics like ampicillin and

aminoglycosides [2,22,24]. *Enterococci* are capable of forming biofilm, a feature that not only facilitates infections but also helps to colonize the GIT [25,26].

Despite the significance of enterococcal colonization of the gut, the FDA has yet to approve a drug that can be used for VRE decolonization [2,19]. Also, the current control methods, including contact precautions and isolation of the patient are costly, have negative psychological impact on the patients and were not proven to have positive clinical outcomes [27]. Overall, there is an unmet and urgent need for a potent decolonizing agent against VRE.

Auranofin is an FDA-approved drug for the treatment of rheumatoid arthritis. It has a well-defined toxicity profile and an acceptable safety for human use [28,29]. Auranofin was reported to have a potent inhibitory activity against Gram-positive bacteria, including *enterococci*, fungi and parasites [7,30,31]. Due to its poor intestinal absorption, about 25% after oral administration, auranofin has been granted orphan-drug status from the FDA for treatment of intestinal amebiasis and is currently in a Phase II clinical trial for treatment of intestinal giardiasis [11,28,30].
In the current study we showed the superior *in vitro* activity of auranofin against a broad panel of *enterococcus* isolates compared to linezolid (the drug of choice for the treatment of VRE infections) and ramoplanin (clinical molecule currently in clinical trials for VRE decolonization). It is worth mentioning that the activity of auranofin did not change between vancomycin-resistant and vancomycin-sensitive strains. Auranofin was also consistent against both *E. faecium* and *E. faecalis*, a property that is lacking in some current antibacterial agents, for example quinupristin-dalfopristin [21].

A time-kill assay was utilized to ascertain whether auranofin is bacteriostatic or bactericidal. Auranofin exerted a bacteriostatic effect against the tested VRE isolate, a result that concurs with the previous report against *staphylococcus aureus* at equal concentration [32]. Although the bacterial count was reduced after 24 hours, it did not reach to the 3-log10 cutoff value that distinguishes bacteriostatics from bactericidals. The bacteriostatic activity of auranofin should not be discouraging, especially in the GIT environment of numerous competing microorganisms.

In previous studies, auranofin exhibited a complex mechanism of action against methicillin-resistant *Staphylococcus aureus* (MRSA), a possible reason for MRSA's inability to develop resistance against auranofin [30]. These results were encouraging for our test of VRE's ability to develop resistance against auranofin. As reported earlier, the MIC of auranofin against

the tested isolate did not change during the 14-day experiment and even up to 25 days (data not shown). This is in agreement with the previous reports of failure to generate auranofin-resistant MRSA [30,33].

As discussed earlier, biofilm is a virulence factor that helps enterococci to establish colonization. Biofilm-positive enterococcal phenotypes are associated with GI colonization. Auranofin is known to inhibit the enterococcal biofilm through the inhibition of selenium metabolism and selenoenzymes, given that biofilm formation is reported to enhance gut colonization of *enterococci* [25, 34]. We sought to test the biofilm inhibition activity of auranofin as well as the ability of auranofin to eradicate established biofilms. As was expected, when auranofin was incubated with the bacteria, auranofin significantly inhibited the biofilm formation at sub-MIC concentrations (Figure 2.3A). While linezolid and ramoplanin had no effect at all or a minimal effect, auranofin inhibited about 50% of the biofilm formation when compared to the untreated control. Additionally, auranofin treatment to the already formed biofilm drastically reduced the biofilm intensity at supra-MIC concentration. This effect was superior to those of linezolid and ramoplanin (Figure 2.3B). Biofilm enhances enterococcal colonization capacity, and the antibiofilm activity is particularly important in evaluating the decolonization efficiency of auranofin [25].

Auranofin's potent *in vitro* activity against numerous multi drug-resistant enterococcus strains and the inhibition of VRE biofilm (combined with the low intestinal absorption of auranofin and the inability of VRE to develop resistance against auranofin *in vitro*) prompted us to investigate the efficacy of auranofin *in vivo* in the VRE colonization mouse model. We were interested in evaluating the ability of auranofin to reduce the VRE shedding in fecal samples as well as reduce the VRE burden in the mice guts. The ampicillin-primed mice were infected with a VRE strain, and treatment began after the VRE colonization was established. At a concentration of 0.5 mg/kg, auranofin significantly reduced the bacterial shedding in fecal samples after only three days of treatment. By the seventh day of treatment, the bacterial shedding was reduced by about 99% compared to the untreated control (Figure 2.4). Auranofin (at the same concentration, 0.5 mg/kg) reduced the bacterial load in cecum and ileum contents (Figure 2.5) by more than 99.9%. This effect was superior to linezolid at a concentration of 10 mg/kg, which had a minimal effect in fecal samples and no significant effect in cecum and ileum contents. Although both auranofin and linezolid are bacteriostatic, linezolid's lack of activity is possibly due to its complete

absorption from the GI tract [35]. The auranofin dose used in this study is lower than the lowest published toxic dose (TD_{Lo}) in humans (0.54 mg/kg) [36], and is far less than the oral LD50 in mice, 84.94 mg/kg [37]. Although auranofin was not as effective as ramoplanin in reducing the VRE burden in the mouse model, auranofin is FDA-approved and ramoplanin—a Phase II clinical molecule—is not. Further investigations are needed to test whether VRE recurrence observed with ramoplanin treatment is also encountered with auranofin treatment [6] as well as the effect of auranofin on the normal bacterial population of the gut. Overall, the current study suggests that auranofin is a good candidate for further investigation as a decolonizing agent of VRE.

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CHAPTER 3. REPURPOSING EBSELEN FOR DECOLONIZATION OF VANCOMYCIN-RESISTANT ENTEROCOCCI (VRE)

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3.1 Abstract

Enterococci represent one of the microbial world's most challenging enigmas. Colonization of the gastrointestinal tract (GIT) of high-risk/immunocompromised patients by enterococci exhibiting resistance to vancomycin (VRE) can lead to life-threating infections, including bloodstream infections and endocarditis. Decolonization of VRE from the GIT of high-risk patients represents an alternative method to suppress the risk of the infection. It could be considered as a preventative measure to protect against VRE infections in high-risk individuals. Though multiple agents (ramoplanin and bacitracin) have been evaluated clinically, no drugs are currently approved for use in VRE decolonization of the GIT. The present study evaluates ebselen, a clinical molecule, for use as a decolonizing agent against VRE. When evaluated against a broad array of enterococcal isolates *in vitro*, ebselen was found to be as potent as linezolid (minimum inhibitory concentration against 90% of clinical isolates tested was 2 μg/ml). Though VRE has a remarkable ability to develop resistance to antibacterial agents, no resistance to ebselen emerged after a clinical isolate of vancomycin-resistant *E. faecium* was serially passaged with ebselen for 14 days. Against VRE biofilm, a virulence factor that enables the bacteria to colonize the gut, ebselen demonstrated the ability to both inhibit biofilm formation and disrupt mature biofilm. Furthermore, in a murine VRE colonization reduction model, ebselen proved as effective as ramoplanin in reducing the bacterial shedding and burden of VRE present in the fecal content (by > 99.99%), cecum, and ileum of mice. Based on the promising results obtained, ebselen warrants further investigation as a novel decolonizing agent to quell VRE infection.

3.2 Introduction

Enterococcal infections represent one of the major challenges facing healthcare providers worldwide, in part because of the uncanny ability of enterococci to acquire or develop resistance to antibiotics. In addition to their intrinsic resistance to many antibiotics, enterococci have developed resistance to many antibiotics either through mutations in the target gene/protein of an antibiotic or through acquisition of foreign genetic material, this is particularly seen in species of *Enterococcus faecium* and *Enterococcus faecalis* [1-3]. The emergence of clinical isolates exhibiting resistance to vancomycin (termed vancomycin-resistant enterococci or VRE) has been troubling as these isolates are often co-resistant to other classes of antibiotics including β-lactams [2]. Though newer antibiotics such as linezolid, daptomycin, tigecycline and quinupristin/dalfopristin remain effective treatment options clinically, several cases of enterococcal resistance against all the aforementioned drugs have been reported [4-9]. As a result, infections caused by drug-resistant enterococci are one of the major and ascending challenges present in healthcare settings [10]. Recently, this was affirmed when the World Health Organization listed vancomycin-resistant *E. faecium* as one of the high priority pathogens for research and development of new antibiotics and novel strategies to combat infections [11]. One novel strategy that warrants further investigation is identifying agents capable of decolonizing VRE from the gastrointestinal tract (GIT) of high-risk patients susceptible to infection [12].

Both *E. faecium* and *E. faecalis* are normal inhabitants of the human GIT, and their count remains constant due to a natural trait of the GIT know as colonization resistance [13]. Colonization resistance is the active ability of the host to eliminate pathogens from the GIT. The most important element that enhances colonization resistance is the presence of healthy gut microbiota. Administration of broad-spectrum antibiotics is a common practice for patients undergoing solid organ transplants and immunocompromised patients at high-risk of bacterial infection. However, these antibiotics are capable of disrupting the integrity of the normal GI bacterial consortium allowing for colonization by antibiotic-resistant enterococci [10]. An established enterococcal colonization can persist for months to years during which the carrier serves as a springboard for infection. Enterococcal colonization of the intestinal mucosal surface has been identified as a key initial step that permitted bacterial invasion of the bloodstream [10, 14, 15]. Given the significance of gut colonization in the development of enterococcal infections, it is quite surprising that no drug is currently approved for the decolonization of multidrug-resistant

Enterococcus [12, 15]. Several antibiotics (including bacitracin) have been investigated for use as decolonizing agents against enterococci but all suffered from poor patient tolerability or limited efficacy [16, 17]. Another molecule, ramoplanin has been investigated in clinical trials to decolonize VRE from the GIT of susceptible patients. Though this molecule did successfully reduce the burden of VRE in the GIT, patients suffered a high rate of recurrence after treatment was stopped; furthermore, ramoplanin had a negative impact on the microbiota as it promoted overgrowth of Gram-negative pathogens [17, 18]. Thus, there represents an unaddressed need to find new, safe molecules and drugs that can be used as decolonizing agents against VRE.

One approach for discovering novel decolonizing agents is via drug repurposing. This approach significantly decreases the high innovation cost and time normally associated with bringing a new drug to the clinic [19]. Using this approach, we recently discovered ebselen, a multifunctional organoselenium molecule in clinical trials, it possesses a potent antibacterial activity against important Gram-positive bacterial pathogens (including VRE) [20, 21]. Ebselen is being evaluated for various applications including cancer, cardiovascular disorders and kidney disorders [22, 23]. The activity of ebselen was established against a wide range of microbes including several *staphylococcus* strains, *Escherichia coli*, *Bacillus subtilis*, *Helicobacter pylori*, *Candida albicans* and *Aspergillus niger* [24]. Despite its known antimicrobial activity, the potential to use ebselen as a decolonizing agent against VRE has not been investigated. Thus, the present study evaluates the activity of ebselen against a wider panel of enterococcal clinical isolates, investigates the ability of VRE to develop resistance to ebselen, examines the efficacy of ebselen to disrupt VRE biofilm, and evaluates ebselen's ability to decolonize VRE from the GIT using a murine VRE colonization reduction model.

3.3 Materials and Methods

3.3.1 Bacterial strains and chemicals

Bacterial isolates (Table 3.1) were obtained from the American Type Culture Collection (ATCC) and Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Ebselen (Ark pharma, Arlington Heights, IL), linezolid (Chem-impex International, Wood Dale, IL), ampicillin (Peosta, IA), vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO), gentamicin sulfate (Fisher Bioreagents, Fairlawn, NJ) and ramoplanin (Sigma-

Aldrich, St. Louis, MO) were purchased from commercial vendors. Brain heart infusion (BHI), tryptic soya broth (TSB), tryptic soya agar (TSA) and enterococcosel broth were purchased from BD (Becton, Dickinson and Company, Cockeysville, MD) and phosphate-buffered saline (PBS) was purchased from Corning (Corning, NY).

3.3.2 Broth microdilution assay

The minimum inhibitory concentration (MIC) of ebselen and control antibiotics (linezolid, ramoplanin and vancomycin) was assessed in accordance with the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI) [49]. Approximately 5 x 10^5 CFU/mL of bacteria, in brain heart infusion broth, was incubated with serial dilutions of drugs at 37 ºC for 16-20 hours. The MIC represents the lowest concentration that inhibited the growth of the bacteria by visual inspection. MIC_{50} and MIC_{90} are the lowest concentration of each agent that inhibited the visible growth of 50% or 90% of the tested isolates, respectively [50, 51].

3.3.3 Time-kill assay

E. faecium HM-952, about 10^6 CFU/mL, in logarithmic growth phase was incubated with either 3×MIC or 6×MIC of ebselen, linezolid, or ramoplanin (in triplicate) at 37 ºC for 24 hours. Samples left untreated served as the negative control. At the indicated time points, samples were taken from the bacterial suspensions, serially diluted in PBS, and plated on BHI agar plates to count bacterial CFU. Plates were incubated at 37 ºC for at least 16 hours before enumerating colonies [52, 53].

3.3.4 Multi-step resistance selection of VRE against ebselen

To assess the ability of VRE to develop resistance against ebselen, *E. faecium* HM-952 was subcultured daily in the presence of subinhibitory concentrations of ebselen or control antibiotics (linezolid, ramoplanin, and gentamicin), using triplicate samples for each agent. At the end of each day the MIC of the tested isolate was determined, via the broth microdilution assay, to check for an increase in the MIC relative to the initial passage. A four-fold increase in the MIC, from the initial sample, was indicative of resistance formation as per previous reports [54, 55].

3.3.5 Anti-biofilm activity of ebselen.

Inhibition of biofilm formation

The ability of ebselen and control antibiotics (linezolid and ramoplanin) to inhibit VRE biofilm formation was tested, as described previously [26, 56]. In brief, an overnight culture of *E. faecalis* NR-31972 in TSB was diluted 1:100 in fresh broth supplemented with 1% dextrose. The bacterial suspension was incubated at 37 ºC with sub-MIC concentrations of all tested drugs (tested in triplicate) for 24 hours. To evaluate the biofilm density, media containing drugs and planktonic bacteria was discarded and the adherent biofilms were washed twice with sterile PBS. The biofilms were stained with 100 µL of crystal violet (0.1%) for 30 minutes. Excess crystal violet was washed out and the adherent stain was extracted using 95% ethanol for 45 minutes. The optical density (595 nm) for each treatment was measured using a microplate reader (SpectraMax i3x, Molecular Devices LLC, Sunnyvale, CA).

Eradication of mature biofilm

The ability of ebselen to disrupt established VRE biofilm was determined via the microtier dish biofilm formation assay, using the protocol described above. An overnight inoculum of *E. faecalis* NR-31972 was diluted 1:100 (in TSB + 1% dextrose) and were permitted to establish biofilm on a 96-well tissue-culture treated plate for 24 hours at 37 ºC. Next, media was removed and drugs were added (in triplicate) and serially diluted. Biofilm was incubated with drugs for 24 hours at 37 ºC. The biofilm mass was stained as described above.

3.3.6 VRE colonization reduction mouse model

To evaluate the ability of ebselen and ramoplanin to decolonize VRE from the GIT of the mice, we followed the protocol proposed by Ubeda *et al* [12, 15] with slight modification. Briefly, 8-week-old female C57BL/6 mice (Envigo, Indianapolis, IN) were housed in groups of five in individually ventilated cages. Mice were given access to food and water *ad libitum*. All the animal procedures were approved and done in accordance with the Purdue Animal Care and Use Committee (PACUC) and following the recommendation of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Ampicillin (0.5 g/l) was added to the drinking water for a week before the animals were orally infected with 10^8 CFU/mL of *E. faecium*

HM-952. Four days later animals were treated orally with ebselen or ramoplanin (10 mg/kg) daily for 20 days, while one group was left untreated. Bedding in the cage was changed regularly to avoid reinfection of mice. Fresh stool samples were collected from the mice on days 0, 5, 10, 15 and 20 post-infection. Mice were humanely euthanized on day 21 post-infection using CO_2 inhalation and the cecum and ileum contents were aseptically collected. Stool samples, the cecum, and ileum were suspended in PBS, serially diluted and plated on enterococcosel agar plates (supplemented with vancomycin, 8 mg/mL) on the same day of collection to assess the bacterial burden present. Agar plates were incubated for 48 hours at 37º C before the colonies were counted.

3.3.7 Statistical analysis

All statistical analysis was conducted using GraphPad Prism (version 7, GraphPad Software, La Jolla, CA). Biofilm inhibition data and data obtained from fecal samples were analyzed via two-way Analysis of Variances (ANOVA) followed by Dunnett's pairwise comparison, while data obtained from cecum and ileum contents was analyzed using one-way ANOVA with post hoc unpaired t test.

3.4 Results

3.4.1 Activity of ebselen against enterococcal isolates *in vitro*

The antibacterial activity of ebselen, linezolid, vancomycin, and ramoplanin was evaluated against 27 strains of enterococci from humans and animals. Most of the tested strains, Table 3.1, were resistant to vancomycin. Utilizing the broth microdilution assay, ebselen was found to possess potent antibacterial activity against all the tested isolates (Table 3.1). Against both vancomycin-resistant and vancomycin-sensitive strains, ebselen inhibited growth of 50% of all the tested isolates (MIC_{50}) at a concentration of 1 μg/mL. Against 90% of the isolates tested (MIC_{90}), ebselen's inhibited growth at 2 μg/mL. The MIC_{50} and MIC_{90} for linezolid were equal to ebselen. The MIC_{50} and MIC_{90} for ramoplanin was two-fold higher than the values obtained for ebselen. Vancomycin's MIC exceeded 128 μg/mL against more than 90% of the clinical isolates tested.

Table 3.1 The minimum inhibitory concentration (MIC, μg/mL) of ebselen and control antibiotics against enterococci clinical isolates.

Strains	MIC (μg/mL)				Source
	Ebselen	Linezolid	Ramoplanin	Vancomycin	
E. faecium, UAA714	0.5	1	2	>128	Aix-en-Provence, France.
E. faecium, HF50106	1	1	0.25	128	Swine feces, Michigan, USA.
E. faecalis, TX0104	0.5	1	2	>128	Blood of a patient with endocarditis, Connecticut, USA.
E. faecalis, S613	1	1	2	>128	Blood of a 64-year-old female hemodialysis patient with fatal bacteremia
E. faecium, E1578	0.5	2	2	4	Feces of a miniature pig in Germany.
E. faecium, UAA945	$\leq$ 0.25	0.5	2	>128	New York, USA.
E. faecium, ERV165	1	1	0.5	>128	Feces collected in Colombia.
E. faecium, Patient #2-1	0.5	1	4	>128	Stool of a human patient prior to bacteremia.
E. faecium, E0269	0.5	1	1	128	Turkey feces in the Netherlands.
E. faecium, HF50104	1	1	0.5	>128	Swine feces, Michigan, USA.
E. faecium, 503	1	1	2	>128	Human isolate from the United States.
E. faecium, E417	1	1	1	>128	Human blood, Ecuador.
E. faecalis, R712	1	1	2	>128	Blood of a 64-year-old female hemodialysis patient with fatal bacteremia.
E. faecium, ERV102	2	1	1	>128	Human oral sputum, Colombia.
E. faecium, Patient #1-1	1	16	2	>128	VRE isolated from the stool of a human patient prior to bacteremia.
E. faecium, E0164	2	1	1	>128	Turkey feces, Netherlands.
E. faecium, HF50105	1	1	0.25	>128	Swine feces, Michigan, USA.
E. faecium, E1620	1	1	2	2	Human blood, Netherlands.
E. faecium, E2620	1	1	2	2	Human Blood, Netherlands.
E. faecalis, SF24413	1	1	2	64	Urine, Michigan, USA.
E. faecium, E0120	1	1	2	>128	Ascites fluid, Netherlands.
E. faecalis, SF28073	1	1	4	>128	VRE isolated in 2003 from a human urine sample obtained in Michigan, USA.
E. faecium, E1071	0.5	1	2	>128	Netherlands, hospital surveillance program.
E. faecium, Patient #3-1	1	1	2	>128	Stool of a human patient.

Table 3.1 continued

E. faecalis, ERV103	0.5	1	4	>128	Human, Bogota, Colombia.
E. faecium, E1604	4	2	2	≤1	Cheese, Norway.
E. faecalis, B3336	2	0.5	4	≤1	Human blood, United States.
MIC50	1	1	2	>128	
MIC90	2	2	4	>128	

3.4.2 Time-kill kinetics of ebselen against VRE

After confirming the potent antibacterial activity of ebselen, we sought to investigate if ebselen is bacteriostatic or bactericidal against VRE. Using a standard time-kill assay, ebselen was found to exhibit a bacteriostatic mode of action at two different concentrations (3×MIC and 6×MIC, against vancomycin-resistant *E. faecium*) (Figure 3.1). Linezolid exhibited a similar pattern of activity to ebselen at both concentrations. In contrast, ramoplanin was found to exhibit rapid bactericidal action, completely reducing the burden of VRE to zero after four hours (at both test concentrations). A drug was only referred to as bactericidal if it could inhibit ≥ 99.9% of the bacterial burden.

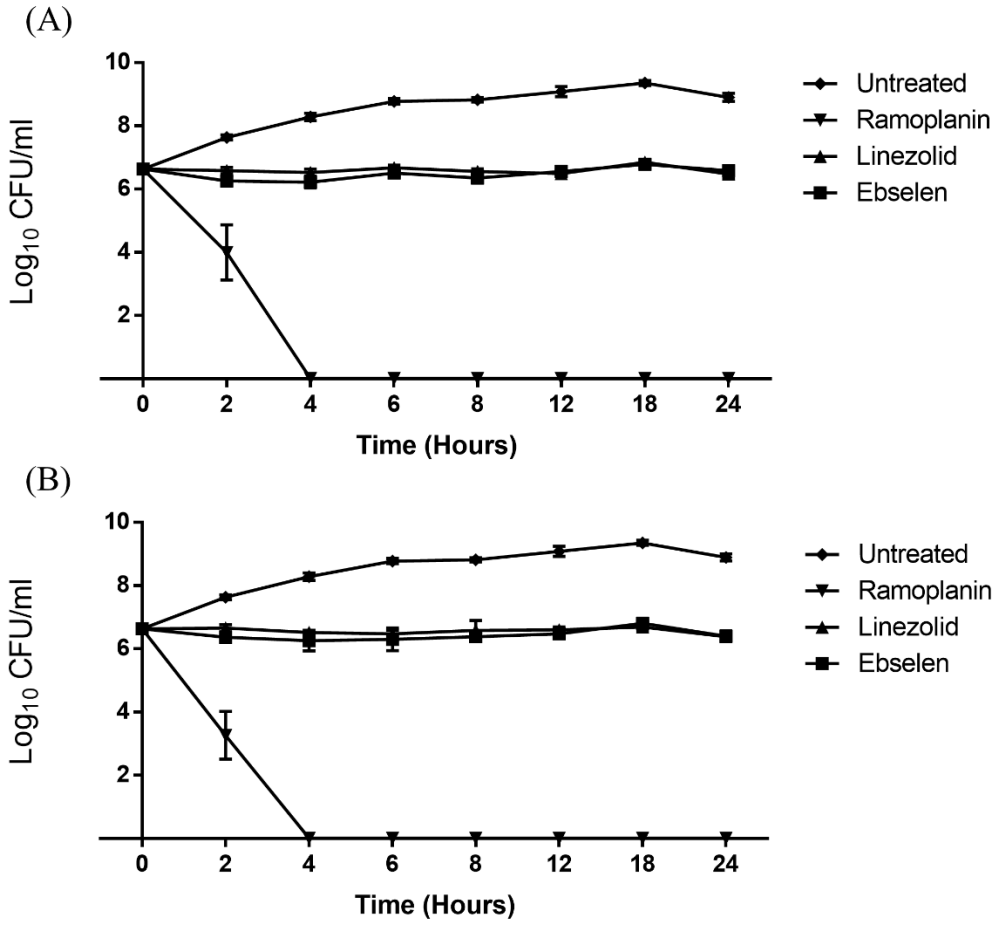

Figure 3.1 Time-kill assay for ebselen and the control antibiotics, linezolid and ramoplanin. Tested at **(A)** 3 × MIC and **(B)** 6 × MIC. *E. faecium* HM-952 was aerobically incubated with the indicated concentrations of the drugs, in triplicate, for 24 hours at 37 ℃ and samples were collected at the indicated time points to enumerate bacteria.

3.4.3 Evaluation of resistance development to ebselen

Recognizing the great propensity of enterococci to develop resistance to antibacterial agents [25], we were curious to test whether or not VRE can develop resistance to ebselen. To investigate this point, ebselen was evaluated via a multi-step resistance selection experiment against vancomycin-resistant *E. faecium*. As depicted in Figure 3.2, *E. faecium* remained sensitive to ebselen even after 14 consecutive passages (no change in the MIC was observed). Similar effects were observed with linezolid and ramoplanin (only one-fold increase in MIC). In contrast, resistance to gentamicin emerged rapidly. After the second passage, the MIC of gentamicin

increased seven-fold. The MIC continued to increase, resulting in a 31-fold change in the MIC of gentamicin at the end of the 14 passages. Although there was 1-fold increase in the MICs of Linezolid and ramoplanin, unlike ebselen, they did not cross the 4-fold cutoff limit that distinguishes sensitivity from resistance [26].

