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Pharmacology of disulfiram as an antibacterial agent

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PHARMACOLOGY OF DISULFIRAM AS AN ANTIBACTERIAL AGENT

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Approval of Thesis

We, the faculty supervising the work of Yogesh Meka, affirm that the thesis, *Pharmacology Of Disulfiram as an Antibacterial agent*, meets the high academic standards for original scholarship and creative work established by the Department of Pharmaceutical Sciences and the School of Pharmacy. The work also conforms to the requirements and formatting guidelines of Marshall University. With our signatures, we approve the manuscript for publication.

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Abstract

Disulfiram (Antabuse®) is an oral prescription drug used to treat alcohol abuse disorder (AUD). Disulfiram (DSF) and its metabolites act on the body by inhibiting multiple enzymes involved in alcohol metabolism. This study aims to understand the pharmacology of disulfiram as an antibacterial medication. Previous studies on DSF showed antibiotic activity against multidrug-resistant *Staphylococcus aureus* strains (MRSA). The initial aim of this study was to decipher the mechanisms involved in inhibiting bacterial growth by DSF, which was done by performing biochemical studies. The studies revealed that DSF had antagonistic effects on redox buffering and energy production. Another aim of this study was to evaluate the *in vitro* pharmacodynamics of DSF in combination with vancomycin (VAN) against *S. aureus* strains with intermediate levels of susceptibility. The data showed that DSF had synergistic potential with VAN, significantly antagonizing the growth of *S. aureus* when combined. Overall, this study established that DSF has significant antibacterial activity against *S. aureus* individually. Moreover, it could be used as an antibiotic adjuvant with VAN against several *S. aureus* strains with intermediate levels of VAN susceptibility.

Chapter 1

Review of Disulfiram as an Antibacterial Agent

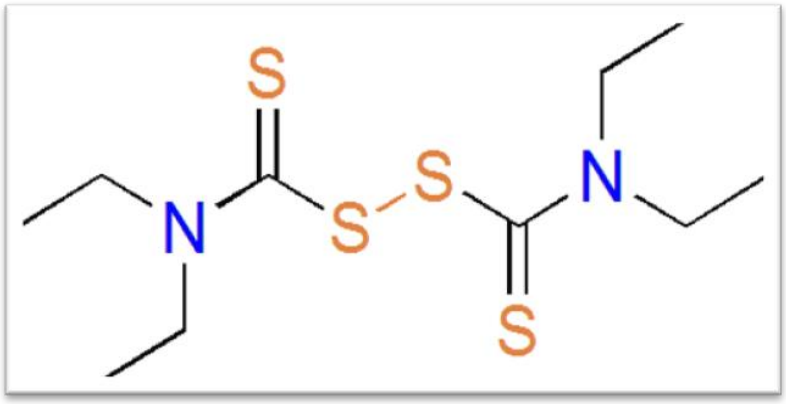
Introduction

Disulfiram (DSF) was the first drug authorized by the United States Food and Drug Administration (FDA) for the effective treatment of alcoholism and is a second-line choice to acamprosate and naltrexone for patients with adequate physician supervision ("Disulfiram," 2012). DSF, in its pure state, is a white to off-white, odorless, tasteless powder that is soluble in water and alcohol. As an alcohol-aversive or alcohol-sensitizing drug, DSF causes an acutely toxic physical reaction when mixed with alcohol (Center for Substance Abuse, 2009). Alcohol consumption during DSF therapy results in diaphoresis, palpitations, face flushing, nausea, vertigo, hypotension, and tachycardia (hangover symptoms) that manifest as an accumulation of acetaldehyde due to inactivation of aldehyde dehydrogenase (ALDH). This collection of symptoms resulting from acetaldehyde toxicity or DSF-Ethanol Reaction (DER) reaction makes drinking alcohol harmful. For example, when the patient drinks alcohol and has recently taken a DSF tablet, the concentration of acetaldehyde in the blood can be up to 5–10 times higher than normal (Guerzoni, Pellesi, Pini, & Caputo, 2018). The DSF-ethanol response typically starts about 10 to 30 minutes after consuming alcohol, ranging from mild to severe, with negative repercussions. Based on the characteristics of each patient, intensity varies, and the response is typically inversely correlated with the intake of alcohol and DSF. With blood alcohol levels of 5 to 10 mg/100 ml, mild effects could manifest. Effects are often fully developed at 50 mg/100 ml. Unconsciousness could happen when the concentration exceeds 125–150 mg/100 ml. Therefore, DSF does not affect the neurobiological mechanism of alcohol addiction, nor is it an anti-craving medication (Stokes & Abdijadid, 2022). However, DSF has proven to be an

effective adjunct for more than 60 years when used in conjunction with biopsychosocial alcoholism therapy (Krampe & Ehrenreich, 2010). Although DSF-alcohol reactions can be fatal, they are relatively uncommon today due to the low dosages and thorough patient medical screening (Center for Substance Abuse, 2009).

Figure 1

Chemical Structure of Disulfiram (DSF)



The History Behind the Discovery of DSF

DSF was synthesized in 1881 by a German chemist M. Grodzki. The discovery made its comeback into the limelight by finding its use as an antioxidant that accelerates polymerization processes in the rubber industry (i.e., the process of stiffening rubber). A coincidental finding mentioned by Williams (1937) in a letter to the Journal of the American Medical Association noticed the adverse effects of subsequent alcohol use by personnel who handled thiuram products. He mentioned that the drug had no other physiological effects except when combined with alcohol uptake and could be a cure for alcoholism which was ignored at that time (Ellis & Dronsfield, 2013). Later in the 1940s Dr. Erik Jacobsen and Dr. Jens Hald who were working on copper metabolism of intestinal parasites, observed that DSF could form chelates with copper leading to the death of organisms. DSF immediately gained popularity during the second

world war, when scabies, and intestinal worms were major public health problems at that time. With a habit of testing out new medications, Dr. Jacobsen exposed himself to DSF in 1945, just before he consumed alcohol while investigating the drug's effectiveness in treating intestinal worms, and the result was very unpleasant. Dr. Hald observed similar effects, and they thought of using DSF as a treatment for patients with alcohol use disorder. However, the idea was least considered as alcohol dependence was not a big problem in society at that time (Pal et al., 2015).