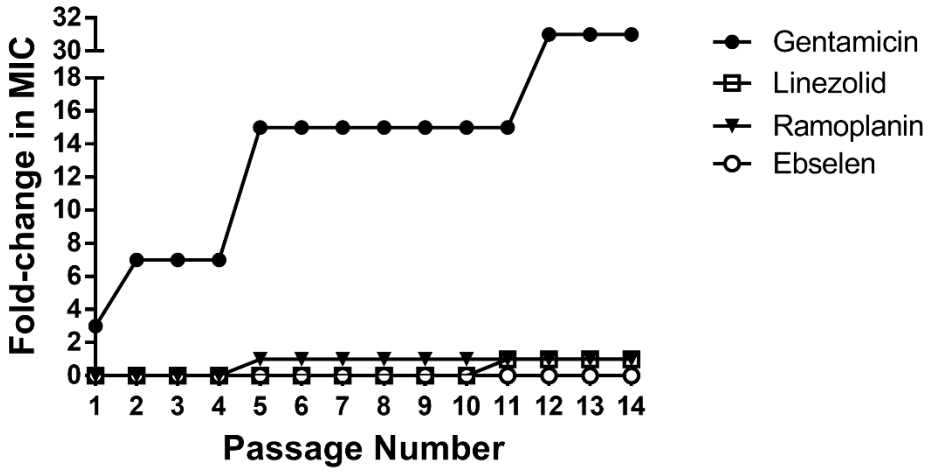

Figure 3.2 Multi-step resistance selection of *E. faecium* HM-952 in presence of ebselen, gentamicin, linezolid, or ramoplanin

The MIC of all the test agents was determined daily (for 14 passages) to test for the development of resistance (increase in MIC) to the tested isolate. A 4-fold increase in the MIC is indicative of resistance formation.

3.4.4 Antibiofilm activity of ebselen

We next moved to investigate if ebselen would be capable of interfering with a key virulence factor, biofilm formation, important for GIT colonization by VRE. *E. faecalis* NR-31972 was used given it forms strong, mature biofilm in microtiter plates. Interestingly, ebselen was found to exhibit a concentration-dependent inhibition of VRE biofilm formation. Ebselen was found to inhibit about 30% of VRE biofilm formation at 0.25×MIC and 0.5×MIC (Figure 3.3A). Linezolid, in contrast, was only effective at inhibiting biofilm formation at 0.5×MIC. Ramoplanin inhibited biofilm formation by 55% (at 0.25×MIC) and 70% (at 0.5×MIC).

We also investigated the ability of ebselen to disrupt mature, adherent VRE biofilm. Ebselen was superior to all other tested drugs in eradicating established VRE biofilm. In a

concentration-dependent manner, ebselen reduced mature VRE biofilm by 55% (at 4×MIC), 65% (at 8×MIC), and 95% (at 16×MIC), respectively (Figure 3.3B). At the same three concentrations, linezolid reduced biofilm mass by 30% (4×MIC), 40% (8×MIC), and 45% (16×MIC) while ramoplanin reduced the biofilm mass by 55% (4×MIC), 55% (at 8×MIC), and 65% (16×MIC). (*) indicates significant difference from linezolid-treated wells, while (#) indicates significant difference from ramoplanin-treated wells using 2-way ANOVA with Dunnett's post hoc comparison test at P<0.001.

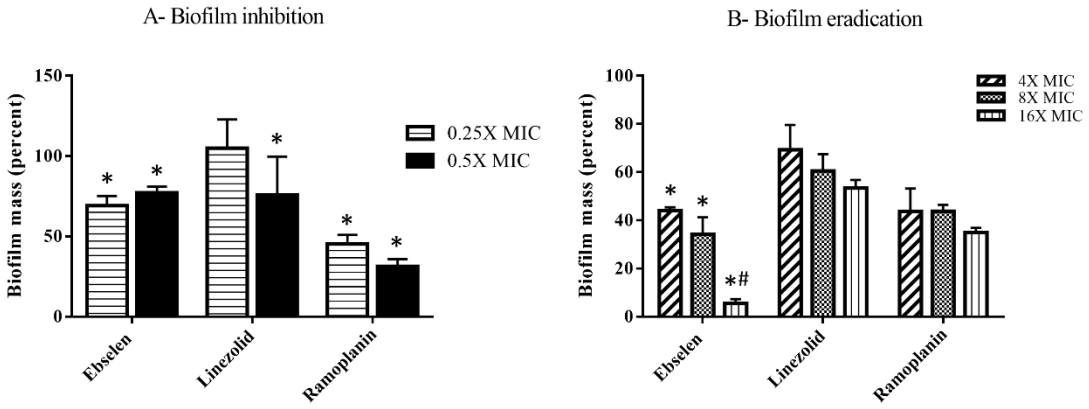

Figure 3.3 The anti-biofilm activity of ebselen against *E. faecalis* NR-31972

(A) Biofilm inhibition activity of ebselen. Sub-inhibitory concentrations of each drug were added to bacteria in tryptic soya broth (TSB) + 1% glucose and incubated for 24 hours at 37 ℃. The biofilm mass (OD_{595}) was measured after staining with crystal violet and destaining with ethanol. Data is presented as biofilm mass relative to DMSO-treated wells, (*) indicates significant difference from DMSO treated wells using 2-way ANOVA with Dunnett's pairwise comparison (P< 0.001). **(B) Biofilm eradication activity of ebselen**. Bacteria were incubated for 24 hours in TSB + 1% glucose to allow for the formation of mature biofilm. Supra-inhibitory concentrations of the drugs were then added and incubated with the bacterial biofilm for additional 24 hours before the biofilm density was measured (OD_{595}) by crystal violet staining. Data is presented as biofilm mass relative to DMSO-treated wells, (*) indicates significant difference from linezolid-treated wells, while (#) indicates significant difference from ramoplanin-treated wells using 2-way ANOVA with Dunnett's post hoc comparison test at P<0.05.

3.4.5 *In vivo* assessment of ebselen in a VRE colonization reduction mice model

After confirming the potent *in vitro* effect of ebselen against both planktonic VRE and VRE biofilm, we moved to confirm ebselen's ability to decolonize VRE from the GIT of infected

mice. Guided by the protocol of Ubeda *et al*. [15], ebselen was used to treat mice colonized with VRE. The effects of ebselen (10 mg/kg) and ramoplanin (10 mg/kg) were evaluated based on their ability to decrease bacterial burden in the stool samples of infected mice. Both ebselen (0.8-$\log_{10}$ reduction in CFU/mL) and ramoplanin (2.5-$\log_{10}$ reduction in CFU/mL) significantly reduced the burden of VRE in the stool, relative to mice in the untreated group, starting at day five (Figure 3.4). Ebselen continued to reduce the burden of VRE by 2.4-$\log_{10}$ reduction by day 15 and additional 1.7-$\log_{10}$ reduction by day 20. This was similar to the result obtained for ramoplanin which reduced the burden of VRE (relative to untreated mice) in fecal samples by 2.5-$\log_{10}$ on day 5, 2.4-$\log_{10}$ reduction (day 10), 1.4-$\log_{10}$ reduction (day 15), and 2.1-$\log_{10}$ reduction by day 20.

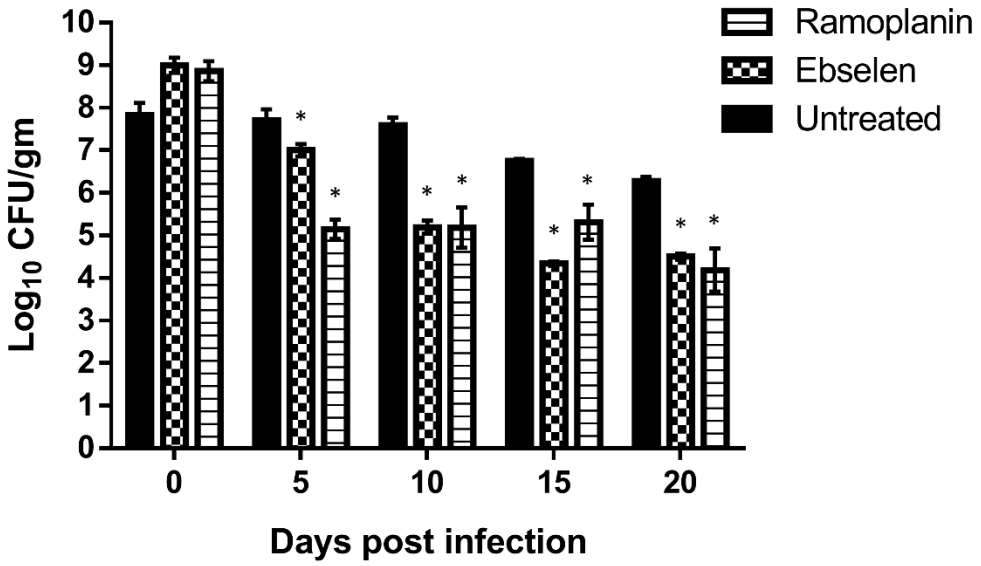

Figure 3.4 Bacterial counts of *E. faecium* HM-952 in the fecal samples of infected mice

Infected mice were orally treated with ebselen (0.5 mg/kg) and ramoplanin (10 mg/kg) daily for 8 days. One group was left untreated. Fecal samples were freshly collected from mice in each group on days 0, 5, 10, 15, and 20 post treatment. (*) denotes significant difference from the untreated group ($P < 0.001$).

In addition to examining the presence of VRE in stool samples of infected mice, the burden of VRE present in the cecum and ileum of mice was determined. One day after the final treatment was administered, mice were humanely euthanized and the cecum and ileum were aseptically removed and homogenized to determine viable bacterial CFU. Similar to results obtained from the

fecal samples, ebselen and ramoplanin significantly diminished the burden of VRE in both the cecal and ileal contents (Figure 3.5). Ebselen reduced the burden of VRE by 0.9-$\log_{10}$ in the ceca and generated a one-$\log_{10}$ reduction in the ilea of mice. Ramoplanin generated a 2.1-$\log_{10}$ reduction of VRE in the ceca and 1.5-$\log_{10}$ reduction in the ilea of infected mice.

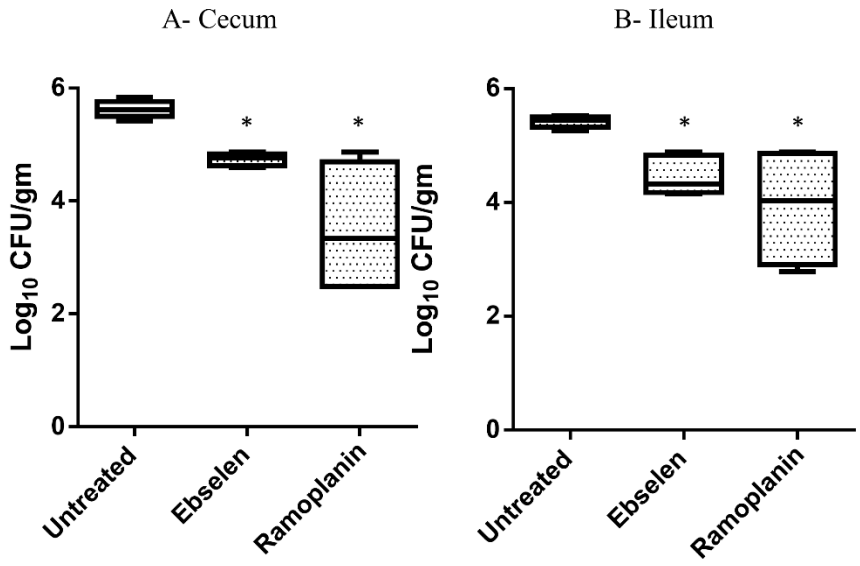

Figure 3.5 Bacterial counts of *E. faecium* HM-952 in (A) cecum and (B) ileum contents of mice

Infected mice (n = 5 per group) were orally treated with ebselen (0.5 mg/kg) or ramoplanin (10 mg/kg) daily for eight days. One group was left untreated. Cecum and ileum contents were collected one day after the last treatment was administered (day 21 of experiment). Asterisk (*) denotes significant difference from the untreated group ($P < 0.05$). No significant difference was found between ebselen-treated and ramoplanin-treated groups.

3.5 Discussion

The challenge of multidrug-resistant enterococcal infection continues to pose a threat to patients in healthcare facilities. Enterococcal infections represent about 9% of all the healthcare-associated infections in the United States of America alone. Due to their broad tissue tropism, enterococci can infect a wide variety of human organs. Enterococci, principally *E. faecium* and *E. faecalis*, are the causative agent of about 15% of bloodstream infections, urinary tract infections and surgical site infections. Moreover, bloodstream infections can advance to cause infective endocarditis that can be fatal in up to 46% of the cases [10, 27]. Treatment of enterococcal

infections has become increasingly challenging given the remarkable ability of enterococci to develop resistance to antibacterial agents [3, 25]. The emergence of clinical isolates of *E. faecium* and *E. faecalis* exhibiting resistance to vancomycin presents a formidable challenge given these strains are often co-resistant to other antibiotics. Though newer antibiotics such as linezolid have become the mainstays of treatment for VRE infections, these antibiotics are not immune to resistance development. As highlighted in a recent study by Bi *et al*, enterococci exhibiting resistance to linezolid represents an emerging problem globally [28]. Interestingly, the ability of enterococci to develop resistance against antibiotics is more prominent in strains of *E. faecium* moreso than *E. faecalis*. About 79% percent of *E. faecium* infections are vancomycin-resistant while only nine percent of *E. faecalis* infections are vancomycin-resistant [29]. Given the potential challenge of treating VRE infections once they arise, alternative approached to combating infection are needed. One such strategy is decolonizing the GIT of patients susceptible to infection by VRE.

One of the leading events that heralds enterococcal infections is gastrointestinal colonization. Enterococci normally reside in the human GIT as a member of the gut microflora. In normal settings, the population of enterococci remains in balance with the other members of the healthy bacterial consortium of the gut. However, administration of broad-spectrum antibiotics can disrupt the integrity of the normal gut flora leading to diminished ability to resist enterococcal overgrowth including strains of antibiotic-resistant enterococci. This GIT colonization has two major consequences: infection of the colonized individual and cross-transmission of enterococci to other patients residing within the same healthcare facility [15, 29-31]. Decolonization is not typically performed for patients with VRE because the current decolonization strategies, including antibiotics, bowel washing and administration of probiotics, suffer poor tolerability and/or limited efficacy [16, 17, 32]. Important qualities to seek in a decolonizing agent for VRE include potent antibacterial activity against VRE, stability to resistance development, safety to humans, and efficacy to decrease the burden of VRE in the intestinal tract. To date, no agent exists that possesses all of these qualities. Thus there remains a need to identify and develop new decolonizing agents effective against VRE.

Ebselen is an organoselenium compound that is being investigated for the treatment of various conditions and has been proven to be safe for human use [33, 34]. Ebselen is/has been evaluated for its preventive and treatment activates against several diseases such as cancer,

ischemic stroke, hearing loss, diabetes-related atherosclerosis and nephropathy and bipolar disorder [22, 23, 35-37]. More recently, ebselen was found to possess potent antibacterial activity against both VRE and methicillin-resistant *Staphylococcus aureus* (MRSA). While the activity of ebselen against MRSA has been intensively studied [20, 21, 38, 39], ebselen's activity against VRE still needs further evaluation. Herein, the capability of ebselen to serve as a novel decolonizing agent against VRE was investigated.

Initially, the antibacterial activity of ebselen was evaluated against more than 20 clinical isolates of VRE. Ebselen inhibited growth of the vast majority of these isolates at concentrations as low as 2 μg/mL, similar to linezolid. It is important to highlight that ebselen was effective against both *E. faecalis* and *E. faecium*, unlike some of the anti-enterococcal agents that have been used previously (such as quinupristin-dalfopristin) [4, 25]. Likewise, ebselen was active against both VRE and vancomycin-sensitive strains. When evaluated against VRE in a time-kill assay, ebselen was found to exert a bacteriostatic activity, similar to linezolid.

Given enterococci's remarkable ability to develop resistance against antibacterial agents, we were compelled to test whether ebselen-resistant mutants against VRE could be generated. Although bacteria are more likely to develop resistance against bacteriostatic drugs [40], no change in MIC for ebselen was observed in a multi-step resistance selection experiment. This is similar to a previous report where resistant mutants to ebselen could not be isolated for other Gram-positive bacteria, including *S. aureus* and *Bacillus subtilis* [41]. We suspect the inability of bacteria, such as VRE, to develop resistance to ebselen may be due to its multifaceted mechanism of action against bacteria that involves inhibition of several biochemical pathways in VRE as ebselen does in MRSA [21]. However further investigation is needed to corroborate this hypothesis. The failure to develop resistance *in vitro* is a predictive measure of low resistance development *in vivo*, although it is not a guarantee. This explains the rare cases of linezolid-resistant VRE observed in clinical blood and urine isolates from hospital patients [42].

As highlighted earlier, colonization of the GIT by VRE is an important precursor to subsequent invasion and infection. A key virulence factor that permits VRE to colonize to the GIT is the formation of biofilms. Biofilms are complex structures composed of bacteria and extracellular material that protect bacteria from the effect of antibiotics and the host immune system. Inhibition of VRE's ability to form biofilms or disrupting adherent biofilms could potentially be advantageous to disrupting VRE from colonizing and expanding in the GIT. The

ability of enterococci to colonize the GIT of mice has been previously correlated with the microorganism's ability to form biofilm [43]. Thus, agents capable of interfering with biofilm formation would be advantageous for a drug intended to be used for decolonization of VRE. We thus investigated if ebselen could interfere with biofilm formation against VRE. Ebselen significantly inhibited VRE biofilm formation by 30%, at a concentration as low as 0.25×MIC. Furthermore, ebselen disrupted mature VRE biofilm by nearly 95% (at 16×MIC). The antibiofilm activity of ebselen against VRE is similar to ebselen's antibiofilm activity against two other Gram-positive bacterial pathogens, *S. aureus* and *S. epidermidis* [21, 44]. In addition, ebselen was previously reported to be a potent antivirulence agent against *Clostridium difficile* infection in mice through biochemical inhibition of *C. difficile* toxin B [45]. This could be an added advantage knowing that *C. difficile* coinfection is common in patients with VRE colonization and that *C. difficile* infection is a significant risk factor for VRE bacteremia in colonized patients [46, 47].

After confirming the above *in vitro* activities, we sought to test the *in vivo* activity of ebselen in a VRE colonization reduction mouse model. Infection of ampicillin-pretreated mice resulted in colonization of the GIT of mice with VRE. Upon treatment with ebselen, the bacterial burden of VRE in fecal samples was significantly reduced after only three days of treatment. The effect extended and was more significant until the twentieth day of treatment. On day 20, ebselen treatment resulted in 4.5 $\log_{10}$-reduction (~99.99%) in VRE when compared to the initial bacterial load. Additionally, ebselen reduced about 90% of the burden of VRE in both the cecum and ileum content of infected mice. The dose of ebselen used in the study was 10 mg/kg. Interestingly, ebselen was proven to be safe for human use up to about 20 mg/kg [33, 48]. This suggests a higher dose of ebselen could be investigated in a future study to determine if complete eradication of VRE from the GIT can occur.

The fact that ebselen has an established safety profile in humans will potentially shorten the development process and reduce its cost. Further studies need to be conducted to evaluate the effect of ebselen on the composition of the human gut microbiota. Also, the protective effect of ebselen against VRE colonization and the probability of recurrence after cessation of ebselen treatment are yet to be evaluated. However, the data presented above indicate that ebselen has auspicious *in vitro* and *in vivo* activity and supports further investigation as a novel decolonizing agent to curb VRE infection in high-risk patient populations.

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CHAPTER 4. SCREENING FOR POTENT AND SELECTIVE ANTICLOSTRIDIAL LEADS AMONG FDA-APPROVED DRUGS

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4.1 Abstract

Clostridium difficile is a leading cause of morbidity and mortality particularly in hospital settings. In addition, treatment is very challenging due to the scarcity of effective therapeutic options. Thus, there remains an unmet need to identify new therapeutic agents capable of treating *C. difficile* infections. In the current study, we screened two FDA-approved drug libraries against *C. difficile*. Out of almost 3,200 drugs screened, 50 drugs were capable of inhibiting the growth of *C. difficile*. Remarkably, some of the potent inhibitors have never been reported before and showed activity in a clinically achievable range. Structure-activity relationship (SAR) analysis of the active hits clustered the potent inhibitors into four chemical groups; nitroimidazoles (MIC_{50}= 0.06 – 2.7 μM), salicylanilides (MIC_{50}= 0.2 – 0.6 μM), imidazole antifungals (MIC_{50}= 4.8 – 11.6 μM) and miscellaneous group (MIC_{50}= 0.4 – 22.2 μM). The most potent drugs from the initial screening were further evaluated against additional clinically relevant strains of *C. difficile*. Moreover, we tested the activity of potent inhibitors against representative strains of human normal gut microbiota to investigate the selectivity of the inhibitors towards *C. difficile*. Overall, this study provides a platform that could be used for further development of potent and selective anticlostridial antibiotics.

Keywords: *Clostridium difficile*, Imidazole anticlostridials, salicylanilides anticlostridials, Gut microbiota, Drug library screening.

4.2 Introduction

Clostridium difficile infection (CDI) has recently drawn a significant worldwide attention. In 2011, CDI afflicted nearly half a million people and was a direct cause of death of over 29,000 patients in the United States alone [1]. In Europe, the European Centre for Disease Prevention and

Control estimated there were nearly 124,000 cases of healthcare-associated CDIs in acute care hospitals alone between 2011 - 2012 [2]. Further, CDIs are not restricted to the healthcare setting only; community-acquired infections represent about 41% of all CDIs while 9% transpired in the residents of long-term care services such as retirement homes [3].

The United States Centers for Disease Control and Prevention (CDC) has classified *C. difficile* and CDIs as an urgent public health threat that necessitates immediate and rigorous action [4]. However, despite numerous calls for effective preventive measures and potent treatments, only three drugs are used for the treatment of CDI, vancomycin, metronidazole and fidaxomicin. A challenge with treating CDI with either vancomycin or metronidazole is that both agents harm the gut microflora. A second limitation with metronidazole is this drug is completely absorbed from the intestinal tract leaving a very minute concentration at the site of infection. These drawbacks contribute to a high percentage of treatment failure and relapse [5]. Fidaxomicin, FDA-approved for the treatment of CDI in 2011, has a better profile than both vancomycin and metronidazole as pertaining to bacterial specificity and oral bioavailability [6]. Nonetheless, the clinical outcome is still unsatisfactory regarding treatment failure and relapse especially against the more virulent NAP1 strains of *C. difficile*. Furthermore, treatment with fidaxomicin costs 150 times more than metronidazole thus compounding the cost to treat CDI. This highlights the need to identify and develop new, safe, and effective anticlostridial drugs.

In spite of the massive advances in drug discovery technologies, developing a *de novo* drug takes up to 15 years and can cost up to 2 billion US dollars. Drug repositioning, or finding a novel indication for a known drug, is a way to lessen the time and cost of drug discovery since these drugs have well-characterized toxicity and pharmacokinetic profiles. Many successful drugs being used now are repurposed from their original indication [7-14]. We used this approach in the current study to conduct a screening of about 3,200 drugs and clinical molecules to identify drugs or lead molecules with potent anticlostridial activity and limited effect against important bacterial species that comprise the gut microflora.

4.3 Materials and Methods

4.3.1 Bacterial strains and reagents

C. difficile and human gut microbiota strains used in this study (Supplementary Table 4.1) were acquired from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and the American Type Culture Collection (ATCC, Manassas, VA). Strains were cultured in brain heart infusion supplemented broth (BHIS, Brain heart infusion medium from Becton, Dickinson and Company, Cockeysville, MD), supplemented with yeast extract, L-cysteine, vitamin K1 and hemin (Sigma-Aldrich, St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Corning (Corning, NY).

4.3.2 Compounds and libraries

The Pharmakon 1600 repositioning compound library was purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT) and the Johns Hopkins library was provided by Johns Hopkins University (Baltimore, MD). Both libraries were supplied in 96-well plates of 10 mM stocks of the compounds in either dimethyl sulfoxide or water and stored in -80° C. Ornidazole, miconazole nitrate, econazole nitrate, tioconazole, butoconazole, clotrimazole, metoprolol tartrate, metoclopramide hydrochloride, chloroquine diphosphate, miconazole, dimetridazole, nithiamide and methylthiouracil (Alfa Aesar, Ward Hill, MA), dichlorophen, triclabendazole, closantel, nitazoxanide (Ark Pharm Inc, Libertyville, IL), ronidazole, oxyclozanide (Sigma-Aldrich, St. Louis, MO), tinidazole (TCI, Portland, OR) and niclosamide (Cayman Chemical, Ann Arbor, MI) were all purchased separately to confirm the results of both libraries. Vancomycin hydrochloride (Gold Biotechnology, Olivette, MO) and metronidazole (BTC, Hudson, NH) were used as positive controls.

4.3.3 Screening assay

Libraries were screened at a fixed concentration of 16 µM against one strain of *C. difficile* (*C. difficile* NAP07) to identify active compounds (Table 4.1). Briefly, bacteria were streaked on BHIS agar plates and incubated anaerobically at 37 ºC for 48 hours. Colonies were scraped off from the agar plates, suspended in PBS and diluted in BHIS broth at a concentration of about 5×10^5 CFU/mL. Compounds, at a concentration of 16 µM, were mixed with the bacterial suspension

in 96-well plates and incubated anaerobically for 48 hours at 37 ºC. Drugs that inhibited bacterial growth visually were considered as “hits”. Active drugs hits were purchased from commercial vendors and their activity was confirmed against *C. difficile* NAP07. Commercial drugs that did not show activity were excluded from the study.

4.3.4 Microdilution assay against *C. difficile* strains

The most potent hits were divided into four groups based on their chemical structure (Table 4.2) and tested for their minimum inhibitory concentrations (MICs) against a panel of ten *C. difficile* strains according to the Clinical and Laboratory Standards Institute guidelines (CLSI, M11-A8) [15]. Drugs, at the required concentrations, were anaerobically incubated with bacterial suspensions (5×10^5 CFU/mL) at 37 ºC for 48 hours in 96-well plates. After incubation, plates were examined for bacterial turbidity. MIC was defined as the lowest concentration where bacterial growth was halted and turbidity was unnoticeable.

4.3.5 Activity against human microbiota

Active compounds were evaluated for antibacterial activity against representative strains of human normal gut flora previously described [16]. Different types of bacteria were used in this experiment; for anaerobic bacteria (*Bifidobacterium* and *Bacteroides*) and *Lactobacillus*, bacteria were first streaked on agar plates and incubated for 48 hours at 37 ºC (anaerobically using BHIS agar for anaerobes and in 5% CO_2 using MRS agar plate for *Lactobacillus*). Bacterial colonies were suspended in BHIS broth (for anaerobes) or in MRS broth (for *Lactobacillus*) to achieve a bacterial concentration of approximately 10^5 CFU/ml. Bacteria were then added to 96-well plates containing serial dilutions of the compounds and incubated as mentioned above for 48 hours. Regarding *Escherichia coli* and *Enterococcus faecalis*, bacteria were scraped off tryptic soy agar plates and suspended in tryptic soy broth to achieve a bacterial concentration of 5×10^5 CFU/mL. The bacterial suspensions were aerobically incubated with serial dilutions of the drugs at 37 ºC for 16 – 20 hours. Reported MICs are the minimum concentration of the compounds that could inhibit visual growth of bacteria.

4.4 Results and Discussion

4.4.1 Screening assay and Structure-activity relationship (SAR) analysis:

Two drug libraries consisting of approximately 3,200 FDA-approved drugs and clinical molecules were evaluated against one strain of *C. difficile* (NAP07, CDC#2007054, a reference strain in the human microbiota project). All molecules were initially tested at a single concentration, 16 µM, in order to pinpoint active compounds or "hits". The initial screening revealed 116 compounds from Johns Hopkins library and 111 compounds from Pharmakon library that inhibited *C. difficile* at 16 µM (Supplementary Figure 4.1, Supplementary Tables 4.2 and 4.3). After excluding antiseptic and antibacterial agents and combining drugs from both libraries, 50 compounds were identified (Table 4.1). To confirm the screening results, the minimum inhibitory concentrations (MICs) of these 50 hits were determined against *C. difficile* NAP07. As depicted in Table 4.1, the MIC values for the active hits ranged from 0.06 µM to 16 µM.

Table 4.1 Active drugs "hits" identified from initial screening against *C. difficile* NAP07

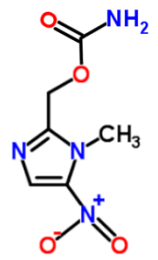
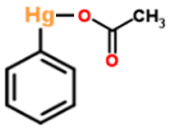
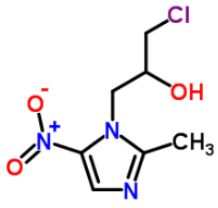
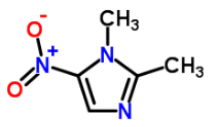
	Compound name	Chemical structure	MIC (µM)	Main use
1	Ronidazole		0.06	Anthelmintic
2	Phenylmercuric acetate		0.25	Antifungal, antimicrobial
3	Ornidazole		0.25	Anthelmintic
4	Dimetridazole		0.25	Anthelmintic

Table 4.1 continued

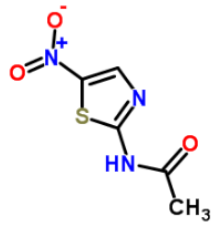
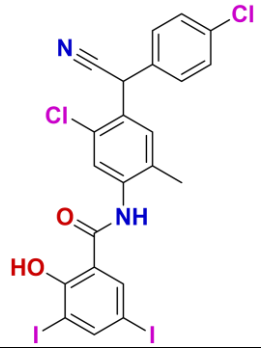
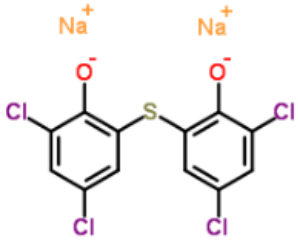
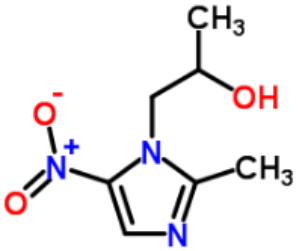
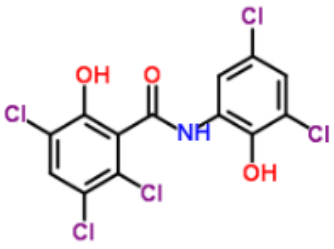
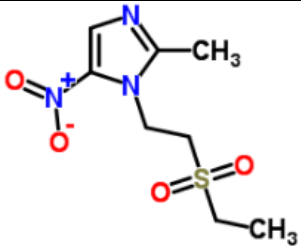
5	Nithiamide		0.25	Antiprotozoal (trichomonas).
6	Closantel		0.25	Anthelmintic.
7	Bithionate sodium		0.50	Anthelmintic, antiseptic
8	Secnidazole		0.50	Anthelmintic, antitrichomonas
9	Oxyclozanide		0.50	Anthelmintic
10	Tinidazole		0.50	Antiprotozoal

Table 4.1 continued

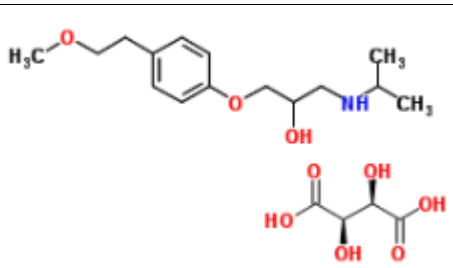
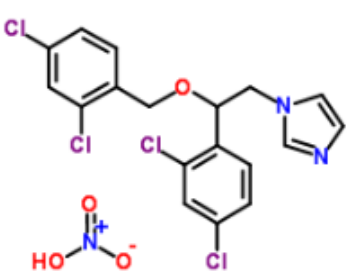
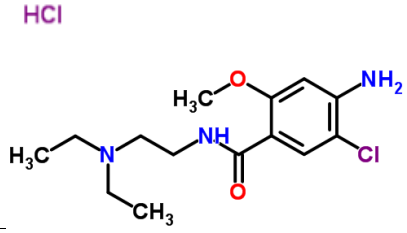
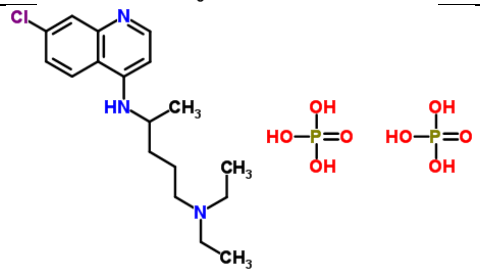
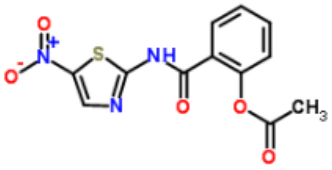
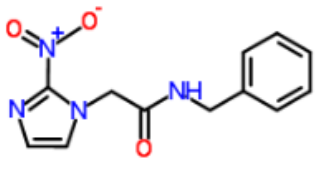
11	Metoprolol tartrate		1	Antihypertensive, antianginal
12	Miconazole nitrate		1	Antifungal (topical)
13	Metoclopramide hydrochloride		2	Antiemetic
14	Chloroquine diphosphate		2	Anthelmintic, antirheumatic, intercalating agent
15	Nitazoxanide		2	Anthelmintic
16	Benznidazole (n-benzyl-2-nitro-1h-imidazole-1-acetamide)		2	Antiprotozoal (trypanosoma)

Table 4.1 continued

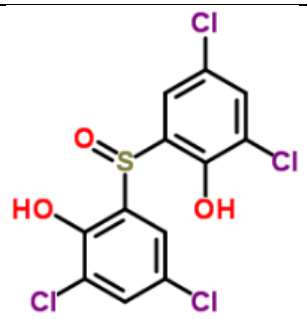
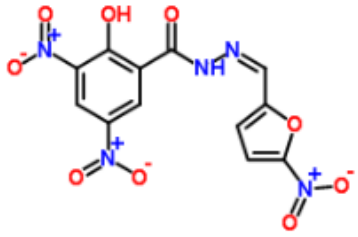
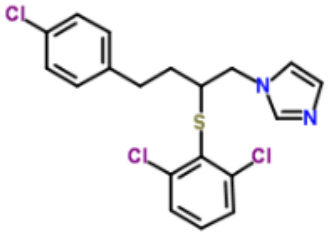
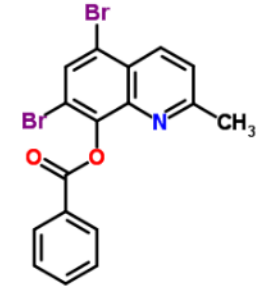
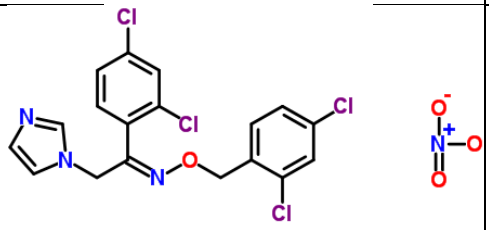
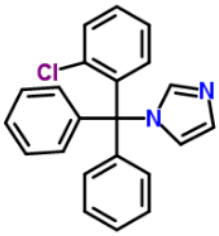
17	Bithionol oxide (2,2'-sulfinyl-bis(4,6-dichlorophenol))		2	Anthelmintic
18	Nifursol		4	Anthelmintic
19	Butoconazole		4	Antifungal
20	Broxaldine		4	Anthelmintic, antifungal
21	Oxiconazole nitrate		4	Antifungal
22	Clotrimazole		8	Antifungal

Table 4.1 continued

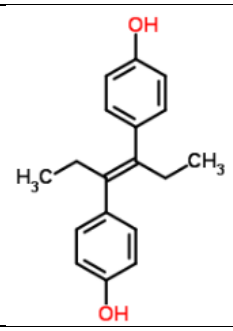
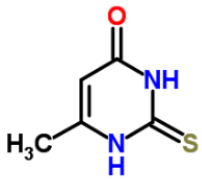
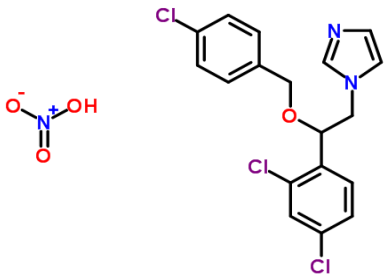
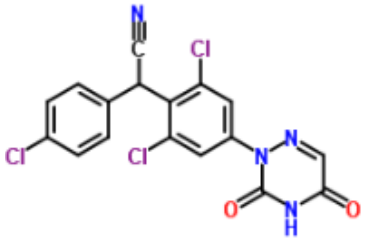
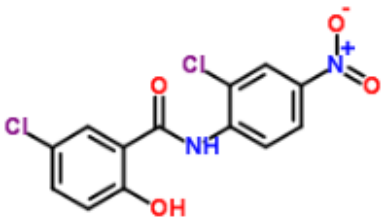
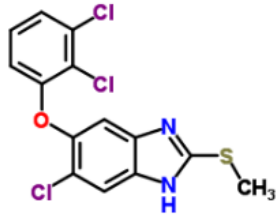
23	Diethylstilbestrol		8	Estrogen
24	Methylthiouracil		8	Antithyroid agent
25	Econazole nitrate		8	Antifungal
26	Diclazuril		8	Coccidiostat
27	Niclosamide		8	Anthelmintic, teniacide
28	Triclabendazole		8	Anthelmintic

Table 4.1 continued

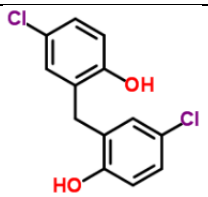
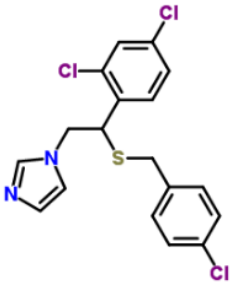
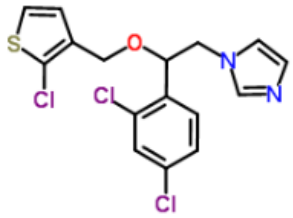
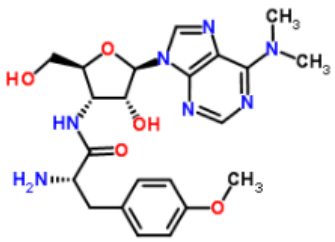
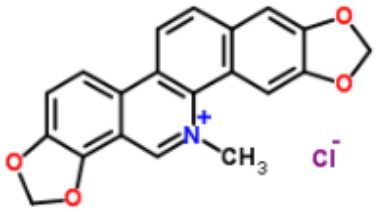
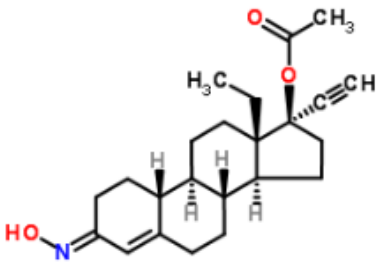
29	Dichlorophene		8	Anthelmintic (cestodes)
30	Sulconazole		8	Antifungal
31	Tioconazole		8	Antifungal (topical)
32	Puromycin		8	Antineoplastic
33	Sanguinarium chloride		16	Antineoplastic, antiplaque agent
34	Norgestimate		16	Progestin

Table 4.1 continued

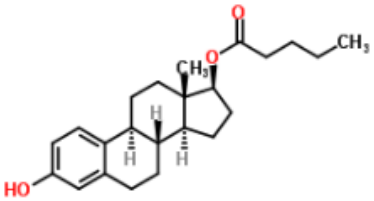
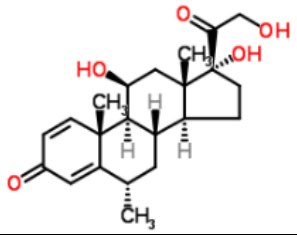
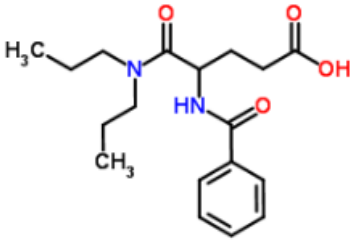
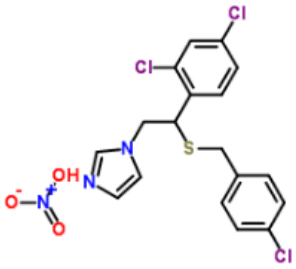
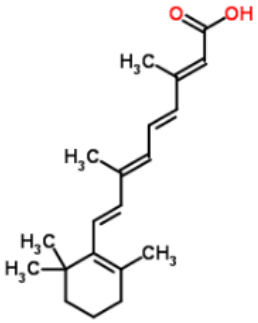
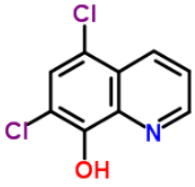
35	Estradiol valerate		16	Estrogen
36	Methylprednisolone		16	Anti-inflammatory, glucocorticoid
37	Proglumide		16	Anticholinergic and cholecystokinin antagonist
38	Sulconazole nitrate		16	Antifungal
39	Tretinoin		16	Keratolytic, antiacne, antineoplastic
40	Chloroxine		16	Chelating agent, antiseborrheic

Table 4.1 continued

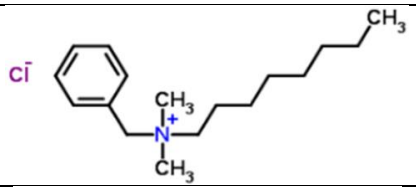
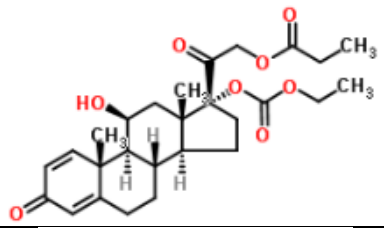
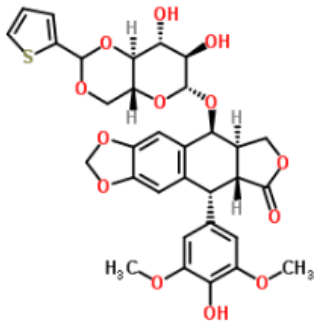
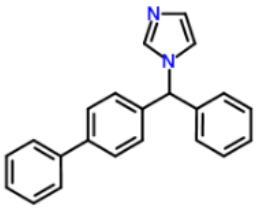
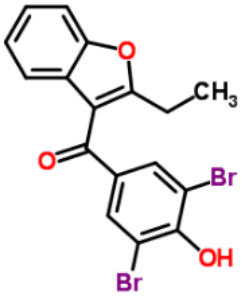
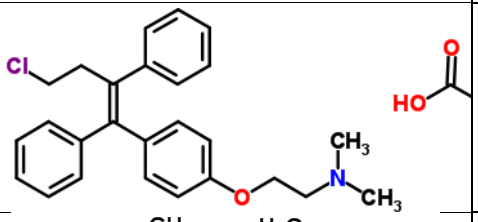
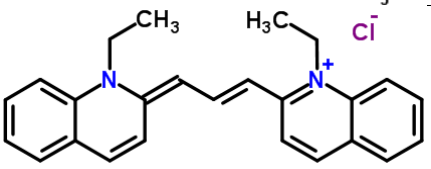
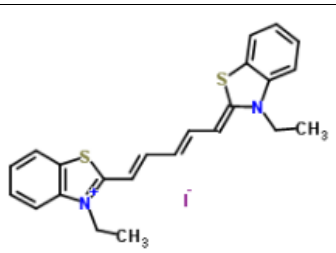
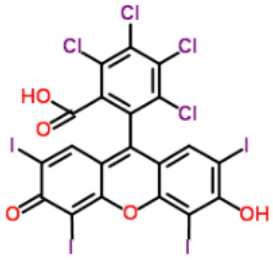
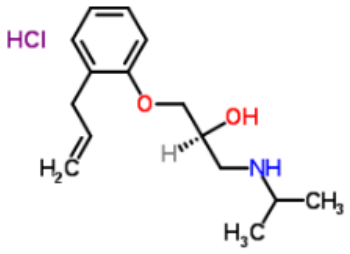
41	Benzalkonium chloride		16	Preservative
42	Prednicarbate		16	Anti-inflammatory, glucocorticoid
43	Teniposide		16	Antineoplastic
44	Bifonazole		16	Antifungal, calmodulin antagonist
45	Benzbromarone		16	Uricosuric
46	Toremifene citrate		16	Antineoplastic, anti-estrogen
47	Quinaldine blue (pinacyanol chloride)		16	Antineoplastic

Table 4.1 continued

48	Dithiazanine iodide		16	Anthelmintic (nematodes)
49	Rose bengal		16	Diagnostic aid (corneal trauma indicator).
50	Alprenolol hydrochloride		16	Antihypertensive

At first glance, the active compounds seem to be highly scattered structurally. However, the vast majority of the active molecules are imidazole-containing structures. Among the imidazole-containing structures, the nitroimidazoles seem to be the most efficient as six nitroimidazoles (dimetridazole, secnidazole, ronidazole, ornidazole, tinidazole and benznidazole) possessed MIC values below 2 µM against *C. difficile* NAP07 (Table 4.1). Additionally, the anticlostridial activity was impacted by the position of the nitro group on the imidazole ring. In this vein, the 2-nitroimidazole derivative benznidazole was remarkably less active than all 5-nitromidazole analogs. This is also in accordance with the potent activity of the 5-nitrimidazole metronidazole which was used for a long time as a first-line therapy for CDIs [17]. Moreover, the alkyl substitution seems to have less effect on the anticlostridial activity as dimetridazole (with only two methyl groups at positions 1 and 2) was nearly equipotent to the 5-nitroimidazole derivatives carrying more complex and bulkier substituents at positions 1 and 2, such as ornidazole and secnidazole (Figure 4.1). Apart from the nitroimidazole ring system, 5-nitrothiazole, a 5-nitroimidazole close bioisostere heterocyclic system, revealed very promising anticlostridial

activity. In particular, nithiamide inhibited the growth of *C. difficile* NAP07 at a sub-micromolar concentration (the MIC value was 0.25 μM, Table 4.1). Increasing the bulkiness at thiazole position-2 (like in nitazoxanide) decreased the anticlostridial activity by a factor of 8 in where the MIC value of the antiprotozoal nitazoxanide was 2 μM (Figure 4.1).

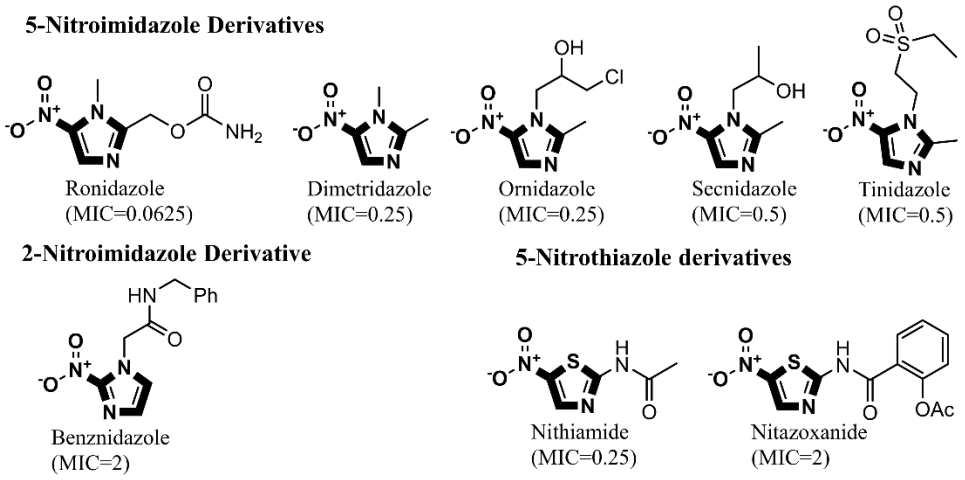


Figure 4.1 Effect of the nitro group position and alkyl substitution on the anticlostridial activity of nitroimidazoles and nitrothiazoles

5-nitroimidazoles were found to be more potent than 2-nitroimdazle. Contrarily, variation in the alkyl substitution of 5-nitroimdazole did not significantly affect anticlostridial activity. Additionally, Increasing the size of the substitution at position 2 of the nitrothiazole ring increased the MIC from 0.25 μM in the case of nithiamide to 2 μM in case of nitazoxanide. MIC values are against *C. difficile* NAP07 and are expressed in μM.

The last group of compounds with a similar scaffold was the imidazole antifungals as seven of them were active against *C. difficile* NAP07 with MIC values ranging between 1 and 8 μM (Table 4.1). This set of compounds shares more than the imidazole ring since most of them are 1-(2,4-dichlorophenylethyl)imidazole derivatives. From a structure-activity relationship position, the second chlorination at the side chain, benzyloxy moiety, drastically improves anticlostridial activity as observed with the dichlorinated miconazole (MIC value is 1 μM) and its monochlorinated analog econazole (MIC value is 8 μM). On the other hand, the type of linker

seems to have less effect on anticlostridial activity. In this regard, econazole with an ether linker possessed the same MIC value against *C. difficile* NAP07 as its thioether analog sulconazole (MIC = 8 μM). This value is identical to the MIC for the first-generation imidazole-antifungal clotrimazole (Table 4.1). Additionally, the oxime linker seems to reduce the anticlostridial activity as observed with oxiconazole (MIC = 4 μM) whereas the ether analog miconazole had a MIC of 1 μM (Figure 4.2).

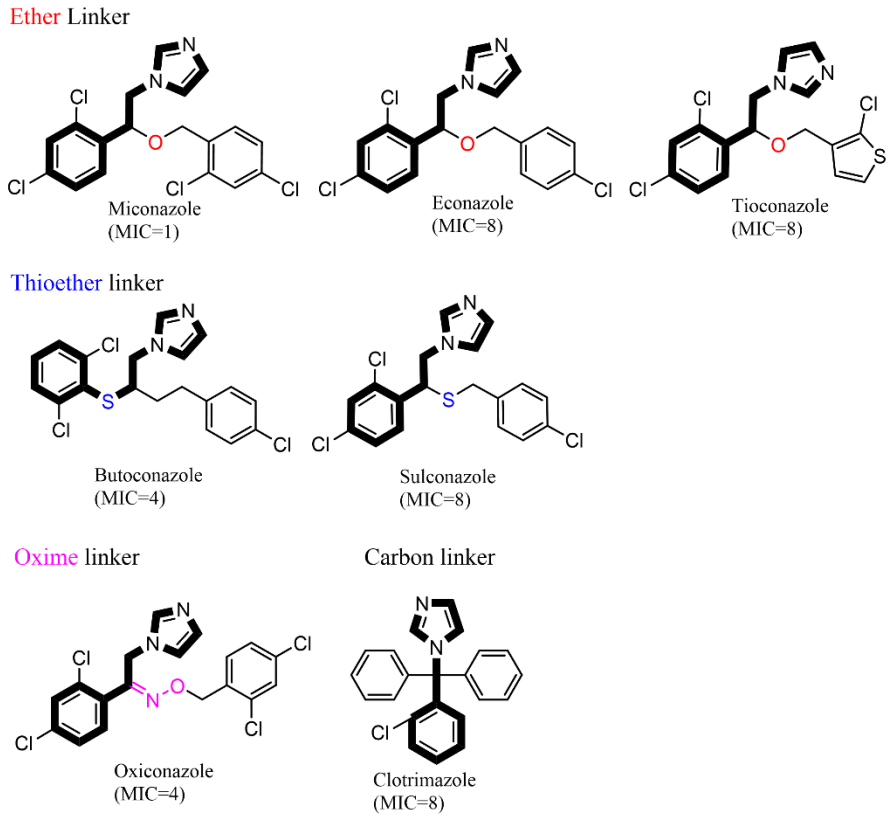


Figure 4.2 Effect of chlorination and linker type on the anticlostridial activity of imidazole antifungals

Dichlorinated miconazole is more potent than monochlorinated econazole. Additionally, the type of linker between the imidazole and phenyl rings does not affect anticlostridial activity. MIC values are against *C. difficile* NAP07 and are expressed in μM.