Clinical trials on DSF were later established when Dr. Hald met Oluf Martensen-Larsen, a physician who had experience in treating alcoholics, to determine the drug's effectiveness and its mechanism of action. In 1948, after conducting a few clinical trials, Jacobsen and Larsen presented DSF as an alcoholism therapy (Banys, 1988). Immediately after that, Jacobsen and his group discovered more potential formulations of DSF while attempting to purify a batch contaminated with copper which was later introduced as "Antabuse." DSF prescribed in very high doses (of up to 3000 mg per day) caused hypotension, and neurological, cutaneous, and hepatotoxic reactions, which were extremely severe and even fatal because of the toxic metabolites of DSF, particularly diethyldithiocarbamate (DDTC) formed from DSF- alcohol reaction (Ellis & Dronsfield, 2013). Besides these undesired effects, DSF continues to be used in typical alcoholism therapeutic interventions because findings suggest it is helpful for some alcoholic patients, despite being harder to justify in the absence of well-controlled research. (Banys, 1988).

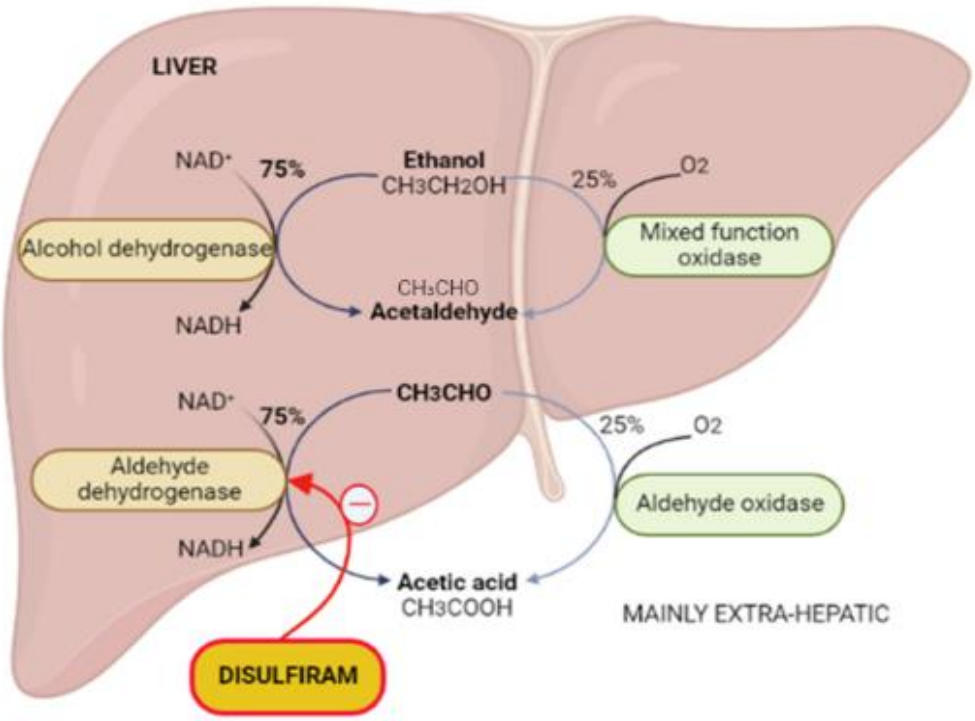
Mechanism of Action and Metabolism of DSF

Investigations established that ethanol is oxidized to acetaldehyde by the enzyme alcohol dehydrogenase (ADH), and the formed acetaldehyde is subsequently transformed into

acetic acid by the action of enzyme aldehyde dehydrogenase (ALDH) in the liver. The principal mechanism of action of DSF is to block the action of ALDH, which results in the accumulation of acetaldehyde (Jacobsen, 1952). Figure 2. ALDH is irreversibly inhibited by DSF by interacting with nicotinamide adenine dinucleotide (NAD) at the cysteine residue in the active region of the enzyme, presumably through a thiol-disulfide exchange reaction (Stokes & Abdijadid, 2022; Shen, Lipsky, & Naylor, 2000).