4.4.2 Activity against clinical *C. difficile* strains

The most potent drugs from the initial screening were further evaluated against additional clinically-relevant strains of *C. difficile*. These compounds were grouped based on their chemical

structure and tested against additional ten *C. difficile* strains. For each compound, we calculated the minimum concentration that inhibited the growth of 50% of the tested strains, MIC_{50}, in order to use it to compare the activities of different drugs. Grouping of the hits yielded four distinct structural classes of molecules, namely nitroimidazole, imidazole antifungals, salicylanilide and a fourth group of potent inhibitors that did not belong to a specific chemical class (Table 4.2).

Table 4.2 Classification of the most potent anticlostridial hits

Class			Drug	Use(s)
A	**Nitroimidazole**	1	Ronidazole	Antiprotozoal in veterinary medicine
		2	Dimetridazole	Antiprotozoal in veterinary medicine
		3	Tinidazole	Antiparasitic
		4	Ornidazole	Antiparasitic
		5	Secnidazole	Antiparasitic
B	**Salicylanilides**	5	Closantel	Anthelmintic and pesticide
		6	Oxyclozanide	Anthelmintic in veterinary medicine
		7	Niclosamide	Anthelmintic in humans and animals
C	**Imidazole antifungal**	8	Miconazole nitrate	Antifungal
		9	Econazole	Antifungal
		10	Tioconazole	Antifungal
		11	Butoconazole	Antifungal
		12	Clotrimazole	Antifungal
D	**Miscellaneous**	13	Dichlorophene	Antiparasitic in veterinary medicine
		14	Triclabendazole	Antiparasitic in human and animal medicine
		15	Nitazoxanide	Antiparasitic and antiviral
		16	Nithiamide	Antiprotozoal

The first group we investigated was nitroimidazole-containing compounds. Members of this group included ronidazole, dimetridazole, ornidazole, secnidazole and tinidazole in addition to metronidazole, the positive control. Nitroimidazoles inhibited the growth of all tested *C. difficile* strains at low concentrations (MIC_{50}s ranged from 0.3 to 2.7 μM, Table 4.3). Nitroimidazole-containing compounds, and nitroheterocyclic drugs in general, are known to exert potent inhibitory activity against anaerobic bacteria [18]. Although nitroimidazoles can diffuse into both aerobic and anaerobic bacterial cells, reductive activation occurs only in obligate anaerobes by pyruvate:ferredoxin oxidoreductase system. As a result, nitro group reduction produces imidazole

radical and nitrite, both of which damage bacterial DNA leading to cell death. In addition, reduction reserves the concentration gradient around the bacterial cell envelop and allows more diffusion of the drug into the bacterial cells [19]. Metronidazole was previously used as a first-line treatment for mild to moderated CDIs and is still recommended when vancomycin and fidaxomicin are not attainable [17, 20]. However, the activity of metronidazole is limited by the high bioavailability of the drug, leaving a minute concentration of drug in the gut lumen where the infection is localized [21]. As a result, the treatment outcome is not satisfactory. In addition, several cases of metronidazole-resistant CDIs have been reported [22]. Still, nitroimidazole represents an attractive scaffold that could be modified in order to obtain a better anticlostridial drug (with decreased oral bioavailability). The activities of 5-nitroimidazole-containing compounds were thoroughly studied against parasites in comparison to metronidazole. In most cases, several nitroimidazoles (whether FDA-approved or not) were found to be more effective than metronidazole and some of them possessed activity against metronidazole-resistant strains [23]. On the contrary, fewer comparative studies have been conducted to evaluate the activity of 5-nitroimidazoles against anaerobic bacteria (only a handful of these studies involved *C. difficile*) [24]. Tinidazole was previously shown to possess excellent *in vitro* activity against *C. difficile* and was found to be more effective than metronidazole against metronidazole-resistant strains [25]. However a limitation with this study is that tinidazole was not assessed in an *in vivo* model of CDI [26]. Ornidazole is an alternative therapy to metronidazole in the treatment of giardiasis and bacterial vaginosis. [27]. Though ornidazole is not recommended for treatment of CDIs, it is reported to be used as a treatment for CDI in certain parts of the world [28]. The most potent anticlostridial nitroimidazole was ronidazole, a veterinary antiprotozoal drug [29]. Ronidazole inhibited the growth of all the tested *C. difficile* strains at a concentration of 0.6 μM or less. Indeed, ten out of the eleven tested strains were inhibited at 0.3 μM. Although ronidazole is anecdotally reported to have carcinogenic and embryotoxic effects, it was shown to be safe in albino rats and pigs at very high concentrations and for prolonged periods of time [30]. Overall, nitroimidazoles warrant further investigation as more potent anticlostridial alternatives to metronidazole.

The second group to possess a potent anticlostridial activity was salicylanilide-related drugs/clinical molecules. Three drugs were included in this group, namely niclosamide, oxyclozanide and closantel. Salicylanilides inhibited the growth of *C. difficile* at concentrations that ranged from 0.2 to 1.2 μM, while their MIC_{50}s were 0.2 μM for closantel, 0.4 μM for

niclosamide and 0.6 μM for oxyclozanide (Table 4.3). Salicylanilides were recently reported to exert potent activities against several Gram-positive bacterial pathogens including *C. difficile* and vancomycin-resistant *Enterococci* (VRE) [7, 31]. Niclosamide and oxyclozanide exhibited activity against methicillin resistant *Staphylococcus aureus* through compromising the integrity of the bacterial cell envelope without causing cell lysis [32]. Against *Helicobacter pylori*, niclosamide disrupted the bacterial proton motive force resulting in growth inhibition [33]. Similar activity was observed against *C. difficile*, whereby salicylanilides were found to inhibit the growth of both logarithmic and stationary phase bacteria via dissipation of their membrane potential [31]. However, the *in vivo* activity of this group of compounds in an animal model of CDI is yet to be tested. On the other hand, salicylanilides exhibited potent *in vitro* activity against VRE, while niclosamide was very effective in reducing the bacterial burden in a VRE colonization reduction mouse model [7]. Knowing that VRE overgrowth is a major side effect of vancomycin and metronidazole when used to treat CDI [5, 34], the activity of salicylanilides against VRE can be exploited in the treatment of CDI.

The third group of *C. difficile* inhibitors included imidazole antifungal compounds, miconazole, econazole, tioconazole, butoconazole and clotrimazole. The MICs for these drugs varied from 2.4 to 23.2 μM and the MIC_{50} was 4.8 μM for miconazole, 4.9 μM for butoconazole, 10.3 μM for tioconazole, 10.5 μM for econazole and 11.6 μM for clotrimazole (Table 4.3). Azoles in general, and imidazoles in particular, represent an attractive scaffold for drug discovery. They can easily interact with enzymes through a wide array of noncovalent interactions. Imidazole compounds exert their antifungal activity through inhibition of ergosterol biosynthesis. Ergosterol depletion is primarily due to inhibition of cytochrome P-450-dependant 14α-demethylase activity and results in mitigating membrane integrity and fungal inhibition [35]. On the contrary, two mechanisms have been proposed for imidazoles activity as antibacterial agents. The first one is through the inhibition of enoyl acyl carrier protein reductase (FabI) with a resultant inhibition of bacterial fatty acid synthesis [36]. Although this mechanism of action applies to several bacterial pathogens, e.g. *S. aureus* and *E. coli*, it cannot be expected in *C. difficile* due to the absence of FabI as a catalyst in fatty acid biosynthesis [37]. The second proposed mechanism of bacterial inhibition by imidazoles is blocking of flavohaemoglobins-mediated metabolism of nitric oxide leading to bacterial cell death [38]. Nevertheless, more investigation is required to confirm this activity in *C. difficile*. Although the antibacterial activity of imidazoles has been reported against

other bacteria, this is the first report, to our knowledge, of the anticlostridial activity of imidazole antifungals.

Table 4.3 MICs (μM) of the active compounds against clinical strains of *C. difficile*

***C. difficile* Strain**	**NR number**	**Nitroimidazoles**					**Imidazole antifungals**				
		Ronidazole	**Dimetridazole**	**Ornidazole**	**Tinidazole**	**Secnidazole**	**Miconazole**	**Econazole**	**Tioconazole**	**Butoconazole**	**Clotrimazole**
P 3	32884	0.3	1.8	0.6	2	2.7	4.8	10.5	10.3	4.9	23.2
P 5	32885	0.3	1.8	0.6	2	2.7	4.8	5.2	5.2	4.9	5.8
P 6	32886	0.3	1.8	0.3	2	1.4	4.8	10.5	10.3	4.9	11.6
P 7	32887	0.3	1.8	0.6	16.2	5.4	4.8	10.5	10.3	4.9	11.6
P 19	32895	0.3	1.8	0.6	4	5.4	4.8	10.5	10.3	4.9	11.6
P 30	32904	0.3	1.8	0.6	8.1	5.4	2.4	10.5	10.3	4.9	23.2
Isolate 7	13433	0.3	1.8	0.6	4	2.7	2.4	10.5	5.2	4.9	11.6
Isolate 11	13437	0.3	1.8	0.3	2	2.7	4.8	10.5	10.3	9.7	11.6
Isolate 13	13553	0.6	1.8	0.6	16.2	2.7	9.6	5.2	20.6	4.9	11.6
ATCC BAA 1801		0.3	1.8	0.6	2	1.35	9.6	21	20.6	19.4	23.2
MIC_{50}		**0.3**	**1.7**	**0.6**	**2**	**2.7**	**4.8**	**10.5**	**10.3**	**4.9**	**11.6**

***C. difficile* Strain**	**NR number**	**Salicylanilides**			**Miscellaneous**				**Vancomycin**	**Metronidazole**
		Closantel	**Oxyclozanide**	**Niclosamide**	**Nitazoxanide**	**Dichlorophene**	**Triclabendazole**	**Nithiamide**		
P 3	32884	0.4	1.2	0.8	0.8	7.4	22.2	0.7	0.7	1.5
P 5	32885	0.2	0.3	0.2	0.8	7.4	22.2	0.7	0.7	0.7
P 6	32886	0.2	1.2	0.8	0.8	3.7	11.1	0.3	0.7	0.7
P 7	32887	0.4	1.2	0.8	1.6	7.4	22.2	0.7	0.7	0.7
P 19	32895	0.2	0.6	0.4	0.2	14.9	11.1	0.7	1.4	1.5
P 30	32904	0.2	0.6	0.4	0.2	7.4	22.2	0.3	0.3	1.5
Isolate 7	13433	0.2	0.3	0.4	0.4	7.4	22.2	0.7	0.3	1.5
Isolate 11	13437	0.4	1.2	0.4	0.4	14.9	22.2	0.7	0.7	1.5
Isolate 13	13553	0.8	0.6	0.4	0.8	14.9	22.2	0.7	0.7	1.5
ATCC BAA 1801		0.2	0.3	0.4	0.2	7.4	11.1	0.3	0.3	0.7
MIC_{50}		**0.2**	**0.6**	**0.4**	**0.4**	**7.4**	**22.2**	**0.7**	**0.7**	**1.5**

The last group of *C. difficile* inhibitors contained compounds from scattered chemical classes. The most potent two compounds among this group were nitazoxanide and nithiamide. Both drugs contain a nitrothiazole ring structure. The MICs for nitazoxanide ranged from 0.2 to 1.6 μM and the MIC_{50} was 0.4 μM. On the other hand, nithiamide's MICs were between 0.3 and 0.7 μM against all the tested *C. difficile* strains and its MIC_{50} was 0.7 μM (Table 4.3). Nitazoxanide is an antiprotozoal drug that possesses, along with its active metabolite tizoxanide, a potent antibacterial activity against both aerobes and anaerobes. Although it is not included in CDI treatment guidelines, nitazoxanide has been reported to possess potent anticlostridial activity both *in vitro* and *in vivo* [39]. In addition, nitazoxanide proved to be noninferior to both vancomycin and metronidazole in clinical studies against CDI [40]. Despite the similarity between the spectrum of activity of metronidazole and nitazoxanide, studies have shown that nitazoxanide is mechanistically distinct from metronidazole. The anticlostridial activity of nitazoxanide is attributed to its noncompetitive inhibition of pyruvate:ferredoxin/flavodoxin oxidoreductases which in turn blocks the oxidative decarboxylation of pyruvate to acetyl coenzyme A and lead subsequently to bacterial killing [41]. Nithiamide, on the other hand, has been used as an antiparasitic agent in both human and animal medicine [42]. However, nithiamide has never been tested against *C. difficile* and the data about its antibacterial activity is very erratic [43]. In addition, nithiamide is structurally similar to nitazoxanide, hence it is expected that they might share the same mechanism of action, although this hypothesis will need to be confirmed experimentally. The last two drugs in the miscellaneous group (dichlorophene and triclabendazole) had less potent activity than nithiamide and nitazoxanide *in vitro*. Dichlorophene and triclabendazole are both antiparasitic drugs with no significant known antibacterial activity [44, 45]. Dichlorophene inhibited the growth of *C. difficile* strains at concentrations ranging between 3.7 and 14.9 μM while triclabendazole inhibited the same strains at concentrations between 11.1 and 22.2 μM. The MIC_{50} values for dichlorophene and triclabendazole were 7.4 and 22.2 μM, respectively (Table 4.3).

4.4.3 Activity of the potent hits against human microbiota

Intestinal microbiota protects against *C. difficile* colonization through the production of short chain fatty acids (SCFAs). SCFAs stimulate the growth of gut epithelium, reduce inflammation through the induction of regulatory T cells (Tregs), induce antimicrobial peptides like thuricin CD and augment the mucus barrier through increasing production of mucin [46].

Furthermore, resident bacteria compete with invading *C. difficile* cells for intestinal niches and nutrients. In addition, the microbiota is involved in the transformation of primary bile acids, a germinant for *C. difficile* spores, into secondary bile acids resulting in a reduction of spore germination and inhibition of vegetative growth [46]. Based on that, we sought to test the selectivity of the active anticlostridial hits by assessing their activity against representative strains of commensal bacteria present in the human gastrointestinal tract. Evaluated strains included anaerobic bacteria (*Bifidobacteria* and *Bacteroides*), microaerophilic bacteria (*Lactobacilli*), Gram-positive (*Enterococci*) and Gram-negative (*Escherichia coli*) bacteria.

In accordance with their reported activity against anaerobes, nitroimidazoles inhibited the growth of *Bifidobacteria* and *Bacteroides* [24, 47]. Only one strain of *Bifidobacterium*, *B. breve* HM-856, was not inhibited by nitroimidazoles including metronidazole, the positive control (Table 4.4). Nitroimidazoles had minimal or no activity against the rest of the tested bacterial strains. Imidazole antifungals had a similar pattern of activity against *Bifidobacteria* and *Bacteroides*, in addition, they inhibited the growth of both enterococcal strains tested. The activity of the imidazole antifungal miconazole was reported previously against several Gram-positive bacteria e.g. *Staphylococcus*, *Streptococcus* and *Enterococcus* [48]. Similarly, salicylanilides inhibited both anaerobes and had some activity against *Enterococcus*. Additionally, they inhibited growth of several *Lactobacillus* strains (Table 4.4). Closantel was the most potent inhibitor amongst salicylanilides potentially due to the difference in its physicochemical properties relative to other salicylanilides [7]. All four drugs in the miscellaneous group inhibited the growth of anaerobes and some Gram-positive strains. The most selective drug in this group was nitazoxanide; it inhibited the growth of the microbiota at concentrations that were several folds higher than the drug's MIC_{50} against *C. difficile*. Nithiamide also exhibited a good selectivity profile against most of the tested strains of microbiota. The MIC of nithiamide against most of the inhibited microbiota strains was much higher than its MIC_{50} against *C. difficile* (Table 4.4).

Table 4.4 MICs (μg/mL) of the active compounds against human normal gut flora

Bacterial Strain	Strain ID	Nitroimidazole				Imidazole antifungals				
		Ronidazole	Dimetridazole	Ornidazole	Tinidazole	Miconazole	Econazole	Tioconazole	Butoconazole	Clotrimazole
Lactobacillus casei	ATCC-334	>640	>907	>583	>518	308	335	330	311	371
Lactobacillus acidophilus	ATCC-314	640	>907	>583	>518	308	335	>330	>311	371
Bifidobacterium bifidum	ATCC-11863	≤5	≤7	≤5	32	10	21	21	19	23
Bifidobacterium breve	ATCC-15700	≤5	227	146	518	38	42	41	39	46
Bifidobacterium longum	HM-845	≤5	≤7	36	16	38	42	41	5	46
Bifidobacterium breve	HM-856	>640	>907	>583	>518	38	42	>330	10	46
Bacteroides fragilis	HM-711	≤5	28	≤5	≤4	38	42	5	10	46
Bacteroides fragilis	HM-709	≤5	28	≤5	≤4	19	21	≤3	10	46
Lactobacillus crispatus	HM-371	640	907	>583	>518	308	335	>330	>311	371
Lactobacillus gasseri	HM-407	320	907	>583	>518	308	335	>330	>311	371
Escherichia coli	ATCC-35150	640	113	>583	>518	>308	>335	>330	78	>371
Escherichia coli	1411	640	113	>583	>518	>308	>335	>330	78	>371
***Enterococcus faecalis* -- TX0104**	HM-201	640	454	>583	>518	38	42	>330	19	>371
***Enterococcus faecalis* -- TX1322**	HM-202	>640	907	>583	>518	19	42	>330	19	46
Strain	**Strain ID**	**Salicylanilides**			**Miscellaneous**				**Vancomycin**	**Metronidazole**
		Closantel	Oxyclozanide	Niclosamide	Nitazoxanide	Dichlorophene	Triclabendazole	Nithiamide		
Lactobacillus casei	ATCC-334	193	80	196	>417	>476	>356	>684	>88	>748
Lactobacillus acidophilus	ATCC-314	97	40	>391	>417	>476	>356	>684	0.7	>47
Bifidobacterium bifidum	ATCC-11863	≤1.5	40	≤3	≤3	30	11	≤5	0.7	5.8
Bifidobacterium breve	ATCC-15700	≤1.5	10	≤3	≤3	30	22	≤5	2.8	47
Bifidobacterium longum	HM-845	≤1.5	≤2.5	≤3	52	30	5.5	≤5	0.3	23
Bifidobacterium breve	HM-856	3	40	≤3	26	30	22	≤5	0.3	>748
Bacteroides fragilis	HM-711	3	5	6	26	30	44	43	88	≤5.8
Bacteroides fragilis	HM-709	3	5	≤3	26	30	22	11	44	≤5.8

Table 4.4 continued

Lactobacillus crispatus	HM-371	12	>319	49	417	476	>356	342	1.4	>748
Lactobacillus gasseri	HM-407	12	>319	49	417	476	>356	342	1.4	>748
Escherichia coli	ATCC-35150	>193	>319	>391	>417	>476	>356	>684	ND	ND
Escherichia coli	1411	193	>319	>391	>417	>476	>356	>684	ND	ND
Enterococcus faecalis **-- TX0104**	HM-201	24	>319	>391	26	59	22	11	>88	ND
Enterococcus faecalis **-- TX1322**	HM-202	24	>319	>391	26	59	11	11	0.7	ND

To summarize, we screened two libraries consisting of FDA-approved drugs and clinical molecules against *C. difficile* in order to identify potent and selective inhibitors. We identified three distinct chemical classes of molecules that have potent inhibitory activity against *C. difficile*, nitroimidazoles, salicylanilides and imidazole antifungals. Additionally, we identified four drugs that do not belong to any of the previous chemical categories, nitazoxanide, nithiamide, dichlorophene and triclabendazole. All the active compounds were tested against a panel of *C. difficile* strains and were found to exhibit potent inhibitory activity. In addition, they were tested against normal intestinal microflora strains to investigate their selectivity for *C. difficile* over other beneficial bacteria. Overall, the current study can serve as a reference for anti-*C. difficile* drug developers and can provide leads for further development for the treatment of CDIs.

4.5 References

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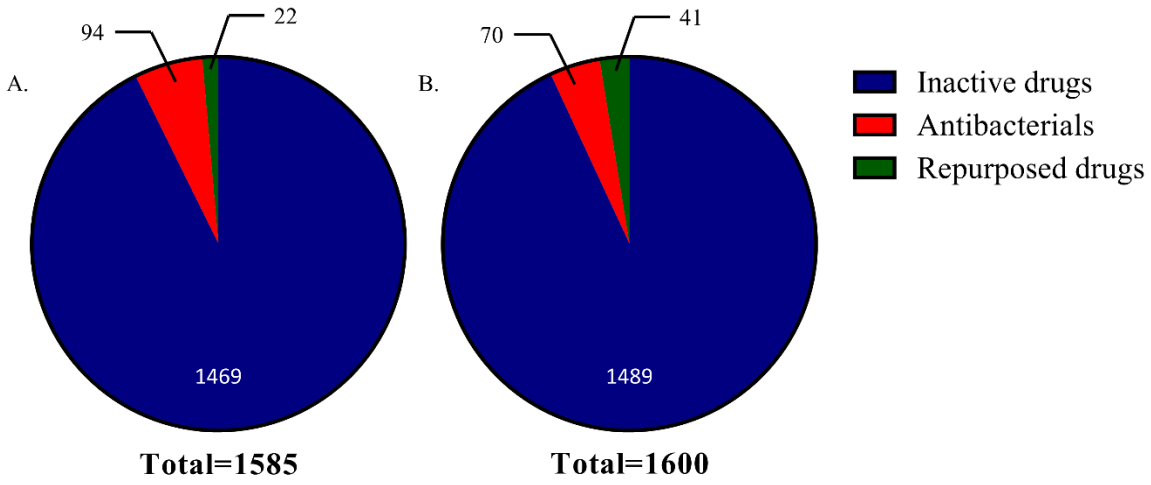

Supplementary Figure 4.1 Anticlostridial activity of drugs in Johns Hopkins and Pharmakon drug libraries

Both Johns Hopkins (A) and Pharmakon (B) Libraries were screened against a single strain of *C. difficile* (Strain NAP07) at a concentration of 16 μM. Active drugs were divided into two classes; drugs with known antibacterial activity (red) and repurposed drugs (green). Repurposed drugs from both libraries were combined for further testing.

Supplementary Table 4.1 Bacterial strains used in the study

Bacterial Strain	ID number	Source and comments
C. difficile NAP07 (CDC#2007054)	HM-88	Isolated from human feces.
C. difficile ATCC BAA 1801	-----	Non-toxigenic strain isolated from the feces of an adult with diarrhea in Belgium.
C. difficile P 3	NR-32884	Toxigenic strain obtained in 2001 from fecal material of a human patient with a CDI in western Pennsylvania, USA.
C. difficile P 5	NR-32885	Toxigenic strain obtained in 2001 from fecal material of a human patient with a CDI in western Pennsylvania, USA.
C. difficile P 6	NR-32886	Toxigenic strain obtained in 2001 from fecal material of a human patient with a relapsing CDI in western Pennsylvania, USA.
C. difficile P 7	NR-32887	Toxigenic strain obtained in 2001 from fecal material of a human patient with a CDI in western Pennsylvania, USA.
C. difficile P 19	NR-32895	Toxigenic strain obtained in 2005 from fecal material of a human patient with a relapsing CDI in western Pennsylvania, USA.
C. difficile P 30	NR-32904	Non-toxigenic strain obtained in 2009 from fecal material of an asymptomatic human patient in western Pennsylvania, USA.
C. difficile Isolate 7	NR-13433	It was obtained in 2008/2009 from a human patient in the mid-Atlantic region of the United States.

Supplementary Table 4.1 continued

C. difficile Isolate 13	NR-13553	It was obtained in 2008/2009 from a human patient in the mid-Atlantic region of the United States.
C. difficile Isolate 11	NR-13437	It was obtained in 2008/2009 from a human patient in the mid-Atlantic region of the United States.
Lactobacillus casei	ATCC-334	Isolated from dairy products.
Lactobacillus acidophilus	ATCC-314	Quality control strain
Bifidobacterium bifidum	ATCC-11863	---
Bifidobacterium breve	ATCC-15700	Isolated from the intestinal tract of an infant.
Bifidobacterium longum	HM-845	Isolated from a one-year-old human patient.
Bifidobacterium breve	HM-856	Isolated from a gastrointestinal biopsy of a human subject in the United States.
Bacteroides fragilis	HM-711	obtained from healthy adult feces in Massachusetts, USA.
Bacteroides fragilis	HM-709	obtained from healthy adult feces in Massachusetts, USA.
Lactobacillus crispatus	HM-371	obtained from a human mid-vaginal wall in 2010 in Virginia, USA.
Lactobacillus gasseri	HM-407	obtained from a human mid-vaginal wall in 2010 in Virginia, USA.
Escherichia coli	ATCC35150	Isolated from human feces.
Escherichia coli	1411	University of Nottingham, UK.
Enterococcus faecalis -- TX0104	HM-201	Isolated in 2002 from the blood of a patient with endocarditis in Connecticut, USA.
Enterococcus faecalis -- TX1322	HM-202	Obtained in 1994 from the feces of a volunteer in Houston, Texas.

Supplementary Table 4.2 Active drugs from Pharmakon library

	Compound name	Indication
1	Amoxicillin	Antibacterial
2	Bacitracin	Antibacterial
3	Carbenicillin disodium	Antibacterial
4	Demeclocycline hydrochloride	Antibacterial
5	Furazolidone	Antibacterial
6	Fusidic acid	Antibacterial
7	Gramicidin	Antibacterial
8	Hetacillin potassium	Antibacterial
9	Minocycline hydrochloride	Antibacterial
10	Nafcillin sodium	Antibacterial
11	Oxytetracycline	Antibacterial
12	Penicillin g potassium	Antibacterial
13	Penicillin v potassium	Antibacterial
14	Piperacillin sodium	Antibacterial
15	Vancomycin hydrochloride	Antibacterial
16	Flumequine	Antibacterial
17	Methacycline hydrochloride	Antibacterial
18	Meclocycline sulfosalicylate	Antibacterial
19	Monensin sodium	Antibacterial
20	Marbofloxacin	Antibacterial
21	Linezolid	Antibacterial
22	Clinafoxacin hydrochloride	Antibacterial
23	Azlocillin sodium	Antibacterial
24	Bacampicillin hydrochloride	Antibacterial
25	Thiamphenicol	Antibacterial
26	Lasalocid sodium	Antibacterial
27	Cefpiramide	Antibacterial
28	Levofloxacin	Antibacterial
29	Moxifloxacin hydrochloride	Antibacterial
30	Enrofloxacin	Antibacterial
31	Thiostrepton	Antibacterial
32	Gatifloxacin	Antibacterial
33	Sarafloxacin hydrochloride	Antibacterial
34	Nithiamide	Antibacterial
35	Florfenicol	Antibacterial
36	Teicoplanin [a(2-1) shown]	Antibacterial
37	Gemifloxacin mesylate	Antibacterial
38	Nadifloxacin	Antibacterial
39	Pazufloxacin mesylate	Antibacterial
40	Clofoctol	Antibacterial
41	Salinomycin, sodium	Antibacterial
42	Alexidine hydrochloride	Antibacterial
43	Nitroxoline	Antibacterial
44	Nifuroxazide	Antibacterial
45	Chlorhexidine dihydrochloride	Antibacterial (topical), disinfectant
46	Rifampin	Antibacterial (tuberculostatic)

Supplementary Table 4.2 continued

47	Streptomycin sulfate	Antibacterial (tuberculostatic)
48	Gentian violet	Antibacterial, anthelmintic
49	Tetracycline hydrochloride	Antibacterial, antiamebic, antirickettsial
50	Chlortetracycline hydrochloride	Antibacterial, antiamebic, Calcium chelator,
51	Pyrithione zinc	Antibacterial, antifungal, antiseborrheic
52	Clofazimine	Antibacterial, antilepretic, antituberculosis
53	Chloramphenicol palmitate	Antibacterial, antirickettsial
54	Chloramphenicol sodium succinate	Antibacterial, antirickettsial
55	Chloramphenicol	Antibacterial, antirickettsial
56	Narasin	Antibacterial, antiviral
57	Nitromide	Antibacterial, coccidiostat
58	Difloxacin hydrochloride	Antibacterial, dna gyrase inhibitor
59	Rifaximin	Antibacterial, rna synthesis inhibitor
60	Dihydrostreptomycin	Antibacterial, tuberculostatic
61	Doxycycline hydrochloride	Antibacterial
62	Methicillin sodium	Antibacterial
63	Cetrimonium bromide	Antiinfective
64	Methylbenzethonium chloride	Antiinfective
65	Triclosan	Antiinfective
66	Benzethonium chloride	Antiinfective (topical)
67	Cetylpyridinium chloride	Antiinfective (topical)
68	Hexachlorophene	Antiinfective (topical)
69	Nitrofurazone	Antiinfective (topical)
70	Thonzonium bromide	Mucolytic, antibacterial, surface active agent
71	Sanguinarium chloride	Antineoplastic, antiplaque agent
72	Bithionate sodium	Anthelmintic, antiseptic
73	Norgestimate	Progestin
74	Clotrimazole	Antifungal
75	Diethylstilbestrol	Estrogen
76	Estradiol valerate	Estrogen
77	Methylprednisolone	Antiinflammatory, glucocorticoid
78	Methylthiouracil	Antithyroid agent
79	Metoclopramide hydrochloride	Antiemetic
80	Metoprolol tartrate	Antihypertensive, antianginal
81	Metronidazole	Anthelmintic
82	Miconazole nitrate	Antifungal (topical)
83	Chloroquine diphosphate	Anthelmintic, antirheumatic, intercalating agent
84	Phenylmercuric acetate	Antifungal, antimicrobial
85	Proglumide	Anticholinergic
86	Sulconazole nitrate	Antifungal
87	Ronidazole	Anthelmintic
88	Econazole nitrate	Antifungal
89	Tretinoin	Keratolytic, antiacne, antineoplastic
90	Tinidazole	Anthelmintic
91	Nifursol	Anthelmintic

Supplementary Table 4.2 continued

92	Diclazuril	Coccidiostat
93	Chloroxine	Chelating agent, antiseborrheic
94	Niclosamide	Anthelmintic, teniacide
95	Benzalkonium chloride	Preservative
96	Nitazoxanide	Anthelmintic
97	Prednicarbate	Antiinflammatory, glucocorticoid
98	Teniposide	Antineoplastic
99	Butoconazole	Antifungal
100	Bifonazole	Antifungal, calmodulin antagonist
101	Oxiconazole nitrate	Antifungal
102	Tioconazole	Antifungal
103	Benzbromarone	Uricosuric
104	Ornidazole	Anthelmintic
105	Toremifene citrate	Antineoplastic, anti-estrogen
106	Dichlorophen	Anthelmintic
107	Broxaldine	Anthelmintic, antifungal
108	Secnidazole	Anthelmintic, antitrichomonas
109	Oxyclozanide	Anthelmintic
110	Triclabendazole	Anthelmintic
111	Dimetridazole	Anthelmintic

Supplementary Table 4.3 Active drugs from Johns Hopkins library

	Name	Indication
1	Potash, sulfurated	Antibacterial
2	Pyrithione zinc	Antibacterial
3	Acriflavine hydrochloride	Antibacterial
4	Bismuth tribromophenate	Antibacterial
5	Chloramine-t hydrate	Antibacterial
6	Chlorquinaldol	Antibacterial
7	Dapsone	Antibacterial
8	Fenticlor	Antibacterial
9	Diclazuril	Antibacterial
10	Carbadox	Antibacterial
11	Cloxyquin	Antibacterial
12	Dihydrostreptomycin, dihydrostreptomycin sulfate	Antibacterial
13	Ornidazole	Antibacterial
14	Nebramycin	Antibacterial
15	Domiphen bromide	Antibacterial (topical)
16	Cetalkonium chloride	Antibacterial (topical)
17	Clioquinol	Antibacterial (topical)
18	Nifuroxime	Antibacterial (topical)
19	Rifampicin (rifampin)	Antibacterial (tuberculostatic)
20	3-formyl rifamycin	Antibacterial (tuberculostatic)

Supplementary Table 4.3 continued

21	Clofazimine	Antibacterial (tuberculostatic, leprostatic)
22	Amikacin	Antibiotic
23	Benzoylpas calcium	Antibiotic
24	Carbenicillin	Antibiotic
25	Cefpiramide	Antibiotic
26	Daptomycin	Antibiotic
27	Chloramphenicol	Antibiotic
28	Gatifloxacin	Antibiotic
29	Doxycycline	Antibiotic
30	Doxycycline hyclate	Antibiotic
31	Meclocycline sulfosalicylate salt	Antibiotic
32	Mezlocillin sodium	Antibiotic
33	Kanamycin	Antibiotic
34	Levofloxacin hcl	Antibiotic
35	Linezolid	Antibiotic
36	Meclocycline	Antibiotic
37	Nitrofurazone	Antibiotic
38	Minocycline	Antibiotic
39	Minocycline hydrochloride salt	Antibiotic
40	Moxifloxacin hcl	Antibiotic
41	Nafcillin sodium salt monohydrate	Antibiotic
42	Nitrofurantoin	Antibiotic
43	Penicillin g	Antibiotic
44	Penicillin g (procaine salt)	Antibiotic
45	Penicillin g potassium	Antibiotic
46	Penicillin v potassium	Antibiotic
47	Piperacillin-tazobactam	Antibiotic
48	Cefamandole	Antibiotic
49	Cefoxitin	Antibiotic
50	Cefepime hydrochloride	Antibiotic
51	Cefixime	Antibiotic
52	Cefoperazone sodium salt	Antibiotic
53	Ceforanide	Antibiotic
54	Tetracycline	Antibiotic
55	Vancomycin hcl hydrate	Antibiotic
56	Florfenicol	Antibiotic
57	Clinafloxacin hcl	Antibiotic
58	Clofoctol	Antibiotic
59	Dibekacin	Antibiotic
60	Rifamycin sv	Antibiotic
61	Rifaximin	Antibiotic
62	Metampicillin	Antibiotic

Supplementary Table 4.3 continued

63	Nitroxoline	Antibiotic
64	Pazufloxacin	Antibiotic
65	Penimepicycline	Antibiotic
66	Pipemidic acid	Antibiotic
67	Tosufloxacin	Antibiotic
68	Sarafloxacin hcl	Antibiotic
69	Thiamphenicol	Antibiotic
70	Thiostrepton	Antibiotic
71	Amoxicillin	Antibiotic
72	Chlortetracycline, chlortetracycline hydrochloride	Antibiotic
73	Dicloxacillin, dicloxacillin sodium, dicloxacillin sodium salt	Antibiotic
74	Monensin, monensin sodium salt	Antibiotic
75	Brilliant green	Antiseptic
76	5-chlorosalicylanilide	Antiseptic
77	Ammonium citrate (ammonium hydrogencitrate)	Antiseptic
78	Castile soap	Antiseptic
79	Cetylpyridinium	Antiseptic
80	Cetylpyridinium bromide monohydrate	Antiseptic
81	Chlorhexidine gluconate	Antiseptic
82	Methylbenzethonium chloride	Antiseptic
83	Methyldopate hydrochloride	Antiseptic
84	Sodium iodate	Antiseptic
85	Tetradecyl sulfate, sodium salt	Antiseptic
86	Thonzonium bromide	Antiseptic
87	Triclosan	Antiseptic
88	Benzododecinium chloride	Antiseptic
89	Pidolic acid	Antiseptic
90	Trilocarban (3,4,4'-trichlorocarbanilide, 99%)	Antiseptic
91	Bithionol	Antiseptic
92	Gentian violet	Antiseptic
93	Benzethonium chloride	Antiseptic
94	Benzalkonium chloride	Antiseptic
95	Bithionoloxide	Anthelmentic bithionol
96	Dichlorophene	Anthelmintic (cestodes)
97	Dithiazanine iodide	Anthelmintic (nematodes)
98	Closantel	Anthelmintic.
99	Tinidazole	Antiprotozoal
100	Ronidazole	Antiprotozoal
101	Nifursol	Antiprotozoal (poultry)
102	Metronidazole	Antiprotozoal (trichomonas)
103	Nithiamide	Antiprotozoal (trichomonas).

Supplementary Table 4.3 continued

104	Benznidazole	Antiprotozoal (trypanosoma)
105	Secnidazole	Antiamebic
106	Oxiconazole nitrate	Antifungal
107	Clotrimazole	Antifungal
108	Econazole	Antifungal
109	Sulconazole	Antifungal
110	Butoconazole nitrate	Antifungal (topical)
111	Miconazole	Antifungal (topical)
112	Tioconazole	Antifungal (topical)
113	Alprenolol, alprenolol hydrochloride	Antihypertensive
114	Quinaldine blue (pinacyanol chloride)	Antineoplastic
115	Puromycin	Antineoplastic
116	Rose bengal	Diagnostic aid (corneal trauma indicator).

CHAPTER 5. ANTIBACTERIAL AND ANTIVIRULENCE ACTIVITIES OF AURANOFIN AGAINST *CLOSTRIDIUM DIFFICILE*

A version of this chapter has been reprinted with permission. AbdelKhalek, A., Abutaleb, N. S., Mohammad, H., & Seleem, M. N. (2019). Antibacterial and antivirulence activities of auranofin against *Clostridium difficile*. *Int J Antimicrob Agents, 53*(1), 54-62. doi: 10.1016/j.ijantimicag.2018.09.018

5.1 Abstract

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

Keywords: *C. difficile* infection; anti-toxin; spores' formation; auranofin; antibacterial; *C. elegans*; repurposing

5.2 Introduction

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

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

Drug repurposing is a promising approach to find new indications for existing or abandoned drugs. Adopting an old drug with a well-studied safety and pharmacokinetic profile can circumvent some of the pitfalls and costs associated with clinical testing and regulatory approval processes for novel compounds [10-12]. Auranofin [2,3,4,6-tetra-*o*-acetyl-l-thio-β-d-glycopyranp-sato-*S*-(triethyl-phosphine)-gold] is a gold-containing anti-inflammatory oral drug that has been used for treatment of rheumatoid arthritis for over 30 years. Auranofin's safety and pharmacokinetic profile in humans has been well-characterized, which has permitted investigation

of auranofin for other clinical indications [13, 14]. The present study evaluates auranofin's potential to be repurposed as a novel anticlostridial drug to treat CDI.

In the current study, we report that auranofin possesses potent antibacterial activity against a wide panel of *C. difficile* strains. Additionally, auranofin is capable of inhibiting toxin production, spore formation and protects human gut cells against the inflammation induced by sterile *C. difficile* toxins. Taken all together, auranofin is a promising candidate to evaluate further to treat CDI.

5.3 Materials and Methods

5.3.1 Chemicals, media and bacterial strains

Auranofin, linezolid (Chem-Impex International, Wood Dale, IL), vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO) metronidazole (Beantown Chemical Corporation, Hudson, NH), sodium selenite (MP Biomedicals, Santa Ana, CA), and fidaxomicin (Apexbio, Houston, TX) were procured from commercial vendors. Brain heart infusion (BHI) was purchased from Becton, Dickinson and Company (Cockeysville, MD). Phosphate buffered saline, fetal bovine serum and non-essential amino acids (NEAA) were purchased from Fisher Scientific (Waltham, MA). Yeast extract, L-cysteine, vitamin K, hemin, Dulbecco's Modified Eagle's medium (DMEM), and penicillin/streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). *C. difficile* and enterococcal isolates (Table 5.1) were obtained from the American Type Culture Collection (ATCC) and Biodefense and Emerging Infections Research Resources Repository (BEI Resources).

Table 5.1 Bacterial strains used in this study

Bacterial Strain	Alternate Designation	Source	Comments
C. difficile NAP07 (CDC#2007054)	HM-88	Isolated from human feces.	Reference genome for The Human Microbiome Project (HMP).
C. difficile P2	NR-32883	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
C. difficile P3	NR-32884	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.

Table 5.1 continued

C. difficile P4	NR-32889	Isolated from the fecal material of a human patient with a relapsing CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
C. difficile P5	NR-32885	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.
C. difficile P6	NR-32886	Isolated from the fecal material of a human patient with a relapsing CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
C. difficile P7	NR-32887	It was obtained in 2001 from the fecal material of a human patient with CDI in Western Pennsylvania, USA.	Toxigenic strain.
C. difficile P8	NR-32888	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2001.	Toxigenic strain.
C. difficile P13	NR-32891	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2005.	Toxigenic strain.
C. difficile P15	NR-32892	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2005.	Toxigenic strain.
C. difficile P19	NR-32895	It was obtained in 2005 from the fecal material of a human patient with a relapsing *C. difficile* infection in Western Pennsylvania, USA.	Toxigenic strain.
C. difficile P20	NR-32896	Isolated from the fecal material of a human patient with a relapsing *C. difficile* infection in Western Pennsylvania, USA in 2005.	Toxigenic strain.
C. difficile P21	NR-32897	Isolated from the fecal material of a human patient with a relapsing *C. difficile* infection in Western Pennsylvania, USA in 2005.	Toxigenic strain.
C. difficile P29	NR-32903	Isolated from the fecal material of a human patient with CDI in Western Pennsylvania, USA in 2009.	Toxigenic strain and reported to be co-colonized with non-toxigenic *C. difficile*.
C. difficile P30	NR-32904	It was obtained in 2009 from the fecal material of an asymptomatic human patient in Western Pennsylvania, USA.	Non-toxigenic strain.
C. difficile Isolate 1	NR-13427	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 2	NR-13428	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 4	NR-13430	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 5	NR-13431	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--

Table 5.1 continued

C. difficile Isolate 6	NR-13432	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 7	NR-13433	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 9	NR-13435	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 10	NR-13436	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 11	NR-13437	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile Isolate 13	NR-13553	It was obtained from a human patient from the Mid-Atlantic region of the United States in 2008/2009.	--
C. difficile 002-P50-2011	HM-746	Isolated from the stool of a patient with diarrhea in January 2011.	Reference genome for the Human Microbiome Project (HMP).
C. difficile ATCC 700057	VPI 11186	--	Toxinotype tcdA-, tcdB-, Ribotype 038, Nontoxigenic.
C. difficile ATCC 43598	1470	Human feces, asymptomatic neonate, Belgium	Presence of tcdB gene confirmed by PCR, Ribotype 017.
C. difficile ATCC BAA 1801	3232	Human feces (adult with diarrhea), Belgium.	Nontoxigenic, Ribotype 010.
C. difficile ATCC BAA 1870	4118	--	Presence of binary toxin, tcdA and tcdB genes. Toxinotype IIIb, Ribotype 027.
C. difficile Isolate 20100207	NR-49278	Obtained from the stool of an elderly adult male patient with a healthcare-associated (HA) *C. difficile* infection in New York, USA, in 2010.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20100502	NR-49277	Obtained from the stool of an older adult male patient with a community-associated (CA) *C. difficile* infection in Colorado, USA, in 2010.	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* of the PaLoc[5] operon, as well as the CDT[6]
C. difficile Isolate 20110052	NR-49281	Obtained from the stool of an elderly male patient with a healthcare-associated (HA) *C. difficile* infection in northeastern USA in 2010.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20110868	NR-49287	Obtained from the stool of an elderly female patient with a healthcare-associated (HA) *C. difficile* infection in southern USA, in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]

Table 5.1 continued

C. difficile Isolate 20110870	NR-49288	Obtained from the stool of a young adult female patient with a healthcare-associated (HA) *C. difficile* infection in Tennessee, USA, in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20110979	NR-49285	Obtained from the stool of an elderly female patient with a community-associated (CA) *C. difficile* infection in midwestern USA in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20110999	NR-49286	Obtained from the stool of an elderly female patient with a healthcare-associated (HA) *C. difficile* infection in western/midwestern USA in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20120013	NR-49283	Obtained from the stool of a young male patient with a community-associated (CA) *C. difficile* infection in northeastern USA, in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20120184	NR-49289	Obtained from the stool of an elderly female patient with a fatal healthcare-associated (HA) *C. difficile* infection in Tennessee, USA, in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
C. difficile Isolate 20120187	NR-49290	Obtained from the stool of an elderly male patient with a healthcare-associated (HA) *C. difficile* infection in Tennessee, USA, in 2011.	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* of the PaLoc[5] operon, as well as the CDT[6]
C. difficile Isolate 20120236	NR-49291	Obtained from the stool of an older female patient with a community-associated (CA) *C. difficile* infection in midwestern, USA, in 2011.	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] and *tcd*[4] *C* (with 18 base pair deletion) PaLoc[5] operon and the CDT[6]
E. faecium HF50104	NR-32052	Isolated from swine feces in Michigan, USA in 2008.	Resistant to erythromycin, tetracycline and vancomycin.
E. faecium Patient #3-1	NR-31912	Isolated from the stool of a human patient having dominance of vancomycin-resistant *Enterococcus* in the stool but no bacteremia.	Vancomycin-resistant
E. faecium E1071	NR-28978	Hospitalized person free of enterococcal infection in the Netherlands in 2000 during a hospital surveillance program.	Non-infectious fecal isolate. Resistant to vancomycin.
E. faecium ERV165	HM-970	Isolated from human feces in Colombia, in 2008.	Resistant to vancomycin.
E. faecium ERV102	HM-968	Isolated from human oral sputum in Colombia, in 2006.	Resistant to ampicillin, vancomycin and streptomycin.

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

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

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

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

5.3.4 Effect of auranofin on *C. difficile* spore formation

C. difficile HM-88, in stationary phase, was diluted in fresh BHIS broth and incubated anaerobically for 4-6 hours at 37 °C. The bacterial suspension was aliquoted into tubes and drugs

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

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

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

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

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

5.3.7 Activity of auranofin against vancomycin-resistant enterococci (VRE)

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

5.3.8 Statistical analysis

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

5.4 Results

5.4.1 Antibacterial activity of auranofin against *C. difficile*

Auranofin's anticlostridial activity was evaluated against a large panel of *C. difficile* strains including hypervirulent strains (ribotype 027). As shown in Table 5.2, auranofin inhibited growth of the 41 tested *C. difficile* strains at concentrations ranging from 0.25 - 4 μg/mL. Auranofin inhibited 50% of the tested isolates (MIC_{50}) at a concentration of 1 μg/mL and inhibited 90% of the isolates (MIC_{90}) at a concentration of 2 μg/mL. The MIC of auranofin was comparable to values obtained for vancomycin, the drug of choice for treatment of severe CDI. Vancomycin was effective at a range of 0.25 – 2 μg/mL (MIC_{50} = 0.5 μg/mL and MIC_{90} = 1 μg/mL). Metronidazole was active at a range of 0.06 – 0.25 μg/mL (MIC_{50} = 0.0.25 μg/mL and MIC_{90} = 0.25 μg/mL).

Table 5.2 The minimum inhibitory concentration (MIC, μg/mL) of auranofin and control anticlostridial drugs against *C. difficile* isolates

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

Table 5.2 continued

Isolate 20120184	NR-49289	0.5	0.25	0.25
Isolate 20120187	NR-49290	0.5	0.5	0.25
Isolate 20120236	NR-49291	0.25	0.5	0.25
MIC_{50}		1	0.5	0.25
MIC_{90}		2	1	0.25

5.4.2 Auranofin inhibits *C. difficile* toxin production

After confirming the potent *in vitro* anticlostridial activity of auranofin, we next moved to test the inhibitory activity of auranofin against *C. difficile* toxin production. Bacteria in the late log phase were incubated with subinhibitory concentrations of auranofin and control anticlostridial drugs. Auranofin exhibited a dose-dependent inhibition of *C. difficile* toxin production, when compared to the untreated control. As depicted in Figure 5.1, auranofin at $^1/_8$, $^1/_4$ and $^1/_2$ × MIC inhibited 15.6%, 31.2% and 40% of total toxin production, respectively. Fidaxomicin, an anticlostridial antibiotic known to inhibit toxin production and spore formation [22], was found to inhibit 37.2%, 50.1% and 52.3% of toxin production at $^1/_8$, $^1/_4$ and $^1/_2$ × MIC, respectively. As expected, no toxin inhibition was observed when *C. difficile* was treated with either vancomycin or metronidazole; on the contrary, the toxin concentration increased at certain concentrations, in agreement with previous reports [17, 22, 23].

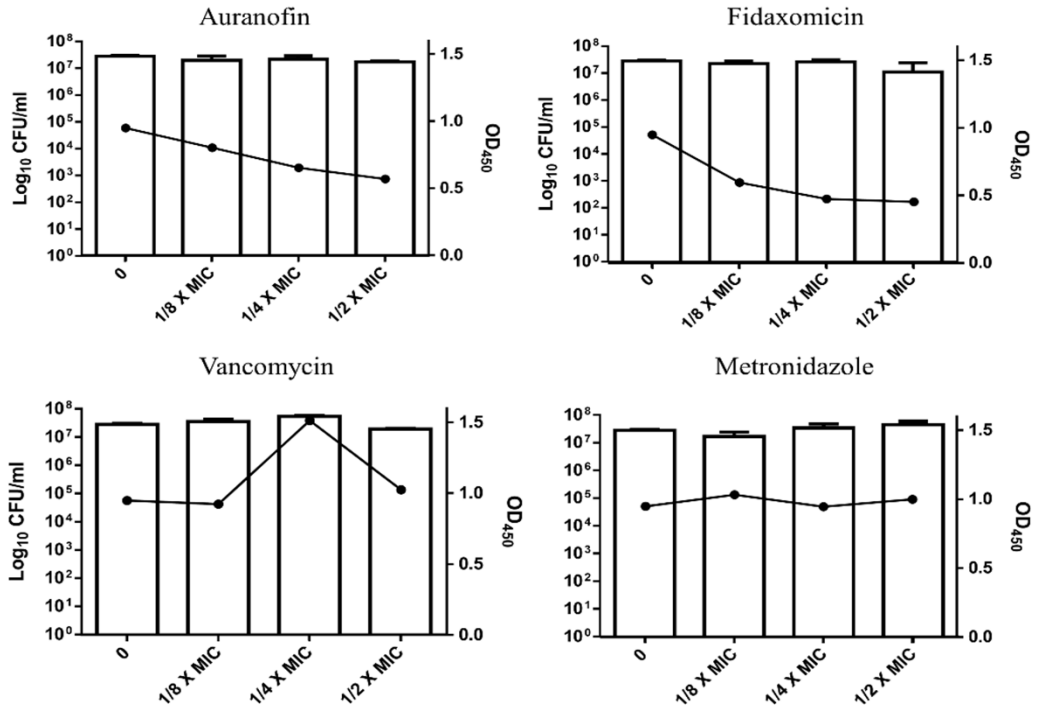

Figure 5.1 Toxin inhibition activity of auranofin and control anticlostridial drugs (vancomycin, metronidazole and fidaxomicin) against *C. difficile*

Drugs, at concentrations of $^1/_8$ ×, $^1/_4$ × and $^1/_2$ × MIC were incubated with a hypervirulent, toxigenic strain of *C. difficile* (strain ATCC BAA-1870). The bacterial counts were determined for each sample, and the toxin levels were assessed in the supernatant using enzyme linked immune fluorescent assay (ELISA). Error bars represent standard deviation values from triplicate samples for each treatment.

5.4.3 Auranofin inhibits *C. difficile* spore formation

C. difficile HM-88, in late exponential growth phase, was incubated with subinhibitory concentrations of auranofin and control anticlostridial drugs. Total vegetative cells and heat-resistant spores were counted in each sample. Spores comprised most of the viable count in the untreated control (Figure 5.2). Auranofin-treated bacteria displayed reduced spore count, ~1.5 $\log_{10}$ at both $^1/_2$ and 1 × MIC. While a similar effect was observed with fidaxomicin, almost no reduction in the spore count was detected with either vancomycin or metronidazole treatment.