Figure 2

Mechanism of Action of DSF and Metabolism of Ethanol



Note. Image created using *BioRender.com*

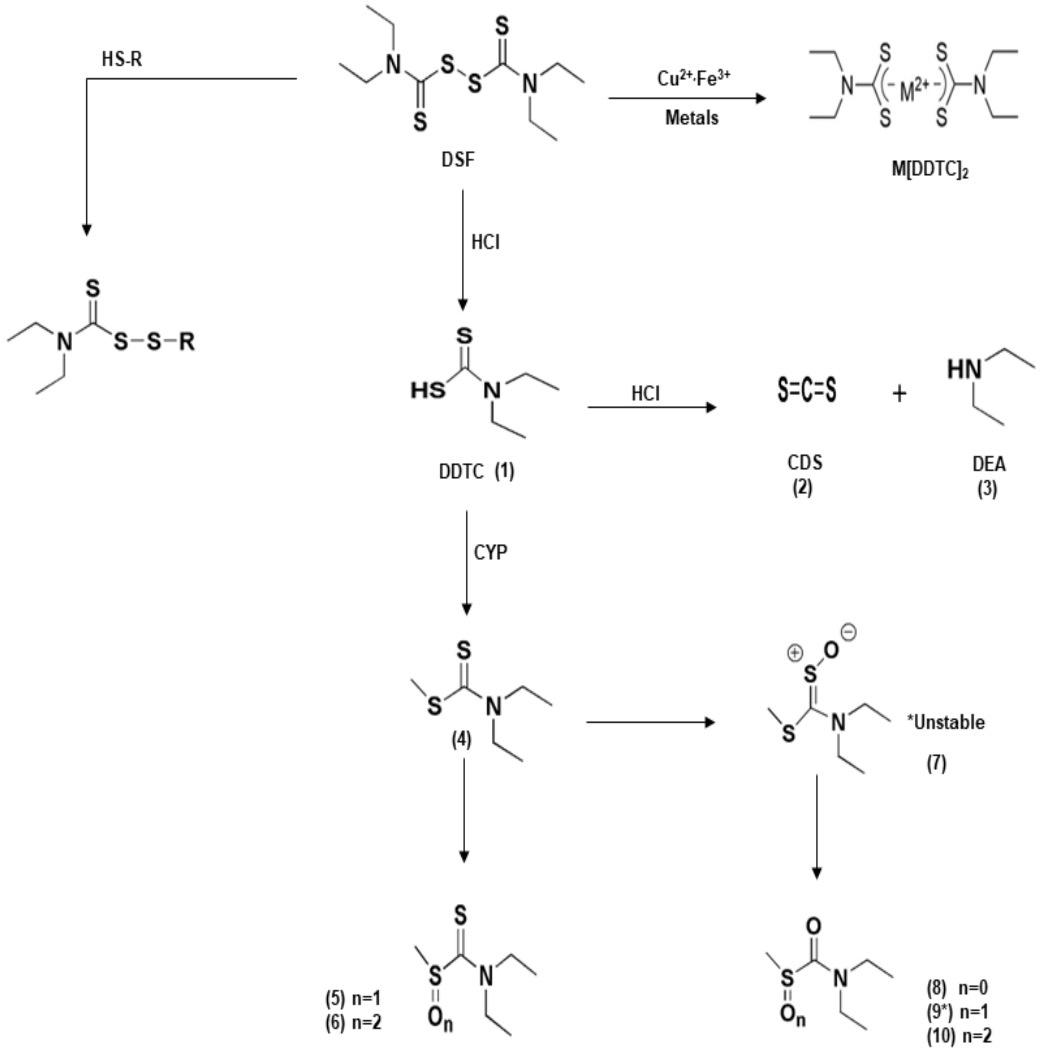
After oral ingestion of DSF, it is rapidly reduced to diethyldithiocarbamate (DDTC,**1**) in the strong acidic condition of the stomach. Formed DDTC is highly unstable in acidic conditions, which may further break down into carbon disulfide (CDS,2) and diethylamine

(DEA,3) by the action of stomach acids (Stromme, 1963), or converted to methyl diethyldithiocarbamate (Me-DDTC,4) by methyl transferases (Glauser, Nelson, Zembower, Lipsky, & Weinshilboum, 1993). Several metabolic studies on liver microsomes revealed that metabolites (5–10) may be formed from oxidation of (4) (Madan & Faiman, 1994; Madan & Faiman, 1995; Nagendra, Madan, & Faiman, 1994; Shen et al., 2000) (Figure 3). Collectively, DSF metabolites (5–10), formed from hepatic metabolism are believed to contribute in ALDH inhibition (Johansson, 1992; Shen et al., 2000; Petersen, 1992).

Reactions of DSF with endogenous thiols (e.g., glutathione), cysteine-containing enzymes (e.g., ALDH), and trace copper (i.e., $Cu[DDTC]_2$) also apparently result in the formation of DDTC outside of the stomach. The initial product of DSF's extensive metabolism DDTC, which has high affinity for endogenous metals, can either bind with them or further metabolize in the body. DDTC is a strong metal chelating compound particularly with heavy metal ions (e.g., cupric ions, ferric, etc.) forms *bis* (diethyl dithiocarbonate) metal complex ($M[DDTC]_2$) (Figure 3). The $Cu[DDTC]_2$ complex formed is more acid-stable, neutral, and highly hydrophobic, enabling further absorption along the upper gastrointestinal tract and increasing its bioavailability. Several radioactive studies with oral administration of ^{35}S-labelled DSF revealed that DSF had more than 80 percent absorption rate (Johansson, 1992). After distribution into the blood from gastrointestinal (GI) mucosa, DSF is rapidly and completely converted to two molecules of DDTC by the action of serum albumin and erythrocyte enzymes. By chemical nature, DSF is highly electrophilic and is readily cleaved by thiol-bearing substances like glutathione, cysteine enzymes, bacillithiol, etc. as a consequence of thiol-disulfide exchange reaction. The suggested process of thiol-disulfide exchange between thiol-containing compounds (R-SH) and DSF is shown in Figure 3.

Figure 3

Schematic Depiction of DSF Metabolism



Note. DSF converted to DDTC under acidic conditions gives CSD and DEA whereas other metabolites 4- 10 are formed using liver microsomes. DSF also reacts with metals, and R-SH-containing compounds through a thiol-disulfide exchange reaction (Custodio, Sparks, & Long, 2022; Frazier, Moore, & Long, 2019; Lewis, Riedel, Kesler, Varney, & Long, 2022).