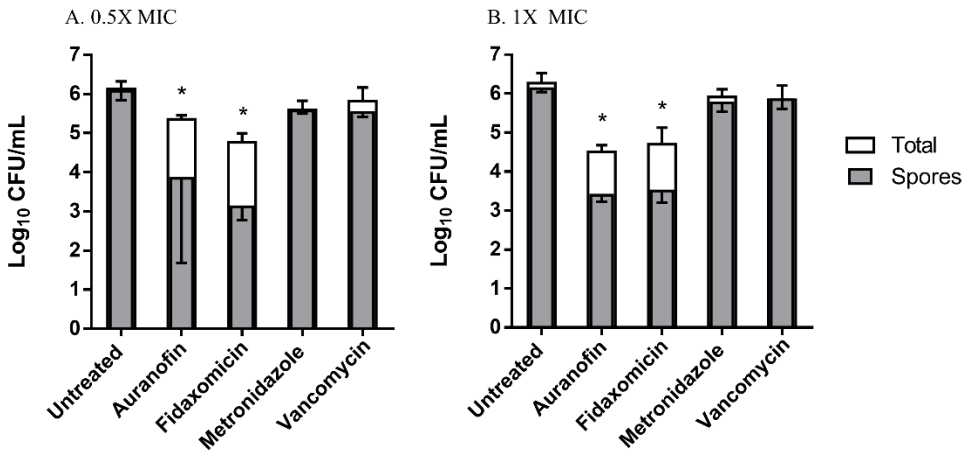

Figure 5.2 Spore inhibition of activity of auranofin against *C. difficile* compared to the control anticlostridial drugs, vancomycin and metronidazole.

Drugs ($^1/_2\times$ and 1 × MIC) were incubated with bacteria for five days followed by serial dilution and plating to count both total bacterial count and heat resistant spores. Error bars represent standard deviation values from triplicate samples for each treatment. (*) denotes significant difference between the total and the spore counts.

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

Auranofin has been previously shown to have anti-inflammatory activity, an important factor for its use in the treatment of rheumatoid arthritis. We thus moved to investigate if auranofin would also have the ability to protect gut cells from inflammation induced by *C. difficile* toxins. Human colorectal cells (Caco-2) were treated with sterile-filtered bacterial supernatant, with or without the addition of auranofin or control anticlostridial drugs. As shown in Figure 5.3, healthy Caco-2 cells display normal morphologic characteristics of enterocytes. Upon treatment with *C. difficile* toxins, cell rounding and detachment occurred [24]. The goal was to inspect if drug treatment will preserve the normal Caco-2 morphology, in the presence of *C. difficile* toxins. Auranofin (1 and 8 μg/mL) protected cells against the inflammatory effect of *C. difficile* toxins and successfully preserved the normal cell morphology. Similar results were obtained with polarized cells (data not shown). On the other hand, cells treated with vancomycin, metronidazole or fidaxomicin (at a concentration ranging from 1 to 128 μg/mL) all exhibited rounding and detachment after exposure to toxins, indicating these drugs were unable to protect the gut epithelial cells.

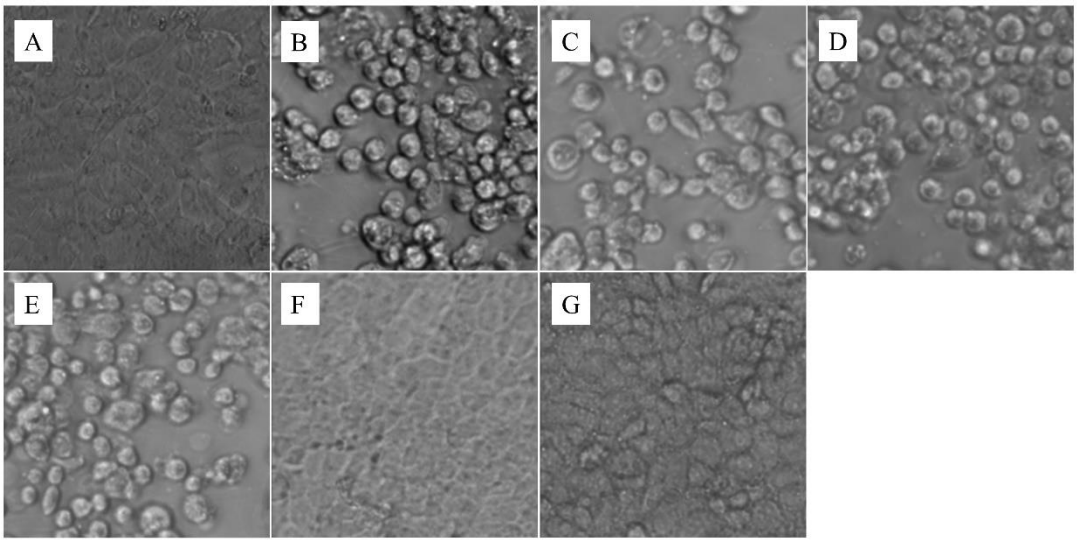

Figure 5.3 Auranofin's effect against *C. difficile* toxin-mediated inflammation of gut epithelial cells

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

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

After confirming auranofin can mitigate *C. difficile* toxin-mediated inflammation, we investigated if auranofin's anti-inflammatory effect was due to inhibition of IL-8. IL-8 is a major proinflammatory cytokine released upon exposure of gut epithelial cells to *C. difficile* toxins [18]. IL-8 was measured in the Caco-2 supernatants after incubation with *C. difficile* toxins with or without auranofin (1 μg/mL). Toxin treatment resulted in a significant increase in IL-8 concentration in the cell supernatant (Figure 5.4). However, when cells were treated with toxins along with auranofin, no significant increase inIL-8 was observed. The OD_{450} is represent to the IL-8 concentration present in the supernatants.

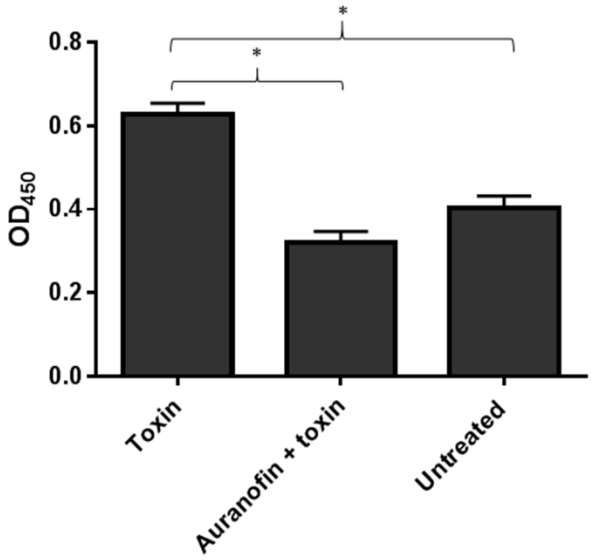

Figure 5.4 Auranofin mediated IL-8 inhibition from gut cells treated with *C. difficile* toxins

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

5.4.6 Activity of auranofin against vancomycin-resistant enterococci (VRE)

To evaluate if auranofin treatment can prompt an overgrowth of VRE, the MIC of auranofin was determined against five VRE strains (Table 5.3). Auranofin inhibited growth of VRE at concentrations ranging from 0.25 – 0.5 μg/mL). Fidaxomicin also inhibited VRE growth albeit at higher concentrations (MIC range was 1 – 2 μg/mL). In contrast, vancomycin and metronidazole were not effective against all the tested VRE strains.

Table 5.3 The minimum inhibitory concentration (MIC, μg/mL) of auranofin and control drugs against vancomycin-resistant *Enterococcus faecium* isolates

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

5.5 Discussion

Clostridium difficile infections (CDIs) are increasing in morbidity and mortality within the healthcare setting. The U.S. Centers for Disease Control and Prevention (CDC) reported about half a million CDI cases in 2011, an increase from 333,000 cases reported in 2007. More worrisome is the relapse rate was 20% and came at a direct cost of $4.8 billion for acute care facilities alone. Moreover, approximately 29,000 deaths occurred within 30 days of the initial diagnosis, with much higher rates reported among elderly patients [2]. The strike in CDI case numbers is associated with the emergence of hypervirulent strains (e.g. NAP1, ribotype 027 strains) which lack negative control on toxin production [1-4]. CDI in the healthcare setting is most likely attributed to administration of broad-spectrum antibiotics that subsequently damages the intestinal microbiota permitting *C. difficile* to expand, attach to epithelial cells and produce toxins. *C. difficile* toxins afflict the colonic epithelium leading to loss of tight junctions, enhanced mucosal permeability and ultimately intense inflammation and neutrophilic infiltration [25].

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

introduction of fidaxomicin, where relapsing CDI occurs [26, 27]. This highlights there remains a need to identify new, effective agents to treat CDI.

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

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

We next sought to investigate the inhibitory activity of auranofin against *C. difficile* toxin production. As mentioned above, toxins are crucial for *C. difficile* to induce inflammation and to provoke disease. As a result, non-toxigenic bacteria are not associated with disease. So, it is conjectured that an inhibition of toxin production will contribute to effective treatment of CDI [17, 33]. Additionally, toxin production occurs during stationary phase of bacterial growth where antibiotics are not as effective. Furthermore, toxin production is increased by environmental stress; therefore, some anticlostridial drugs (e.g. vancomycin and metronidazole) induce *C. difficile* toxin production [34]. Auranofin was previously reported to inhibit protein synthesis, virulence factors and toxin production in *Staphylococcus aureus* [35, 36]. Therefore, we tested the activity of auranofin at subinhibitory concentrations against a hypervirulent, toxigenic strain of *C. difficile*. Auranofin inhibited the total toxin production in *C. difficile*. A similar effect was observed with

fidaxomicin but not with vancomycin or metronidazole. We also tested auranofin's ability to reduce *C. difficile* toxin-mediated inflammation of the gut epithelial cells, given auranofin exhibits potent anti-inflammatory activity. Exposure of human colonic epithelial cells (Caco-2 to supernatant containing toxins produced by *C. difficile* to induced cell rounding similar to that was reported previously [18]. Inclusion of a very low concentration of auranofin (1 µg/mL) with the *C. difficile* culture supernatant protected Caco-2 cells from the deleterious effect of *C. difficile* toxins (Figure 5.4) and suppressed production of the inflammatory cytokine IL-8.

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

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

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

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CHAPTER 6. REPURPOSING THE VETERINARY ANTIPROTOZOAL DRUG RONIDAZOLE FOR THE TREATMENT OF *CLOSTRIDIOIDES DIFFICILE* INFECTION

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6.1 Abstract

Clostridioides difficile infection is currently an unsettling worldwide concern. Hundreds of thousands of people are getting infected and thousands lose their lives every year due to this infection inside and outside the United States. The pressing need for new more efficient anticlostridial agents is far from being satisfied. That said, repurposing can be utilized as a rapid and more cost-efficient method of drug development. Ronidazole, a veterinary antiprotozoal drug, was found to inhibit *C. difficile* growth very potently in a previous screening for anticlostridial agent amongst FDA-approved drugs. The current study further evaluates the activity of ronidazole as a potential treatment for *C. difficile* infection. Ronidazole inhibited the growth of most *C. difficile* strains at a very low concentration (0.125 μg/mL) and showed superior killing kinetics when compared to a known anticlostridial agent from the same chemical category, metronidazole. In addition, ronidazole did not inhibit some of the protective bacteria naturally present in the human intestine. Further, ronidazole was non-toxic to human gut cells and had better pharmacokinetic profile than metronidazole. Finally, ronidazole outperformed metronidazole in a mouse model of *C. difficile* infection by protecting the same number of mice as metronidazole at 10 times lower concentration. Overall, ronidazole merits further investigation as a potential treatment for *C. difficile* infections in both humans and animals.

Keywords: *Clostridioides difficile, Clostridium difficile*, nitroimidazoles, ronidazole, anticlostridial, repurposing veterinary medicines.

6.2 Introduction

Clostridioides difficile (previously known as *Clostridium difficile*) is an eminent perilous nosocomial pathogen. *Clostridioides difficile* infection (CDI) is a leading cause of morbidity and mortality especially in healthcare settings. The Centers for Disease Control and Prevention (CDC) estimated that about 225,000 cases of CDI were reported in 2017 and resulted in an excess healthcare cost of $1 billion in the United States. In the same CDC report, CDI was estimated to be a direct cause of almost 13,000 deaths and was classified as an urgent threat [1, 2]. In Europe, although data is not as accurate, there was a sharp rise in the incidence of CDI in recent years. Surprisingly, this was reported even in countries that had traditionally low incidence of hospital-associated infections [3-5]. Moreover, similar theme of increased incidence of CDI was observed in Asia, Central and South America and the Middle East [6-10]. In addition to the increase in incidence and severity, *C. difficile* was recently reported to infect different classes of patients. Classically, *C. difficile* was known to infect patients after exposure to broad-spectrum antibiotics and was more prevalent in elderly patients. But remarkably, CDIs are progressively diagnosed in atypical patients that were not recently exposed to antibacterials and are relatively younger in age [10-13]. As a result, 41% of all reported CDIs are acquired in non-healthcare settings [14]. Further, several outbreaks of severe community-acquired CDI were recently reported [15-17]. The recent change in the bacterial behavior is mainly attributed to the emergence of the so-called hypervirulent strains. Unlike other *C. difficile* strains, hypervirulent strains produce more robust amounts of toxins at all growth phases. This is the reason why symptoms caused by these strains lie within the severe side of the spectrum of CDI symptoms, which extends from mild diarrhea to pseudomembranous colitis, megacolon and death [10, 18]. Additionally, the hypervirulent strains of *C. difficile* are capable of forming spores more efficiently and at earlier growth stages than other strains. These spores are considered the vehicle for infection, horizontal transmission and persistence [18, 19]. Further, other virulence factors, like biofilm formation and production of binary toxin, are more associated with hypervirulent *C. difficile* [20].

Unfortunately, available treatment options for CDI are not satisfactory. Initially three antibiotics were utilized for the treatment of CDI, namely, metronidazole, vancomycin and fidaxomicin. However, metronidazole was recently withdrawn from the CDI treatment regimen, due to inferiority to vancomycin, and it was recommended only when the other two drugs are not obtainable [21]. As a result, only two effective antibiotics are currently in use for the management

of CDI, vancomycin and fidaxomicin. Vancomycin treatment outcome is not satisfactory, the mean treatment failure and recurrence of about 22% of the treated cases [22, 23]. In addition, using vancomycin to treat CDI increases the likelihood of patients to retract an infection with vancomycin-resistant *enterococci* (VRE), an observation that is also noted with metronidazole treatment [24]. On the other hand, fidaxomicin was approved in 2011 as a narrow-spectrum anticlostridial that spares important gut bacterial species. In addition, fidaxomicin exerted an inhibitory activity against *C. difficile* toxin release and spore formation, an effect that was not observed with either vancomycin or metronidazole [25]. Fidaxomicin had a significant, yet not ample, improve in recurrence rate relative to vancomycin. However, this improvement was only observed with non-hypervirulent strains of *C. difficile* [26]. Furthermore, emergence of *C. difficile* strains that are resistant to one or more anticlostridial agent remain a constant fear. Despite their limited number, resistance to all anticlostridial agents have been reported amongst *C. difficile* strains [27]. On a separate note, the cost of fidaxomicin and vancomycin therapy can be a limiting factor in certain parts of the world. Thus, metronidazole was kept in the guidelines for CDI treatment in case the first two drugs are not attainable [21, 28]. Based on the previous information, there is an imperative demand for new anticlostridial agents.

Repurposing is the use of FDA-approved drugs beyond their initial indication. It is advantageous in the sense that it cuts down the time and cost required for drug discovery when compared to the conventional *de novo* method. The higher success rates and the reduced risk render repurposing attractive for research and drug development [29]. In a recent study, we screened almost 3200 FDA-approved drugs and clinical molecules against *C. difficile*. As expected, nitroimidazole containing drugs inhibited the growth of *C. difficile in vitro*. What was not expected is that some of the drugs inhibited *C. difficile* growth more potently than metronidazole. Ronidazole was one of the nitroimidazole drugs that inhibited *C. difficile* growth at very low concentration [30]. In the current study, we further evaluate the *in vitro* inhibitory activities of ronidazole and some of the drug pharmacokinetic properties. In addition, we investigate the *in vivo* activity of ronidazole in a mice model of *C. difficile* infection.

6.3 Materials and Methods

6.3.1 Bacterial strains and reagents

C. difficile and human gut microbiota strains used in this study (Table 1) were acquired from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and the American Type Culture Collection (ATCC, Manassas, VA). Strains were cultured in brain heart infusion supplemented broth (BHIS, Brain heart infusion medium from Becton, Dickinson and Company, Cockeysville, MD), supplemented with yeast extract, L-cysteine, vitamin K1 and hemin (Sigma-Aldrich, St. Louis, MO). Phosphate buffered saline (PBS), RPMI-1640 and horse serum were purchased from Corning (Corning, NY). For the toxicity study, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was purchased from Promega (Madison, WI). Kanamycin, gentamicin (Chem-Impex, Wood dale, IL), metronidazole, colistin (Cayman, Ann Arbor, MI), ronidazole, (Sigma-Aldrich, St. Louis, MO) were all purchased from commercial vendors. Vancomycin hydrochloride (Gold Biotechnology, Olivette, MO) and metronidazole (BTC, Hudson, NH) were used as positive controls.

Table 6.1 *Clostridioides difficile* isolates tested in the study

***C. difficile* Strain**	**Alternate ID**	**Source**	**Geographical region**	**Year**	**PCR ribotype**	**NAP***	**Toxins**
ATCC 43255	VPI 10463	Abdominal wound	--	--	087	--	A and B
ATCC BAA-1870	4118	Human	--	--	027	NAP1	A, B and CDT
HM-88	CDC#2007054	Human	--	--	07	--	--
NR-13427	Isolate 1	Human	Mid-Atlantic region, USA	2008/ 2009	--	--	--
NR-13431	Isolate 5	Human	Mid-Atlantic region, USA	2008/ 2009	--	--	--
NR-13436	Isolate 10	Human	Mid-Atlantic region, USA	2008/ 2009	--	--	--
NR-32885	P5	Human	Western Pennsylvania, USA	2001	--	--	Toxigenic
NR-32887	P7	Human	Western Pennsylvania, USA	2001	--	--	Toxigenic
NR-32888	P8	Human	Western Pennsylvania, USA	2001	--	--	Toxigenic
NR-32890	P11	Relapsing human	Western Pennsylvania, USA	2001	--	--	Toxigenic
NR-32891	P13	Human	Western Pennsylvania, USA	2005	--	--	Toxigenic
NR-32895	P19	Relapsing human	Western Pennsylvania, USA	2005	--	--	Toxigenic
NR-32896	P20	Relapsing human	Western Pennsylvania, USA	2005	--	--	Toxigenic

Table 6.1 continued

NR-32897	P21	Relapsing human	Western Pennsylvania, USA	2005	--	--	Toxigenic
NR-49277	Isolate 20100502	Elderly male	Colorado, USA	2010	019	NAP1	A, B and CDT**
NR-49278	Isolate 20100207	Elderly male	New York, USA	2010	027	NAP1	A, B and CDT
NR-49281	Isolate 20110052	Elderly male	Northeastern USA	2010	027	NAP1	A, B and CDT
NR-49283	Isolate 20120013	Young male	Northeastern, USA	2011	027	NAP1	A, B and CDT
NR-49285	Isolate 20110979	Elderly female	Midwestern USA	2011	027	NAP1	A, B and CDT
NR-49286	Isolate 20110999	Elderly female	Western USA	2011	027	NAP1	A, B and CDT
NR-49288	Isolate 20110870	Young female	Tennessee, USA	2011	027	NAP1	A, B and CDT
NR-49289	Isolate 20120184	Elderly female	Tennessee, USA	2011	027	NAP1	A, B and CDT
NR-49290	Isolate 20120187	Elderly male	Tennessee, USA	2011	019	NAP1	A, B and CDT
NR-49291	Isolate 20120236	Elderly female	Midwestern, USA	2011	027	NAP1	A, B and CDT

***NAP=** North American pulsed-field gel electrophoresis
****CDT=** *C. difficile* binary toxin

6.3.2 *In vitro* anticlostridial activity of ronidazole

The minimum inhibitory concentrations (MICs) for ronidazole along with the control anticlostridial drugs was determined using the Clinical and Laboratory Standards Institute (CLSI) approved method for broth microdilution assay with slight modification [31]. Briefly, after 48 hours of anaerobic incubation of the *C. difficile* strains on brain heart infusion supplemented (BHIS) agar plates at 37º C, the colonies were scraped off and suspended in BHIS broth at a concentration of approximately 10^5 CFU/ml. The bacterial suspension was incubated with a 2-fold serial dilution of the drugs and placed back in the incubator, anaerobically at 37º C, for 48 hours. At the end of the 48 hours plates were visually checked for turbidity. The MICs reported in table (6.2) are the least concentration of each drug that could inhibit the growth of the bacteria. MIC50 and MIC90 were the lowest concentration that inhibited 50% and 90% of the tested *C. difficile* isolates, respectively.

6.3.3 Killing kinetics of ronidazole against *C. difficile*

Time-kill assay was performed to evaluate bacterial killing kinetics of ronidazole as formerly defined [32]. A Colony of *C. difficile* ATCC BAA1870 was scraped of BHIS agar plate,

suspended in BHIS broth and incubated anaerobically for 18 hours at 37° C. The bacterial suspension was then back-diluted 100 folds to achieve a starting bacterial concentration of about 10^6 CFU/mL. The bacterial suspension was then mixed with the indicated concentrations of ronidazole or control anticlostridial agents in triplicates. At each designated time point, samples were taken from each bacterial suspension tube to assess bacterial count. Count was measured by serially diluting each sample followed by plating on anaerobic BHIS agar plates in duplicates. Next, plates were incubated anaerobically at 37° C for 24 hours before counting the colonies. Colony count was multiplied by a dilution factor to calculate the bacterial concentration in each sample with a theoretical limit of detection of 41.67 CFU/mL.

6.3.4 Activity of ronidazole against human normal microflora

The broth microdilution assay was utilized one more time to determine the *in vitro* activity of ronidazole against human normal gut flora. Bacteria were streaked on agar plates and incubated at 37º C for 48 hours. Colonies were suspended in nutrient broth to achieve a concentration of ~10^5 CFU/ml. Bacterial suspensions were seeded in 96-well plates with serial dilutions of all drugs and incubated for at 37º C for 48 hours. After incubation plates were checked for bacterial growth and the MICs were recorded, table (6.3). For *Lactobacillus* species, MRS agar and broth were used and the incubation was done in 5% CO_2 atmosphere, while for *Bacteroides* and *Bifidobacteria* species BHIS agar and broth were used and the incubation was anaerobic.

6.3.5 Toxicity of ronidazole toward human gut cells:

Ronidazole at concentrations of 32, 64, 128, and 256 µg/ml was tested for its toxicity against human ileocecal colorectal adenocarcinoma cell line (HRT-18) as described previously [33]. Briefly, ~2 x 10^4 cells suspended in 100 µL of RPMI-1640 supplemented with 10% horse serum were seeded in a tissue culture-treated 96-well plate and incubated at 37 °C in a 5% CO_2 atmosphere until ~ 90% confluency was achieved. Afterward, cells were incubated with the aforementioned concentrations of ronidazole, in triplicates, for 2 hours. Then, the culture media were discarded, and the cells in each well were washed with PBS before adding 100 µL of MTS assay reagent. The plates were incubated for 4 hours at 37 °C in a humidified 5% CO_2 atmosphere. The absorbance at 490 nm was recorded using a kinetic ELISA microplate reader (SpectraMax

i3x, Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the control, DMSO-treated, cells.

6.3.6 *In vitro* absorption assay:

Caco-2 permeability analysis was utilized to assess the *in vitro* rate of permeation through a monolayer of gastrointestinal cells. Cells were seeded in well plate with a permeable support bottom and allowed to grow and differentiate for 3 weeks. A known concentration of each drug was applied in the apical compartment and the concentrations of these drugs were tested in the basolateral compartment after 60 minutes at 37º C. The integrity of the monolayer was confirmed by the testing the permeability to a paracellular fluorescent marker, fluorescein, and making sure that it is within acceptable limits. The apparent permeability coefficient (P_{app}) was calculated using the formula: $P_{app} = (V_R \times C_{R,end}/\Delta t) \times (1/(A \times (C_{D,mid} - C_{R,mid}))$, where V_R is the volume of the receiver chamber. $C_{R,end}$ is the concentration of the test compound in the receiver chamber at the end time point, Δt is the incubation time and A is the surface area of the cell monolayer. $C_{D,mid}$ is the calculated mid-point concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minute and the donor concentration at the end time point. $C_{R,mid}$ is the mid-point concentration of the test compound in the receiver side, which is one half of the receiver concentration at the end time point.

6.3.7 *In vivo* activity of ronidazole in a mouse model of CDI

Spore preparation; An overnight culture of *C. difficile* ATCC 43255 was spread on BHIS agar plates and incubated anaerobically at 37° C for 5 days. After incubation, bacterial loans were scrapped off the agar plates and transferred into PBS. The bacterial suspension was heated at 70° C for 25 minutes to eradicate vegetative bacterial cells and to activate the spores. Spore count was determined through 10-fold serial dilution and plating on BHIS agar plates containing 0.1% taurocholic acid as a germinant for the spores. Count was determined before and after infection to ensure consistency.

Animal study; The mice study was performed following the design proposed previously with slight modification [34]. Female C57BL/6 mice, 6-week-old, were divided into groups of five and caged in individually ventilated cages. Mice were left to acclimatize for a week with access to

food and water *ad libitum*. The guidelines of the Purdue Animal Care and Use Committee (PACUC) was followed. Initially, mice were sensitized for *C. difficile* infection by adding antibiotic cocktail into their sterile drinking water. The antibiotic cocktail contained kanamycin (400 mg/L), gentamicin (35 mg/L), colistin (42 mg/L), metronidazole (215 mg/L), and vancomycin (45 mg/L) for 5 days. On the following day, water was switched back to regular sterile water for 2 days then mice were intraperitoneally injected with clindamycin (10 mg/kg). One day later, mice were orally infected with 8 x 10^5 CFU/ml of *C. difficile* ATCC-43255 spores. Two hours post-infection, mice were orally treated with ronidazole and metronidazole (10 and 1 mg/kg) and vancomycin (10 mg/kg) daily for 6 days while control group was treated using sterile PBS. Cage beddings were changed regularly to avoid coprophagia. Mice were monitored for signs of CDI and euthanized upon showing moribund state for extended period of time. On days 7, mice were humanely euthanized using CO_2 inhalation. Kaplan-Meier survival curve is shown in figure (5.3) for the ronidazole-treated mice versus mice treated with the control anticlostridial drugs and PBS.

6.4 Results

6.4.1 *In vitro* activity of ronidazole against clinical *C. difficile* strains

A standard microdilution assay was performed in order to evaluate the inhibitory activity of ronidazole against *C. difficile*. Bacteria were incubated with various concentrations of ronidazole and control antibiotics to assess their minimum inhibitory concentrations (MICs). As depicted in table 6.2, ronidazole potently inhibited the growth of 24 clinical isolates of *C. difficile*. The MICs of ronidazole ranged from 0.0625 to 0.25 µg/mL, while MIC50 and MIC90 were 0.125 µg/mL. On the other hand, the inhibition observed with both vancomycin and metronidazole was weaker relative to ronidazole. Vancomycin inhibited *C. difficile* growth at concentrations that ranged from 0.25 to 2 µg/mL. The MIC50 and MIC90 for vancomycin were 0.25 and 2, respectively. Similarly, metronidazole inhibited the growth of the tested strains between 0.125 and 1 µg/mL, while the MIC50 and MIC90 were 0.25 µg/mL. Finally, fidaxomicin inhibited *C. difficile* growth at a concentration range of 0.0156 – 0.125 µg/mL. The MIC50 and MIC90 of fidaxomicin were 0.0312 and 0.0625 µg/mL, respectively.

Table 6.2 MICs of ronidazole against several strains of *C. difficile* expressed in μg/mL

***C. difficile* Strain**	**Ronidazole**	**Metronidazole**	**Vancomycin**	**Fidaxomicin**
ATCC 43255	0.125	0.5	1	0.0312
ATCC BAA-1870	0.0625	0.25	1	0.0625
HM-88	0.0625	0.125	0.25	0.0312
NR-13427	0.125	0.5	2	0.0312
NR-13431	0.125	0.25	0.25	0.0156
NR-13436	0.25	0.25	0.25	0.0625
NR-32885	0.125	0.25	0.25	0.0312
NR-32887	0.0625	0.25	0.25	0.0312
NR-32888	0.125	0.125	0.25	0.0312
NR-32890	0.0625	0.125	1	0.0156
NR-32891	0.0625	0.25	0.25	0.0312
NR-32895	0.0625	0.25	2	0.0156
NR-32896	0.125	0.25	2	0.0312
NR-32897	0.125	0.25	0.25	0.0156
NR-49277	0.125	0.125	0.5	0.0312
NR-49278	0.125	1	0.25	0.0625
NR-49281	0.125	0.125	0.25	0.0312
NR-49283	0.0625	0.25	0.25	0.0156
NR-49285	0.0625	0.25	0.25	0.0625
NR-49286	0.125	0.25	0.25	0.0625
NR-49288	0.125	0.25	0.25	0.0625
NR-49289	0.125	0.25	0.25	0.0312
NR-49290	0.125	0.25	0.5	0.0625
NR-49291	0.0625	0.25	0.5	0.125
MIC50	0.125	0.25	0.25	0.0312
MIC90	0.125	0.5	2	0.0625

6.4.2 Time-kill assay of ronidazole against *C. difficile*

Bacterial suspensions were incubated with either 5X MIC or 20X MIC of ronidazole and metronidazole. Bacterial count was measured over the course of 24 hours to determine the killing kinetics of the drugs. As presented in figure 6.1, ronidazole diminished the bacterial count to below detection limit after 8 hours of incubation at both concentrations. However, ronidazole reduced more than 3-log of the initial bacterial count after 8 hours when 5X MIC was used (Figure 6.1A). While on the other hand, bacterial killing was achieved in less than 4 hours in case of 20X MIC

(Figure 6.1B). In addition, bacteria could not regrow when 20X MIC of ronidazole was used unlike with 5X MIC where the bacteria regrew, to a lower concentration than untreated control, after 12 hours. Metronidazole did not reduce the initial bacterial count when 5X MIC was used and the bacteria grew to a similar concentration to the untreated control after 12 hours. When 20X MIC of metronidazole was used, bacterial killing (>3-log reduction) was achieved in less than 4 hours of incubation. In addition, bacterial count was below detection limit after 8 hours.

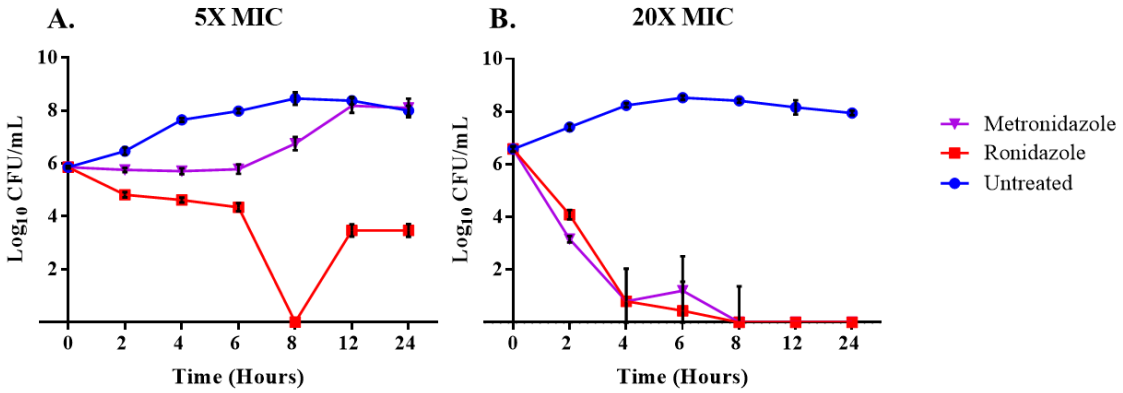

Figure 6.1 Time-kill assay of ronidazole against *C. difficile* ATCC BAA-1870

Ronidazole and metronidazole were anaerobically incubated with *C. difficile* ATCC BAA-1870 (~10^6 CFU/mL) at 37 °C. Samples were taken at the indicated time points and bacterial count was evaluated in each sample through serial dilution and plating on BHIS agar. The mean and standard deviation of three technical replicates is resented.

6.4.3 Activity of ronidazole against human normal microflora

The activity of ronidazole against human microbiota was assessed and compared to standard anticlostridial drugs. Ronidazole showed a pattern of activity similar to that of metronidazole. Generally, both drugs inhibited anaerobic microbiota and spared aerobic ones. Against anaerobes, both ronidazole and metronidazole inhibited the growth of *Bacteroides dorei*, *Bacteroides fragilis* and *Bifidobacterium longum* at a concentration of 1 µg/mL or less. Nevertheless, both drugs did not inhibit the growth of *Bifidobacterium breve* up to 128 µg/mL. On the contrary, ronidazole and metronidazole did not potently inhibit the growth of aerobes, including *Lactobacillus casei*, *Lactobacillus crispatus* and *Lactobacillus gasseri*. Vancomycin inhibited the growth of all tested isolates except *Lactobacillus casei*. The inhibition was at a higher concentration (16 µg/mL) for *Bacteroides dorei* and *Bacteroides fragilis*, while it was more potent

(≤ 2 µg/mL) against the remaining tested isolates. Lastly, fidaxomicin did not inhibit *Bacteroides dorei* and *Bacteroides fragilis* and *Bifidobacterium breve* HM-856 up to 128 µg/mL. However, fidaxomicin inhibited *Lactobacillus casei* at 4 µg/mL while all the other strains were inhibited at a concentration of 1 µg/mL or less (Table 6.3).

Table 6.3 The MICs (µg/mL) of ronidazole against human microbiota

Strain	Ronidazole	Metronidazole	Vancomycin	Fidaxomicin
Bacteroides dorei HM-29	≤1	≤1	16	>128
Bacteroides dorei HM-719	≤1	≤1	16	>128
Bacteroides fragilis HM-710	≤1	≤1	16	>128
Bacteroides fragilis HM-714	≤1	≤1	16	>128
Bifidobacterium breve HM-1120	>128	>128	0.5	≤1
Bifidobacterium breve HM-411	>128	>128	0.5	≤1
Bifidobacterium breve HM-412	>128	>128	0.5	≤1
Bifidobacterium breve HM-856	>128	>128	0.5	>128
Bifidobacterium longum HM-845	≤1	2	0.25	≤1
Bifidobacterium longum HM-847	≤1	2	0.25	≤1
Lactobacillus casei ATCC 334	>128	>128	>16	4
Lactobacillus crispatus HM-375	32	>128	0.5	≤1
Lactobacillus crispatus HM-422	64	>128	1	≤1
Lactobacillus gasseri HM-398	>128	>128	1	≤1
Lactobacillus gasseri HM-399	>128	>128	1	≤1
Lactobacillus gasseri HM-400	32	>128	1	≤1
Lactobacillus gasseri HM-404	>128	>128	1	≤1
Lactobacillus gasseri HM-407	>128	>128	2	≤1
Lactobacillus gasseri HM-409	>128	>128	1	≤1
Lactobacillus gasseri HM-410	>128	>128	1	≤1

6.4.4 Toxicity of ronidazole against human gut cells:

Human ileocecal colorectal adenocarcinoma (HRT-18) cells were incubated with ronidazole to test the potential toxic effect of the veterinary drug against human cells. As presented in figure 6.2, ronidazole showed no toxic effects against HRT-18 cells. Compared to DMSO-treated control, the relative viability of the cells was 94%, 101%, 100% and 113% at ronidazole concentrations of 256, 128, 64 and 32 µg/mL, respectively.

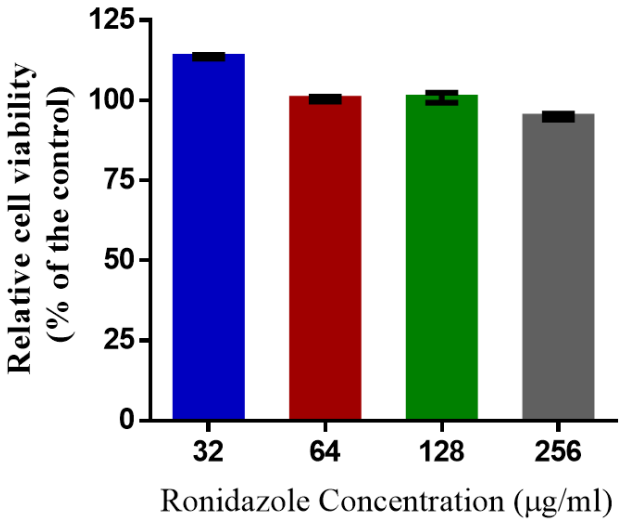

Figure 6.2 Toxicity of ronidazole against HRT-18 cells

Ronidazole was incubated with human ileocecal colorectal adenocarcinoma cell line (HRT-18) at 4 different concentrations, 32, 64, 128, and 256 µg/mL. The viability of the cells was evaluated and compared the DMSO-treated control cells. Data represents the percentage of the viability of ronidazole-treated cells relative to control.

6.4.5 Apparent permeability of ronidazole through human intestinal cells:

A two-compartment system separated by a monolayer of human colorectal adenocarcinoma (Caco-2) was used to assess the transepithelial transport of ronidazole through gut cells. Known concentrations of ronidazole and control drugs were added to the apical compartment and received from the basolateral compartment to calculate the apparent absorption of each drugs. Although ronidazole was highly permeable, it passed through the monolayer of Caco-2 cells at a lower rate than metronidazole. The mean apparent rate of permeability of ronidazole was 56.6×10^{-6} cm/sec whereas for metronidazole it was 69.9×10^{-6} cm/sec. In addition, results indicated that neither drugs is a substrate for efflux transporters, like P-glycoprotein (data not shown). Colchicine, labetalol, propranolol and ranitidine were added as controls for the assay. Colchicine is a poorly permeable drug and a substrate for P-glycoprotein, ranitidine is a poorly permeable drug, labetalol is intermediate and propranolol is a highly permeable drug. Data is depicted in figure 6.3.

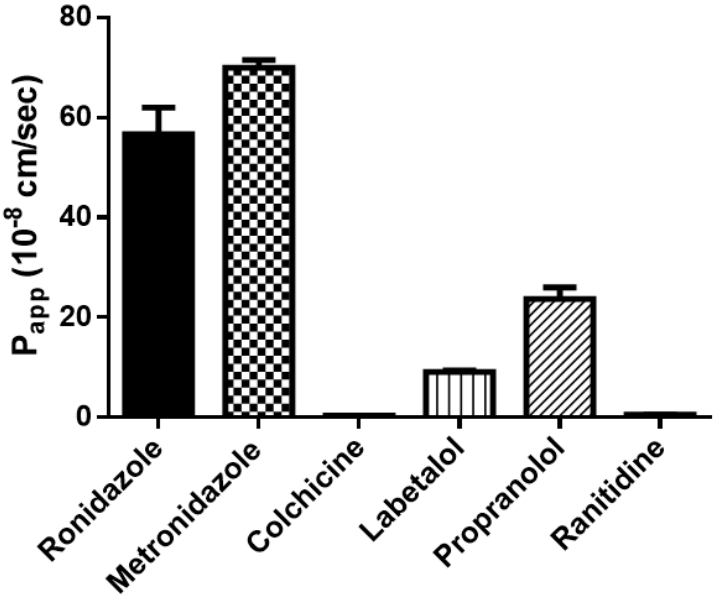

Figure 6.3 Caco-2 Permeability of ronidazole and control compounds

The permeability of ronidazole through a mono layer of Caco-2 cells was measured and compared to metronidazole. Compounds, at a known concentration, were added to the apical compartment a monolayer of Caco-2 cell supported by a permeable support. After incubation, the amount of each compound was measure in the basolateral compartment of the cells.

6.4.6 Activity of ronidazole in an animal model of CDI

Antibiotic-primed mice were infected with a virulent strain of *C. difficile*, afterward, mice were treated with ronidazole and control anticlostridial agents to evaluate the potential of ronidazole to treat CDI *in vivo*. As depicted in figure 6.4, 80% of the untreated mice died within the first 6 days of the infection whereas mice treated with vancomycin (10 mg/kg, the positive control) totally survived the infection. As per ronidazole and metronidazole, both drugs protected 60% of the infected mice at a concentration of 10 mg/kg. Additionally, ronidazole also protected 60% of the mice at 1 mg/kg. But surprisingly, metronidazole at 1 mg/kg did not protect any of the treated mice. Indeed, mice treated with metronidazole (1 mg/kg) died at a faster rate, within 2 days, than the untreated control group of mice.

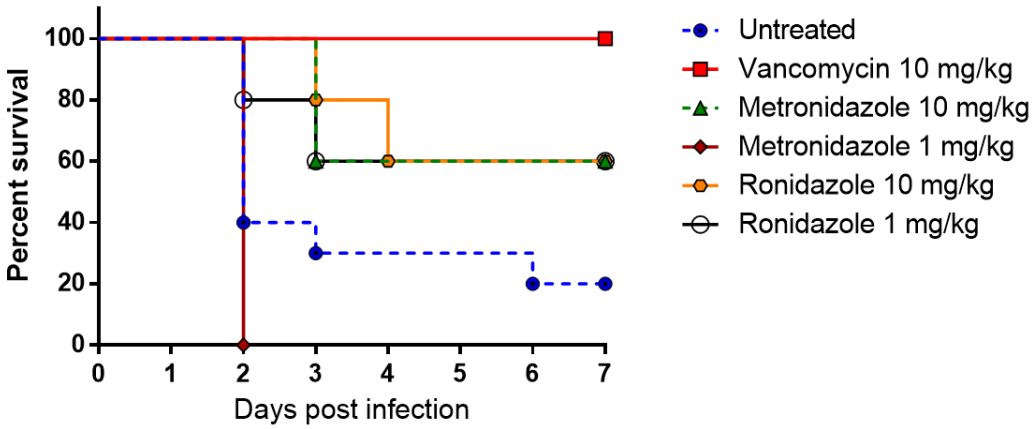

Figure 6.4 Activity of ronidazole in a CDI mice model

The protective effect of ronidazole was evaluated in a mice model of CDI and compared to metronidazole and vancomycin. Antibiotic-primed mice were infected with *C. difficile* ATCC 43255 spores. Treatment started 2 hours post infection and continued daily for 6 days while untreated mice received PBS. Mice were checked several times per day and euthanized when showing morbidity. All survived animals were euthanized on the 7th day by CO_2 inhalation.

6.5 Discussion

CDI is a common complication of antibiotic administration specifically in elderly patients. The recent emergence of hypervirulent strains of *C. difficile* have rendered CDI the most occurring infection in healthcare settings. These hypervirulent strains are capable of producing toxins more efficiently than normal *C. difficile* strains. As a result, disease symptoms associated with hypervirulent strains are more severe and exaggerate to fatality more frequently [35]. The problem of CDI is further complicated by the scarcity of effective treatments. As mentioned previously, only two anticlostridial agents are currently recommended for the treatment of CDI after metronidazole was removed from the treatment guidelines. Considering the great number of causalities and the potential of resistant development among *C. difficile* strains towards the current medications, there is an immense need for developing new anticlostridial agents. One disadvantage of classical drug development techniques is the huge cost associated with their lengthy processes. Economically, going through research and development (R&D) of a new antibiotic is not always a cost-efficient process for pharmaceutical companies. Further, effective antibiotics are usually subject to antibiotic stewardship practice which can further reduce the financial profit out of this product. Added to that, there remains the probability of the bacteria evolving resistance against

any given drug which curtail its efficacy [36]. As a result, antibacterial drug discovery is not an attractive venue for pharmaceutical companies to pursue. Indeed, several leaders in pharmaceutical industry suspended their antibacterial development programs leaving only a handful of big manufacturers currently with active antibacterial programs [37]. One way to minimize the time and cost of drug discovery is repurposing FDA-approved drugs. Several FDA-approved drugs have displayed potent inhibitory activities against various bacterial species [38, 39]. Having thoroughly investigated toxicity, safety and in some cases pharmacokinetic profiles, reduce the time and cost associated with evaluating these parameters in case of a *de novo* molecule. Therefore, repurposing these de-risked drugs is a more attractive process of antibacterial drug discovery [29]. Along the same line, veterinary medicines represent a huge pool of FDA-approved drugs to be investigated. Veterinary drugs are of approved safety to be used in animals and are expected to possess minimal toxicity against human cells. Notably, several veterinary drugs have been investigated for their potential applications in human medicine [40-44]. The current study is an extension to a previous study conducted by our group. In that study, we screened about 3200 FDA-approved drugs and clinical molecules for their inhibitory activity against *C. difficile in vitro* [30]. Out of the active hits, we selected ronidazole to be evaluated further against *C. difficile in vitro* and *in vivo*. Ronidazole is a veterinary antiparasitic drugs used in the treatment of several different animal species. It is marketed for the treatment of Giardiasis, Trichomoniasis, Hexamitosis and Cochlosomosis in pigeons and aviary birds. In addition, ronidazole was found to treat swine dysentery when mixed with drinking water at a low concentration [45]. Furthermore, ronidazole is used off-label to treat cats infected with *Tritrichomonas foetus*. Although ronidazole and metronidazole belong to the same chemical class, nitroimidazole containing compounds, ronidazole shows better *in vitro* and *in vivo* activity profile against parasites than metronidazole [46]. Additionally, ronidazole remains active against metronidazole-resistant strains [47]. Consequently, we thought ronidazole might act in similar fashion against *C. difficile* displaying better activity than metronidazole.

In the current study, ronidazole activity was initially evaluated against numerous clinical strains of *C. difficile* (Table 6.1). As seen with parasites, ronidazole showed more potent inhibitory activity than metronidazole. It was also more potent than vancomycin but not fidaxomicin (Table 6.2). In addition, the killing kinetics study revealed superior activity of ronidazole relative to metronidazole at a lower concentration. Although both drugs completely eradicated the bacteria at

20X their MIC, only ronidazole could clear the bacteria at 5X its MIC. Whereas metronidazole could momentarily inhibit the bacterial growth before the bacteria grew back to reach to the untreated level (Figure 6.1). The superiority of ronidazole activity to metronidazole's impelled us to explore more of its activities as a potential *C. difficile* medication. We next sought to investigate the activity of ronidazole against essential bacterial strains of the human normal microbiota. Indeed, ronidazole retained the same profile of metronidazole. It only inhibited some of the anaerobic bacteria whilst spared the aerobic ones (Table 6.3). The diversity of the human gut microbial community is an essential protective factor against CDI. Different bacterial strains in the gut contribute to the colonization resistance against *C. difficile* in a plethora of mechanisms [48]. That said, an antibiotic with limited activity against *C. difficile* would be ideal for the treatment of *C. difficile* infection. Unfortunately, this aim has not completely been achieved through any of the anticlostridial agents known to date. *Lactobacillus* and some *Bifidobacterium* species were inhibited by vancomycin and fidaxomicin but not with ronidazole or metronidazole. These genera represent two of the protective genera amongst gut microbiota. In this vein, reduction in the abundance of bifidobacteria has been observed with CDI patients. In addition, probiotics containing bifidobacteria and lactobacilli reduce CDI relapse and antibiotic-associated diarrhea in general [48]. Although evidence regarding the health benefits of these two genera is contradicting, sparing these two genera and conserving the bacterial diversity of the gut is a preferred trait in an anticlostridial agent. Next, the safety of ronidazole towards human gut cells was yet to be confirmed. As seen in figure 6.2, ronidazole did not cause any reduction of cell viability up to 256 µg/mL. Taking in consideration the MIC of ronidazole against most of the tested isolates, 0.125 µg/mL, the toxicity data suggest safety of ronidazole towards human gut cells at a concentration that is > 2000 folds higher than its MIC. Additionally, we were curious to investigate the absorption pattern of ronidazole and compare it to metronidazole (Figure 6.3). Initially, both drugs showed higher permeability when compared to the test control drugs. However, ronidazole demonstrated a slight reduction in permeability across Caco-2 monolayer relative to metronidazole. This reduction in absorption rate can allow ronidazole to stay in contact with the bacteria at the site of infection for longer time and hence, more efficient bacterial killing. In addition, ronidazole is a structural analog to metronidazole and expected to behave similarly. Accordingly, ronidazole is expected to be distributed to the gastrointestinal tract, after being absorbed, through mucosal permeability and enterohepatic circulation [49]. Notably, ronidazole demonstrated slow

elimination form cats after both oral and intravenous administration [50]. Finally, In a mouse model of CDI, ronidazole showed equal activity to ronidazole at a concentration of 10 mg/kg. But interestingly, ronidazole completely outperformed metronidazole at 1 mg/kg. Indeed, ronidazole at 1 mg/kg protected equal number of mice as metronidazole and ronidazole at 10 mg/kg (Figure 6.4). The superior activity of ronidazole over metronidazole at a lower concentration might be due to the different killing pattern seen with either drugs (Figure 6.1). Additionally, the increased potency and reduced absorption might have a role in the improved activity of ronidazole at lower concentration (Table 6.2 and Figure 6.3). However, the fact that ronidazole activity did not improve when the concentration was increase from 1 to 10 mg/kg is yet to be investigated.

One limitation of using ronidazole in the treatment of CDI is its presumed toxicity after systemic administration. The major side effect observed with ronidazole administration in cats is neurotoxicity. Several cases have been reported of feline neurotoxicity after oral administration of ronidazole. Symptoms include loss of appetite, lethargy and ataxia [51]. However, neurotoxicosis is associated with higher dose of the drug and is observed only in some, not all, ronidazole-treated cases. In addition, the signs of neurotoxicity are reversible and resolve after drug cessation within 1-2 weeks [52]. Moreover, ronidazole is anecdotally reported to be carcinogenic and teratogenic after prolonged administration. Nevertheless, administration of ronidazole in Albino rats for the whole period of pregnancy and at a dose that is 6 times greater than the maximum therapeutic dose did not cause any teratogenic or embryogenic effects [53]. Notably, CDI treatment takes between 10 to 20 days and hence, short-term studies are required to evaluate the potential toxic effect of ronidazole during this period.

Taken altogether, nitroimidazoles still represent an attractive scaffold for anticlostridial drug development. In this study ronidazole was shown to possess superior *in vitro* and *in vivo* activities against *C. difficile* when compared to metronidazole. Further, ronidazole showed preferred pharmacokinetic properties for CDI treatment. Therefore, ronidazole represents a potential candidate for the treatment of CDI either solely or as an odd-on therapy and warrant further investigation. The study also hints to the importance of veterinary medicine as an additional pool of safe drugs that can repurposed for human indications.

6.6 References

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CHAPTER 7. EVALUATION OF SECNIDAZOLE AS AN ALTERNATIVE THERAPY FOR THE TREATMENT OF *CLOSTRIDIOIDES DIFFICILE* ASSOCIATED DIARRHEA

A version of this chapter has been submitted to the Journal of Antimicrobial Agents and Chemotherapy. AbdelKhalek, A., Pal, R. & Seleem, M. N. (2020). Evaluation of Secnidazole as an Alternative Therapy for the Treatment of *Clostridioides difficile* Associated Diarrhea.