Other Pharmacological Effects of DSF

DSF is an electrophile that easily forms disulfide bonds with compounds that contain thiols (Long, 2017). The ability of DSF to modify sulfhydryl groups and chelate metals suggests the importance of repositioning it as a drug to treat several infectious diseases, cancer, etc. A study revealed that DSF could act as a DNA demethylating agent (Lin et al., 2011) suggesting its novel treatment application for cancer. DSF may be used as a primary or adjunctive therapy for cancer and fungal infections by blocking the ATPS-binding cassette (ABC) drug transport protein responsible for the development of multiple drug resistance (Sauna, Shukla, & Ambudkar, 2005). Recent anti-cancer studies also established that the Cu-chelation complex of DSF can impede the activation of 26S proteasome, resulting in polyubiquitinated and cytotoxin protein aggregation and death of cancer cells (Celik et al., 2016). Moreover, an enhanced antitumor effect was observed when DSF was combined with antitumor drugs (e.g., 5-fluorouracil) (Wang, McLeod, & Cassidy, 2003).

Antibacterial Activity of DSF

Gram-Positive Spectrum

Bacteria have a variety of intracellular cofactors (e.g., co-enzyme A), antioxidants (e.g., glutathione, mycothiol, and bacillithiol) and enzymes (e.g., thioredoxin) that contain thiophilic residues that DSF may modify through a thiol-disulfide exchange to produce antimicrobial effects (Long, 2017). A couple of *in vitro* investigations examined the antibacterial activity of DSF in Gram-positive *Staphylococcus, Streptococcus, Enterococcus*, *Bacillus* and *Pseudomonas aeruginosa* (Sheppard et al., 2018; Serafin et al., 2022). In addition, it has recently been shown that methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) were susceptible at a minimum inhibitory concentration (MIC)

range of 4-32 µg/ml. Moreover, DSF was found to reduce the MIC of *S. aureus* for intermediate vancomycin-resistant (VISA), and vancomycin-resistant (VRSA) isolates (Long, 2017).

Table 1

Reported MICs (µg/ml) of DSF and its Metabolite (DDTC) Against Some Gram-positive and Gram-negative Bacteria (Custodio et al., 2022)

Type of bacteria	Species	DSF	DDTC
Gram-positive	*Bacillus anthracis*	≤0.5	1 - 4
	Bacillus cereus	4	32
	Corynebacterium striatum	8	>32
	Enterococcus faecalis	32	64
	Enterococcus faecium (VRE)	16 - 32	32 - 64
	Staphylococcus epidermidis	1 - 4	32 - >64
	Streptococcus agalactiae	32	>32
	Streptococcus gallolyticus	>32	>32
	Streptococcus pneumonia	32	>32
	Streptococcus pyogenes	16	32
	Streptococcus mutans	16	—
	Staphylococcus aureus (VSSA)	8 - 32	16 - >32
	Staphylococcus aureus (VISA)	4 - 32	16 - 64
	Staphylococcus aureus (VRSA)	4 - 32	8 - 64
	Micrococcus luteus	8	32
	Listeria monocytogenes	32	>32
	Rhodococcus erythropolis	4	>32
Gram negative	*Acinetobacter baumannii*	>32	>32
	Bordetella bronchiseptica	>32	>32
	Brucella neotomae	16	>32
	Burkholderia cepacia	>32	>32
	Burkholderia multivorans	32	>32
	Citrobacter freundii	>32	>32
	Pasteurella multocida	>32	>32
	Proteus mirabilis	>32	>32
	Pseudomonas aeruginosa	>32	>32
	Enterobacter cloacae	>32	>32
	Rhodococcus erythropolis	4	>32
	Salmonella typhi	>32	>32
	Shigella dysenteriae	>32	>32
	Vibrio cholerae	32	>32
	Yersinia enterocolitica	32	>32
	Yersinia pestis	8	16
	Yersinia pseudotuberculosis	>32	>32

As the most prevalent isolate of skin, bone, and joint infections, *S. aureus* has been the subject of interest in the research on DSF as a therapy for Gram-positive bacterial infections. In 1991, a MIC of 1.33 μg/ml was reported against MRSA infections for DSF (Phillips, Malloy, Nedunchezian, Lukrec, & Howard, 1991). Recent research in the field of infectious disease has shown that both the disulfides DSF and DDTC possess narrow-spectrum antibacterial activity against *S. aureus* and other Gram-positive bacteria. An MIC_{90} of 16 μg/ml against 30 isolates of *S. aureus*, which included vancomycin-sensitive *S. aureus* (VSSA; MIC range of 8 - 32 μg/ml) and VISA, VRSA (4 - 32 μg/ml), was reported by (Long, 2017). Furthermore, DSF showed bacteriostatic, as well as bactericidal activity when treated at concentrations 4 x MIC (Long, 2017) and 10 x MIC, respectively, whereas 5 x MIC showed a prolonged post-antibiotic effect of 5 hrs. (Thakare, Shukla, Kaul, Dasgupta, & Chopra, 2019).

The MICs of DSF and its metabolite DDTC against different Gram-positive bacteria are shown in Table 1. *Bacillus anthracis* (anthrax), *Bacillus* cereus, and *Staphylococcus epidermidis* are susceptible bacteria with MICs less than 4 μg/ml. For isolates of *Streptococcus* mutants, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* as well as vancomycin-resistant *Enterococcus faecium* (VRE), moderate to mild susceptibility (MIC 16-32 μg/ml) was noted. On the other hand, apart from *B. anthracis*, DDTC was a poor inhibitor of Gram-positive bacteria growth at (MIC 32-64 μg/ml). Combination drug testing studies have been a potent solution for treating multidrug-resistant MRSA infections and several studies addressed the use of DSF as an antibiotic adjuvant with different antibiotics to tackle these deep-seated infections (Ejim et al., 2011; Frazier et al., 2019; Long, 2017). DSF was found to lower the MIC of VAN in VRSA and VISA to a level observed in VSSA(i.e., ≤ 2 μg/ml) with synergistic to additive effects. However, VAN/DSF combination appeared to have a stronger effect in VRSA

(Sheppard et al., 2018; Turos et al., 2008; Long, 2017; Frazier et al., 2019) and similar effects were observed with DDTC as well. An *in vivo* investigation by (Thakare et al., 2019) further established that DSF treatment reduced *S. aureus* burden in a neutropenic murine thigh infection model.