7.1 Abstract

Clostridioides difficile infection (CDI) is a principal cause of debility and fatiliy worldwide. The current study evaluates the 5-nitroimidazole antibacterial secnidazole as an inhibitor for *C. difficile* growth both *in vitro* and *in vivo*. While sparing some critical protective human microbiota strains, secnidazole demonstrated superior clostridial killing *in vitro*. In addition, it reveald complete protection for *C. difficile* infected mice. Overall, secnidazole's potency and pharmacokinetic properties justify further invistigation for the tratment of CDI.

7.2 Main article

Clostridioides Difficile associated diarrhea (CDAD) is one of the most serious complications of antibiotic administration. It is more prevalent in elderly patients predominantly in healthcare settings and nursing homes. CDAD symptoms can be as simple as mild diarrhea, however, symptom can exaggerate causing severe pseudomembranous colitis and death (1, 2). These symptoms result from the toxin-mediated inflammation and destruction of the intestinal mucosa. While *C. difficile* toxins (TcdA and TcdB) are responsible for disease symptoms, dormant spores, on the other hand, are the transmissible morphotype of the bacteria mediating transmission and recurrence (3-6). Three antibiotics were used in the treatment of *C. difficile* infection (CDI), metronidazole, vancomycin and fidaxomicin. However, metronidazole was opted out of the treatment guidelines recommended by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) in 2018. As per their guidelines, metronidazole is used only when vancomycin and fidaxomicin are not available. The decision was based upon the unsatisfactory cure rate and high recurrence associated with metronidazole

treatment (7). Considering the natural resistance of *C. difficile* to numerous antibiotics and the scarcity of approved anticlostridial agent, there is a pressing demand for more effective anticlostridial agents (8). In a previous *in vitro* screening for anticlostridial agents among FDA-approved drugs, secnidazole was identified as one of the most potent inhibitors of *C. difficile* growth (9). Interestingly, secnidazole is antibacterial agent approved to treat bacterial vaginosis in 2017 and belongs to the same chemical class as metronidazole, 5-nitroimidazoles. In the current study, we aim to further evaluate the activity of secnidazole against *C. difficile* both *in vitro* and *in vivo*.

Initially, the minimum inhibitory concentrations (MICs) of secnidazole, and comparators, were evaluated against 26 clinical isolates of *C. difficile* as describe previously (9, 10). The concentration that inhibited 50 and 90 percent of all tested isolated (MIC_{50} and MIC_{90}, respectively) was calculated and presented in table 1. As expected, similar *in vitro* potency was observed for metronidazole and secnidazole. Both drugs were active between 0.125 and 1 mg/L, in addition, the MIC_{50} and MIC_{90} of both secnidazole and metronidazole were 0.25 and 0.5 mg/L, respectively. In contrast, vancomycin showed weaker profile of *in vitro* inhibition where bacteria were inhibited between 0.25 and 4 mg/L while both MIC_{50} and MIC_{90} were 1 mg/L.

Table 7.1 Minimum inhibitory concentrations (MICs, mg/L) of secnidazole, metronidazole and vancomycin against 26 strains of *C. difficile*

Drug	*MIC range*	MIC_{50}[1]	MIC_{90}[2]
Secnidazole	0.125 - 1	0.25	0.5
Metronidazole	0.125 - 1	0.25	0.5
Vancomycin	0.25 - 4	1	1

[1]MIC50 = Drug concentration that inhibited 50 % of the tested isolates
[2]MIC90 = Drug concentration that inhibited 90 % of the tested isolates

Next, the killing kinetics of both secnidazole and metronidazole were compared in a time-kill assay (11). Drugs were incubated with about 10^6 CFU/mL of *C. difficile* ATCC BAA-1870 for 24 hours and samples were taken for count assessment at the indicated time points (12). As depicted in figure 1, both drugs reduced more than 3 logs of the initial bacterial count within 4 hours at 20X their MICs. On the other hand, only secnidazole eradicated the bacteria at 5X MIC after 8 hours while metronidazole momentarily inhibited the bacterial growth. In both cases, bacteria finally regrew to reach the untreated level after 24 hours. The rapid bactericidal activity

of metronidazole at a higher concentration is in agreement with previous reports (13, 14). In contrast, metronidazole demonstrated bacteriostatic activity at 20X MIC when the starting bacterial inoculum was greater than 10^7 CFU/mL (15). Interestingly, this dual activity of metronidazole was also observed against other organisms, like *Trichomonas vaginalis* (16).

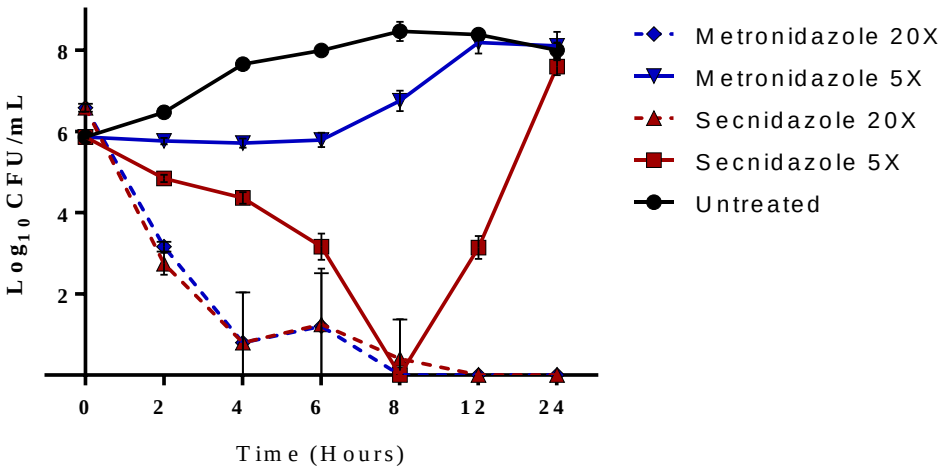

Figure 7.1 Time-kill assay of secnidazole and metronidazole against *C. difficile* ATCC BAA-1870 at 5X and 20X their MICs.

Bacteria (~ 10^6 CFU/mL) were mixed with the indicated concentrations of both drugs and incubated anaerobically for 24 hours at 37 °C in triplicates. At the indicated time points, sample were taken from all the bacterial suspensions, serially diluted and plated to evaluate the bacterial count in each sample.

Afterward, we sought to investigate the activity of secnidazole against representative strain of protective human microbiota as described earlier (9, 17). Human intestine contains a vast number of bacterial species that work in harmony to protect against bacterial invasion. Thus, an ideal anticlostridial agent should exert minimal damage to these beneficial bacterial species, a goal that was not completely achieved by any of the current medications. As presented in table 2, both secnidazole and metronidazole potently inhibited the growth of *Bacteroides* species (MICs $\leq$2 mg/L), an activity that was not observed with vancomycin (MIC $\geq$16). On the contrary, nitroimidazoles generally did not inhibit the growth of both *Bifidobacterium* and *Lactobacillus* species (most MICs are $\geq$128) while vancomycin potently inhibited most of these species (most MICs are $\leq$1). Notably, Bifidobacteria and lactobacilli are particularly important in the protection against CDAD (18-22).

Table 7.2 Minimum inhibitory concentrations (MICs, mg/L) of secnidazole, metronidazole and vancomycin against human normal microbiota

Strain	*Secnidazole*	*Metronidazole*	*Vancomycin*
***Bacteroides fragilis* HM-711**	≤2	1	>16
***Bacteroides fragilis* HM-709**	≤2	2	>16
***Bacteroides dorei* HM-719**	≤2	0.125	16
***Bacteroides dorei* HM-717**	≤2	1	16
***Bifidobacterium longum* HM-845**	32	2	0.5
***Bifidobacterium breve* HM-1120**	>256	>128	0.5
***Lactobacillus gasseri* HM-409**	128	>128	1
***Lactobacillus gasseri* HM-400**	>256	>128	1
***Lactobacillus casei* ATCC-334**	>256	>128	>16

Finally, the activity of secnidazole was yet to be evaluated in a mouse model of CDAD. Antibiotic-primed mice were infected with *C. difficile* VPI 10463 as described elsewhere (23). Mice where treated on daily basis and their survival was recorded and presented in figure 2. While only 20% of the untreated mice survived, vancomycin (10 mg/kg) protected 100% of *C. difficile* infected mice. Additionally, metronidazole at a lower concentration (1 mg/kg) accelerated the mortality of the mice causing them all to die on the second day post-infection. Whereas at 10 mg/kg, metronidazole protected 60% of the infected mice. Interestingly, secnidazole (10 mg/kg) protected 100% of *C. difficile* infected mice, while only 20% survived at 1mg/kg. The activity of metronidazole and vancomycin (10 mg/kg) is in accordance with previously reported data (24-26). In addition, the rapid mortality of mice treated with 1 mg/kg of metronidazole, although not reported before, can be due the induction of *C. difficile* toxin release observed with subinhibitory concentrations of metronidazole (27, 28).

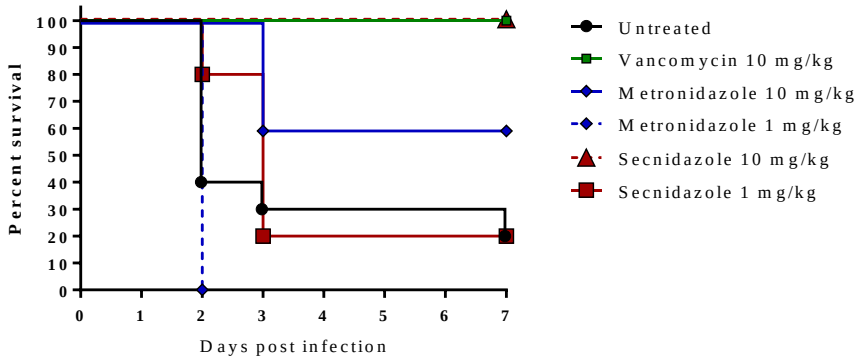

Figure 7.2 Activity of secnidazole in a mouse model of CDAD

Antibiotic-treated mice were infected with the spores of *C difficile* VPI 10463. Secnidazole, metronidazole and vancomycin were used to treat the mice while the untreated mice received saline. Treatment started 2 hours post-infection and continued for 6 days. Mice were humanely euthanized with CO_2 asphyxiation upon showing morbidity or at the completion of the study.

The superior activity of secnidazole versus metronidazole is probably due to the better bacterial killing profile seen in time-kill assay (Figure 1). Additionally, secnidazole has a longer half-life after oral administration (up to 29 hours) compared to metronidazole (~ 8 hours). Furthermore, the plasma protein binding of secnidazole is 15% leaving high plasma concentration for 48 hours after a single oral dose. Therefore, only a single dose of secnidazole (2 grams) is sufficient to treat bacterial vaginosis (29-31). Although secnidazole is recently prescribed in the US for the treatment of bacterial vaginosis, it has been used for long time in other countries for several bacterial and protozoal indications. Thus, availability is not a limiting factor for secnidazole use, unlike vancomycin and fidaxomicin, especially in developing countries. In addition, secnidazole is well-tolerated and is can be used for longer periods in some indications, 7 days for hepatic amoebiasis (1, 30).

Overall, this study present secnidazole with potential application in the management of CDAD either as a first line treatment or as a more potent alternative to metronidazole when vancomycin and fidaxomicin are not attainable.

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Publications

Published journal articles

1- Mohamed F. Mohamed, **Ahmed AbdelKhalek**, Mohamed N. Seleem. Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular *Staphylococcus aureus*. July 2016. Scientific Reports. IF: 4.1

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Accepted manuscripts

13- Aryl-alkyl-lysines: Novel agents for treatment of *Clostridium difficile* infection. Chandradhish Ghosh, **Ahmed AbdelKhalek**, Haroon Mohammad, Mohamed N. Seleem, and Jayanta Haldar. Scientific Reports SREP-18-44189 **(*Co-first author*)**
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Manuscripts under review

15- Jatinder Kaur, Xufeng Cao, Nader S. Abutaleb, Ahmed Elkashif, Amanda L. Graboski, Amer H. Tarawneh, Atul Bhardwaj, **Ahmed AbdelKhalek**, Mohamed N. Seleem, Daniel P. Flaherty. Optimization of acetazolamide-based scaffold as potent and selective Enterococcus inhibitors. Journal of Medicinal Chemistry Manuscript ID: jm-2019-01680u

Conference presentations Oral Presentations [O] & Posters [P] (Presenter *):

1- H. Eldesouky*, W. Younis, **A. AbdelKhalek** and M.N. Seleem. Repurposing FDA approved drugs and clinical molecules for treatment of invasive fungal diseases, Sigma Xi Graduate Student Research Poster Award Competition, March 2nd, 2016, Purdue University, West Lafayette, IN, USA. [P]

2- **A. AbdelKhalek***, W. Younis and M. Seleem. Repurposing drugs for the treatment of multidrug-resistant enterococci. Sigma Xi Graduate Student Research Poster Award Competition, March 2, 2016, Purdue University, West Lafayette, IN, USA. [P]

3- W. Younis*, S. Thangamani, **A. AbdelKhalek** and M.N. Seleem. Repurposing FDA-approved drugs to combat drug –resistant bacteria, ISS Research Symposium March 8, 2016, Armstrong Atrium, Purdue University, West Lafayette, IN, USA. [P]

4- W. Younis*, S. Thangamani, **A. AbdelKhalek** and M.N. Seleem. Repurposing FDA-approved drugs to combat drug –resistant bacteria, health and science symposium, Purdue University. March 28, 2016, West Lafayette, IN, USA. [P]

5- H. Eldesouky*, W. Younis, **A. AbdelKhalek** and M.N. Seleem. Repurposing FDA- approved drugs to combat drug –resistant bacteria, Health and Disease: Science, Technology, Culture and Policy Research symposium, March 28, 2016, Purdue University, West Lafayette, IN, USA. [P]

6- **A. AbdelKhalek***, W. Younis and M. Seleem. Repurposing carbonic anhydrase inhibitors to treat Enterococcal infections. Health and Disease: Science, Technology, Culture and Policy Research symposium, March 28, 2016, Purdue University, West Lafayette, IN, USA. [P]

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8- **A. AbdelKhalek***, W. Younis and M. Seleem. Carbonic Anhydrase Inhibitors; Potential Alternative Solution for Enterococcal Infections. Indiana Branch ASM Meeting, April 1-2, 2016, Fort Wayne (IPFW), IN, USA. [P]

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10- **A. AbdelKhalek***, W. Younis and M. Seleem. Carbonic Anhydrase Inhibitors; Potential Alternative Solution for Enterococcal Infections. The Annual Phi Zeta Research Day, April 11, 2016, Purdue College of Veterinary Medicine, West Lafayette, IN, USA. [P]

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12- **A. AbdelKhalek***, W. Younis and M. Seleem. Carbonic Anhydrase Inhibitors; Potential Alternative Solution for Enterococcal Infections. The Hitchhiker's Guide to the Biomolecular Galaxy Symposium, May 11, 2016, Purdue University, West Lafayette, IN, USA. **P**

13- Y. Hegazy*, W. Younis, **A. AbdelKhalek**, S. Thangamani and M. Seleem. Novel Drugs for Bioterrorism Agents. Purdue institute of inflammation and infectious disease Symposium, August 10, 2016, Purdue University, West Lafayette, IN, USA. **P**

14- **A. AbdelKhalek***, W. Younis and M. Seleem. Repurposing FDA approved carbonic anhydrase inhibitors to treat Enterococcal infections. Purdue institute of inflammation and infectious disease Symposium, August 10, 2016, Purdue University, West Lafayette, IN, USA. **P**

15- Ben Bergstrom*, WM Townsend, W Younis, **A. AbdelKhalek**, GK Hammac, MN Seleem. Antibacterial Activity and Safety of Commercial Cationic Steroid Antibiotics and Neutral Superoxidized Water. 47th Annual American College of Veterinary Ophthalmologists Conference October 26-29, 2016, Monterey, CA, USA. **P**

16- **A. AbdelKhalek***, L. Ha, D. Flaherty and M. N. Seleem. Repurposing approach identifies two novel drugs against *Clostridium difficile*, Sigma Xi Graduate Student Research Poster Award Competition, February 14, 2017, West Lafayette, IN, USA. **P**

17- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies auranofin with novel antimicrobial activities against *Clostridium difficile*, Health and Disease: Science, Technology, Culture and Policy Research Poster Session, March 23, 2017, West Lafayette, IN, USA. **P**

18- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies auranofin with novel antimicrobial activities against *Clostridium difficile*, Indiana branch American society of Microbiology (IBASM) annual meeting, April 1, 2017, Marshall, IN, USA. O

19- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies auranofin with novel antimicrobial activities against *Clostridium difficile*, Indiana branch American society of Microbiology (IBASM) annual meeting, April 1, 2017, Marshall, IN, USA. **P**

20- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies auranofin with novel antimicrobial activities against *Clostridium difficile*, Omicron Chapter of Phi Zeta Abstract & Poster Competition, April 10, 2017, West Lafayette, IN, USA. **P**

21- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies two novel drugs against *Clostridium difficile*, The 34Th Herbert C. Brown lectures in organic chemistry poster session, April 14, 2017, West Lafayette, IN, USA. **P**

22- N. S. Abutaleb*, **A. AbdelKhalek** and M. N. Seleem. Repurposing carbonic anhydrase inhibitors to decolonize and treat Enterococcal infections, The 34th Herbert C. Brown lectures in organic chemistry poster session, April 14, 2017, West Lafayette, IN, USA. **P**

23- **A. AbdelKhalek*** and M. N. Seleem. Repurposing approach identifies auranofin with novel antimicrobial activities against *Clostridium difficile*, The Hitchhiker's Guide to the Biomolecular Galaxy' symposium, May 11, 2017, West Lafayette, IN, USA. O

24- **A. AbdelKhalek*** and M. N. Seleem. Combating *Clostridium difficile* infection using an anti-rheumatic drug, The 4th annual drug discovery symposium, Purdue institute for drug discovery. October 11, 2017, West Lafayette, IN, USA. **P**

25- A. Elkashif*, H. Mohammad, **A. AbdelKhalek**, N. S. Abutaleb, M. N. Seleem. (2018). Evaluation of the Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci. Sigma Xi Graduate Student and Post-Doctoral Research Poster Award Competition, February 21, 2018, West Lafayette, IN, USA. **P**

26- A. Elkashif*, H. Mohammad, **A. AbdelKhalek**, N. S. Abutaleb, M. N. Seleem. (2018). Evaluation of the Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci. Sigma Xi Graduate Student and Post-Doctoral Research Poster Award Competition, February 21, 2018, West Lafayette, IN, USA. **P**

27- **A. AbdelKhalek***, N. S. Abutaleb, W. Younis, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors as a Potential Alternative for Decolonization of Vancomycin-Resistant Enterococci. Sigma Xi Graduate Student and Post-Doctoral Research Poster Award Competition, February 21, 2018, West Lafayette, IN, USA. **P**

28- N. S. Abutaleb*, **A. AbdelKhalek**, M. N. Seleem. (2018). Auranofin combines the antibacterial, antitoxin and anti-inflammatory activities in the treatment of *Clostridium difficile* infections. Sigma Xi Graduate Student Research Poster Award Competition, February 21, 2018, West Lafayette, IN, USA. **P**

29- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization Through a Unique Target. Health and Disease: Science, Technology, Culture and Policy Research Poster Session, March 1, 2018, West Lafayette, IN, USA. **P**

30- N. S. Abutaleb*, **A. AbdelKhalek**, M. N. Seleem. **(**2018). Repurposing an FDA approved drug for the treatment of *Clostridium difficile* infections. Health and Disease: Science, Technology, Culture and Policy Research Poster Session, March 1, 2018, West Lafayette, IN, USA. **P**

31- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization Through a Unique Target. BME-IBSc-PI4D research symposium, Purdue University, April 6, 2018, West Lafayette, IN, USA. **P**

32- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization Through a Unique Target. Indiana branch American society of Microbiology (IBASM) annual meeting, April 7, 2018, Indianapolis, IN, USA. **P**

33- A. Elkashif*, H. Mohammad, **A. AbdelKhalek**, N. S. Abutaleb, M. N. Seleem. (2018). Evaluation of the Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci. Indiana branch American society of Microbiology (IBASM) annual meeting, April 7, 2018, Indianapolis, IN, USA.-**P**

34- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization Through a Unique Target. Indiana branch American society of Microbiology (IBASM) annual meeting, April 7, 2018, Indianapolis, IN, USA. O

35- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization Through a Unique Target. Annual Phi Zeta Research Day, Purdue University, April 9, 2018, West Lafayette, IN, USA. **P**

36- A. Elkashif*, H. Mohammad, **A. AbdelKhalek**, N. S. Abutaleb, M. N. Seleem. (2018). Evaluation of the Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci. Annual Phi Zeta Research Day, Purdue University, April 9, 2018, West Lafayette, IN, USA.

37- **A. AbdelKhalek***, N. S. Abutaleb, L. Ha, W. Younis, D. Flaherty, M. N. Seleem. (2018). Carbonic Anhydrase Inhibitors Curb Vancomycin-Resistant Enterococci Gut Colonization through a Unique Target. The Thirty-fifth Herbert C. Brown lectures in organic chemistry, Purdue University, April 13, 2018, West Lafayette, IN, USA. **P**

38- A. Elkashif*, H. Mohammad, **A. AbdelKhalek**, N. S. Abutaleb, M. N. Seleem. (2018). Evaluation of the Anthelmintic Drug Niclosamide for Intestinal Decolonization of Vancomycin-resistant Enterococci. The Thirty-fifth Herbert C. Brown lectures in organic chemistry, Purdue University, April 13, 2018, West Lafayette, IN, USA. **P**

39- **A. AbdelKhalek***, Nader Abutaleb, Haroon Mohammad, Deepansh Mody and Mohamed N. Seleem. Gold Containing Compounds: Potential Therapeutics for *Clostridium difficile* infection. Purdue graduate student government second student research poster session, Purdue University, November 2018, West Lafayette, IN, USA. **P**

40- **A. AbdelKhalek***, N. S. Abutaleb, Haroon Mohammad, D. Mody, and M. N. Seleem. Anticlostridial Drug Discovery. Health and Disease: Science, Technology, Culture and Policy Research Poster Session, February 28, 2019, West Lafayette, IN, USA. **P**

41- **A. AbdelKhalek***, N. S. Abutaleb, Haroon Mohammad, D. Mody, and M. N. Seleem. *Clostridium difficile* Infection: Fighting Back with Golden Weapons. PVM research day, College of Veterinary Medicine, Purdue University, April 8, 2019, West Lafayette, IN, USA. **P**

42- **A. AbdelKhalek***, N. S. Abutaleb, Haroon Mohammad, D. Mody, and M. N. Seleem. *Clostridium difficile* Infection: Fighting Back with Golden Weapons. Purdue Microbiome Symposium Poster Session, May 13, 2019, West Lafayette, IN, USA. **P**

43- **A. AbdelKhalek***, H. Mohammad, R. Pal, M. N. Seleem. (2020). Nitroimidazoles, Back on Track in the Battle against *Clostridioides difficile* Infection. Sigma Xi Graduate Student and Post-Doctoral Research Poster Award Competition, February 12, 2020, West Lafayette, IN, USA. **P**

44- **A. AbdelKhalek***, H. Mohammad, R. Pal, M. N. Seleem. (2020). Nitroimidazoles, Back on Track in the Battle against *Clostridioides difficile* Infection. Health and Disease: Science, Technology, Culture and Policy Research Poster Session, March 5, 2020, West Lafayette, IN, USA. **P**

Awards

1- **Second place,** The ideal student competition, 2008 – 2009, Al-Azhar University, Cairo, Egypt,

2- **First place**, The ideal student competition 2008 – 2009, College of pharmacy, Al-Azhar University, Cairo, Egypt.

3- **Second place,** Indiana branch American society of Microbiology (IBASM) annual meeting, April 1, 2017, Marshall, IN, USA.

4- **Second place,** Health and Disease: Science, Technology, Culture and Policy Research Poster Session, March 23, 2017, West Lafayette, IN, USA.

5- **First place**, The 4th annual drug discovery symposium, Purdue institute for drug discovery. October 11, 2017, West Lafayette, IN, USA.
6- **First place** (Poster), Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 1, 2018, West Lafayette, IN, USA.
7- **First place, Life science category** Purdue Institute of Inflammation, Immunology and Infectious Disease (PI4D) research symposium, Purdue University, April 6, 2018, West Lafayette, IN, USA.
8- **First place** (Poster), Health and Disease: Science, Technology, Culture and Policy Research Poster Session. March 5, 2020, West Lafayette, IN, USA.

Ad-hoc reviewer for Peer-reviewed journals

1- 2013-2015 Editorial board member for the following journals

- Egyptian Journal of Biotechnology.
- Egyptian Journal of Biomedical Sciences.
- New Egyptian Journal of Microbiology.
- Arab Journal of Laboratory Medicine.

2- July 2018 – present, Scientific reports
3- March 2018 – present, PLoS ONE

Professional Memberships and Community involvement

- 2007, Organization of the 5th International Conference for Pharmaceutical and Biological Sciences.
- 2016-2019, Student representative of the Indiana branch American Society of Microbiology (IBASM).
- August 2016, Indiana University School of Medicine – Lafayette Briarwood Health Fair, presenter.
- 2017-present, alternate CPB Grad Student representative to the Purdue veterinary medicine (PVM) Grade Appeals Committee.
- March 2017, The 65th Annual Lafayette regional science and engineering fair, poster judge.
- August 2017, Summer Undergraduate Research Fellowship (SURF) Symposium, Purdue University, oral presentation judge.
- 2018-present, Member of the American Association for the Advancement of Science (AAAs)
- 2018-present, Student member of the safety committee of the department of Comparative Pathobiology (CPB)
- March 2018, The 66th Annual Lafayette regional science and engineering fair, poster judge.
- 2019-present, Member of the American Society of Microbiology (ASM).