Gram-Negative Spectrum

Most Gram-negative bacteria are non-susceptible to DSF at MIC ≤ 32 g/ml (Table 1), whereas some significant communicable pathogens *Brucella neotomae*, *Rhodococcus erythropolis* and *Yersinia pestis* are susceptible at MICs 16 μg/ml, 4 μg/ml and 8 μg/ml. DSF also inhibited *Helicobacter pylori* growth at a MIC of 1 μg/ml (Kobatake et al., 2021). These results were supported by the evidence of reduced *Helicobacteriaceae* load in a mouse cancer model when DSF was combined with copper (H. Hu et al., 2020). DSF was also found to lower the MICs of imipenem from 64-128 to 8-32 μg/ml in NDM-1 clones of *P. aeruginosa, E. coli*, and *K. pneumoniae* through a possible thiol-disulfide exchange reaction with Cys_{208} in the active site of NDM-1. Moreover, DDTC-Cu chelated complex ($Cu[DDTC]_2$) also lowered the MIC of imipenem in NDM-1 producing bacteria to 4-16 μg/ml (Chen, Yang, Wu, Li, & Sun, 2020).

Mycobacterium

DSF has strong non-tuberculous mycobacterial growth-inhibitory action. According to reports, *M. tuberculosis* (TB) is more sensitive to DSF than nontuberculosis mycobacteria (NTM); however, DSF demonstrated synergistic properties with amikacin and moxifloxacin (second-line antituberculosis medications) against NTM species *M. abscessus* and *M. fortuitum* (Das, Garg, Chopra, & Dasgupta, 2019). Additionally, in the same bacteria, DSF exhibited bactericidal activity when treated with 10 x MIC and enhanced intracellular killing was

observed in macrophages at 5 x MIC. *In vivo* evaluation in a murine bacteremia model has shown that an oral dose of 50 mg/kg/day of DSF significantly reduced *M. fortuitum* load in kidneys after 15 days. Multiple studies revealed that DSF exhibited an MIC_{90} of 1.56 µg/ml and 3.13 µg/ml for TB susceptible and resistant isolates, respectively (Horita et al., 2012; Das, Garg, Chopra, & Dasgupta, 2019).

Previous studies regarding the DSF use as an antibacterial agent showed increased susceptibility against *S. aureus* bacteria (Long, 2017). However, the reason for this antibacterial action of DSF is not apparent. Thus, this study aimed to define the pharmacology of DSF as an antibacterial agent and addresses the following specific aims.

Aim 1: Conduct mechanistic studies on DSF in *S. aureus*. This aim focused on the preliminary understanding of the mechanism of action of DSF on staphylococcal bacteria. This research will test the hypothesis that DSF alters metabolism, disrupts the membrane potential, and induce oxidative stress in *S. aureus*. Previous studies on the antibacterial effect of DSF showed that it exhibited narrow-spectrum antibiotic activity against *S. aureus* and other Gram-positive bacteria (Long, 2017). We believe that DSF has high affinity for thiol bearing substances and could react with them through a thiol-disulfide exchange reaction (Figure 3) altering their level in the cell. *S. aureus* usually has several low molecular weight thiols such as co-factors (e.g., co-enzyme A) and antioxidants (i.e., bacillithiol) which DSF might act on and inhibit. Thus, we will test the hypothesis that DSF antagonizes the metabolism (e.g., respiration) and increases oxidative stress in *S. aureus*.

Aim 2: Define the *in vitro* pharmacodynamic interactions of VAN and DSF in *S. aureus*. This aim focuses on the adjuvant use of DSF to increase VISA susceptibility to VAN. This research will evaluate our hypothesis that the addition of DSF lowers the MIC of VAN in

multiple VISA strains while enhancing the bactericidal capacity of VAN. Previous studies established that DSF lowered the MIC of VAN in VSSA, VISA, and VRSA strains in either an additive or synergistic manner (Sheppard et al., 2018). With this novel finding, we wanted to define the pharmacodynamic interaction of the combination using VISA strains from different genetic lineages. Thus, we will test the hypothesis that the VAN/DSF combination produces enhanced bactericidal effects and lowers MIC of VAN in VISA.

Chapter 2

Mechanistic Studies on Disulfiram in *Staphylococcus aureus*

Chapter Overview

This chapter focuses on testing potential mechanisms by which disulfiram (DSF) inhibits the growth of *Staphylococcus aureus*. We propose that thiol-reactive DSF and its metabolite diethyldithiocarbamate (DDTC) have multiple mechanisms to inhibit bacterial growth. First, it is believed DSF antagonism of redox buffering bacillithiol (BSH) contributes to the decreased growth. Secondly, DSF inhibition of co-enzyme A (CoASH) is thought to decelerate aerobic respiration and energy production. In this chapter, we describe the results of three biochemical assays to the hypothesis that DSF antagonizes the metabolism (e.g., respiration) and increases oxidative stress in *S. aureus*.

Introduction

S. aureus is a serious human pathogen that causes serious skin and soft tissue infections, leading to severe and life-threatening systemic diseases (Cong, Yang, & Rao, 2020). *S. aureus* contains virulence characteristics and the capacity to develop resistance to most antibiotics. The clinical use of methicillin has resulted in the emergence of methicillin-resistant *S. aureus* (MRSA) (Lakhundi & Zhang, 2018). Several investigations revealed that MRSA accounts for between 13 and 74% of all *S. aureus* infections globally (Kock et al., 2010). Compared to methicillin-sensitive *S. aureus* (MSSA), MRSA continues to be associated with worse clinical outcomes (van Hal et al., 2012).

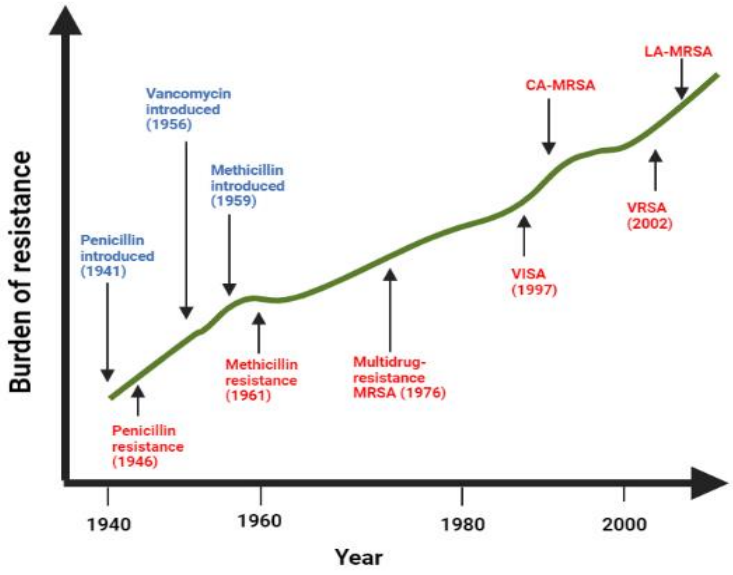
Antibiotics are an efficient way to control these infectious diseases, but excessive use causes multi-drug resistant strains to proliferate across the community. Global public health is at risk from MRSA infections, and antibiotic resistance is a major issue in treating these bacterial

infections. One of the first-line medications for treating MRSA infections remains to be vancomycin (VAN). In recent years, however, isolates of *S. aureus* strains intermediate and complete resistance have begun to appear increasing the need for alternative therapies (Davis, Van Hal, & Tong, 2015) (Figure 4). VAN-resistant *S. aureus* (VRSA) was initially noted in the United States in 2002 and defined as *S. aureus* isolates completely resistant to VAN (MIC ≥ 16 μg/ml), which are thought to have evolved from VAN-susceptible MRSA (Saheed & Rothman, 2016). Although treating VRSA infections can be difficult, there have only been a small number of human VRSA infections in the U.S. In contrast, vancomycin-intermediate resistant *S. aureus* (VISA) has a relatively large load, less clearly understood molecular mechanisms of resistance, and is linked to recurrent infections, ineffective antibiotic therapy, with poor clinical outcomes (McGuinness, Malachowa, & DeLeo, 2017).

VAN-susceptible *S. aureus* (VSSA), VISA, and VRSA continue to be public health threat despite efforts to lower hospital transmission rates. As a result, new treatments must be identified to combat these multidrug-resistant MRSA infections such as VISA (Lewis et al., 2022). One strategy is to develop combination antibiotic therapies that are more potent when given in tandem (i.e., (synergistic). A second approach is to repurpose existing medications that are found have anti-MRSA activity. We have found that DSF could be one such drug, showing antistaphylococcal and synergistic potential with VAN in MRSA (Frazier et al., 2019). Overall, this study focuses on defining the mechanisms by which DSF inhibits bacterial growth

Figure 4

Schematic Diagram Showing the Timeline for the Debut of Antibiotic Treatments and the Subsequent Appearance of S. aureus Resistant to Those Treatments.



Note. Image created using *BioRender.com,* adapted from (Marami et al., 2022).

Methods

ATP Assay

Several *S. aureus* strains (JE2, 25923, RN1) were used to evaluate the bacterial cell viability by measuring the intracellular ATP using Promega BacTiter-Glo™ Microbial Cell Viability Assay. An overnight culture of *S. aureus* was pelleted and resuspended in 1 ml saline. This culture was used to make a 0.5 McFarland standard (10^8 cfu/ml) in 1ml Cation-Adjusted Mueller-Hinton Broth (CAMHB) and was diluted to (10^7 cfu/ml). Aliquots (tubes) were prepared according to the Table 2 with treatments DSF and its metabolite DDTC, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and vehicle (DMSO) in replicates of three. We decided to test DSF and DDTC at 50µM (i.e., ~1 x MIC for DSF).Positive control CCCP, which depletes the membrane potential, causes an immediate drop in cellular ATP levels.

Table 2

Treatment Groups for ATP Assay

Tubes	Bacteria (µl)	5 mM DSF (µl)	5 mM DDTC (µl)	5 mM CCCP (µl)	DMSO (µl)	total vol. (µl)	Treatment
1 - 3	990	10			0	1000	50 µM DSF
4 - 6	990		10		0	1000	50 µM DDTC
7 - 9	990			5	5	1000	25 µM CCCP
10 - 12	990				10	1000	vehicle

After preparation, the tubes were incubated for 1 hr. at 37 ºC in a water-jacketed incubator. The BacTiter-Glo™ reagent was then prepared, and 50 µl was added to all wells in a black 96-well opaque luminescent plate. Afterwards, 50 µl of drug-treated bacteria were transferred from the tubes to the wells in opaque luminescence plate containing the reagent. The relative luminescence units (RLU) were then measured using a Molecular Devices FilterMax™ F3 Plate Reader. Statistical analyses by two-way analysis of variance (ANOVA) following a Tukey's multiple-comparison were performed by Prism 9.0.2 software (GraphPad Software, Inc.).

Similarly, Mu50 viability over time was measured using the Promega BacTiter-Glo™ Microbial Cell Viability Assay with slight modifications. A 10^5 cfu/ml early-log phase VISA Mu50 inoculum in CAMHB was incubated at 37 °C, 150 rpm with DSF (8 µg/ml), VAN (4 µg/ml), VAN/DSF (4/8), or an equal volume of vehicle (DMSO) in replicates of three. At different time points, 1 ml of the treated cultures was chilled on ice for 1 min, combined with Lysing Matrix B 0.1 mm spherical silica beads (MP Biomedicals), and homogenized using a BeadBug™ 6 (Benchmark Scientific) for 5 x 0.5 mins at 4350 rpm. We found that bead-beating

was a necessary step prior to reagent addition to due to its thicker cell wall. Samples of 40 µl were combined with 60 µl of assay reagent in a black 96-well plate and mixed for 1 min in the dark before measuring RLU using a Molecular Devices FilterMax™ F3 Plate Reader. Bacterial samples were standardized based on the ratio of RLU and protein obtained by combining 200 µl Pierce™ BCA Protein Assay reagent with 100 µl of lysate. Viability curves were plotted against time from the mean data of three replicates using Prism 9.0.2 software (GraphPad Software, Inc.)

Membrane Potential Assay

The membrane potential of the bacterial cells was evaluated using an Invitrogen™ BacLight™ Bacterial Membrane Potential Kit, which uses fluorescent dye, 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$). An overnight culture of *S. aureus* strain JE2 (1 ml) was pelleted and resuspended in membrane potential buffer MP buffer (60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 10 mM glucose) and a 6 ml suspension of OD_{600} ~0.3 AU/ml was prepared by using Genova Bio Jenway UV-Visible Spectrophotometer in the MP buffer. Afterwards, 5.9 ml of this suspension was added to 100 µl of 3 mM $DiOC_2(3)$. The mixture was then vortexed and incubated in the dark for 15 mins. In a 96-well opaque (black) plate 5 µl of DSF and DDTC at concentrations (0.5, 0.25, 0.125 mM) and 2, 3, 4 µl of 50 µM CCCP, or/and vehicle DMSO were added into the wells with three replicates of each treatment. Afterwards the label bacteria (95 µl) was added to the wells containing drugs and/or vehicle. The protonophore CCCP was used as a positive control because it eradicates the proton gradient, eliminating the bacterial membrane potential. The relative fluorescence units (RFU) readings were recorded at excitation/emission 485/535 (green) and 485/625 (red) using Molecular Devices FilterMax™ F3 Plate Reader. Graphs were

constructed based on the ratio of red and green RFUs. Statistical analysis was conducted by one-way (ANOVA) followed by a Tukey's multiple-comparison test using Prism 9.0.2 software.

Reactive Oxygen Species (ROS) Assay

To evaluate the reactive oxygen species (ROS) in a bacterial culture, we used Invitrogen™ cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye. An overnight culture of *S. aureus* strain JE2 was pelleted and resuspended in 1 ml saline; from that, a 2 ml of 0.5 McFarland (10^8) standard of JE2 was prepared in Hank's balanced salt solution (for 0.5 L HBSS, 0.14 M NaCl, 0.005 M KCl, 0.001 M $CaCl_2$, 0.0004 M $MgSO_4$ anhydrous, 0.0005 M $MgCl_2$•$6H_2O$, 0.0003 M $H_{15}Na_2O_{11}P$, 0.0004 M KH_2PO_4, 0.0004 M $NaHCO_3$). Afterwards, 5 µl of 1 mM H_2DCFDA was added to 1 ml of bacterial culture in HBSS, incubated in the dark with shaking for 30 min, and DCFA-labeled bacteria was further diluted to 10^7 cfu/ml in HBSS. A 96-well opaque (black) plate containing the appropriate concentration of drugs was prepared according to the steps from **Table 3.** The number of replicates for each treatment were six and as a positive control H_2O_2 was used. After adding the DCFDA-labelled bacterial culture, the samples were excited at 485 nm and the RFUs were recorded at 535 nm. Graphs were constructed based on RFUs, and statistical analysis by two-way (ANOVA) followed by a Tukey's multiple-comparison test using Prism 9.0.2 software.

Table 3

Procedure for ROS assay

Steps	DSF A-D (1-6)	DDTC A-D (7-12)	CCCP E-H (1-6)	H_2O_2 E-G (7-12)	DMSO H (7-12)
1. Addition of buffer	95 µl in A 50 µl from B-D	95 µl in A 50 µl from B-D	95 µl in E 50 µl from F-H	96 µl in E 50 µl from F-G	47.5 µl in H
2. Addition of drugs	5 µl 1 mM drug to row A	5 µl 1mM drug to row A	5 µl 1mM drug to row E	4 µl of 5 mM drug to row E	2.5 µl in H
3. Serial dilution	Serially dilute 50 µl from A-D and discarded	Serially dilute 50 µl from A-D and discarded	Serially dilute 50 µl from E-H and discarded	Serially dilute 50 µl from E-G and discarded	-
4. Addition of culture	50 µl of culture	50 µl of culture	50 µl of culture	50 µl of culture	50 µl of culture

Note. A-H, 1-12 represents rows, columns in a 96-well microtiter plate, respectively.

Results

ATP Assay

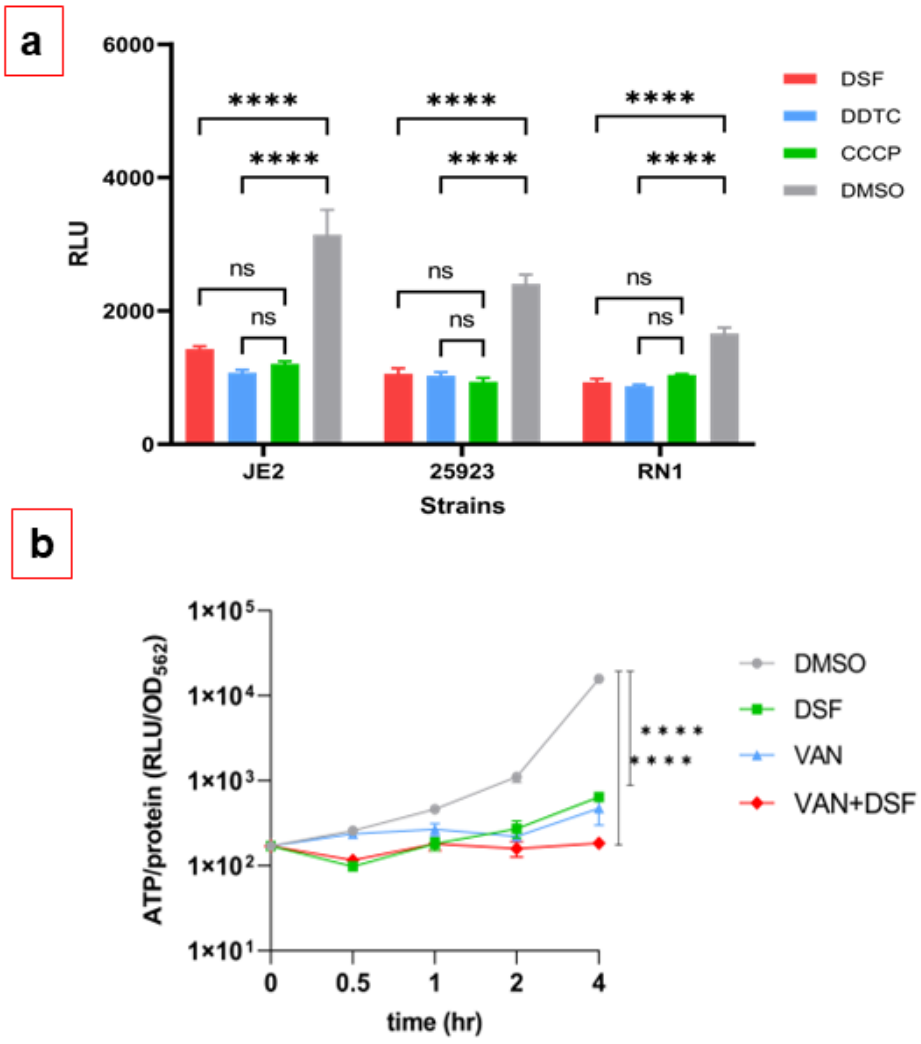
The ATP assay was conducted against different strains of *S. aureus* and revealed that the cells treated with DSF and DDTC showed a significant decrease in the intracellular ATP levels compared to the untreated. Two-way ANOVA indicated a statistically significant effect of drugs DSF, DDTC, and CCCP compared to untreated on the *S. aureus* cells irrespective of the strain ($p < 0.0001$) (Figure 5a). Tukey's post hoc analysis further indicated no significant difference between the positive control CCCP and DSF or DDTC in all *S. aureus* strains ($p > 0.05$).

In a study comparing intracellular ATP/protein levels of Mu50 as a viability measure vs time, there was an initial decrease till 0.5 hr. At the same time, there was a consistent increase in

the ATP levels with VAN. The combination of VAN/DSF had an initial decrease in the ATP levels but maintained constant over time and was the only treatment to suppress energy production over the initial 4 hr. period (Figure 5b).

Figure 5

ATP Assay Results



Note. **(a)** *S. aureus* strains (JE2, 25923, RN1) assessed with DSF, DDTC at 50 µM and CCCP (25 µM), and statistical significance depicted with asterisks (****p < 0.001). **(b)** Mu50

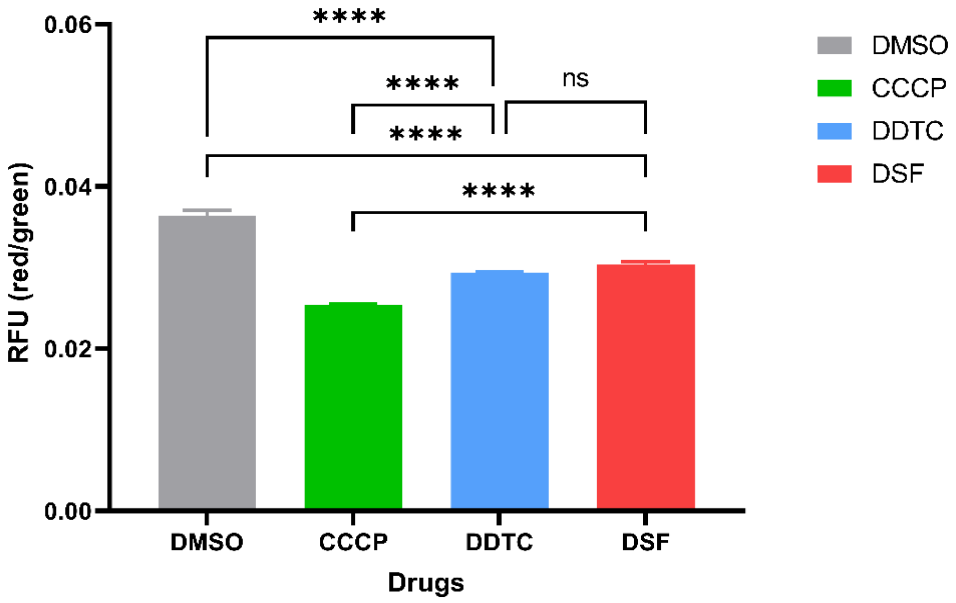
ATP/Protein plotted against time (hr.) with statistical significance with asterisks (****p < 0.001)

Membrane Potential Assay

The membrane potential across the bacterial cell membrane was assessed by comparing the ratio of the RFU of red and green. One-way ANOVA showed a statistically significant decrease in the membrane potential with the treatments DSF, DDTC, and CCCP compared to the untreated cells (DMSO) ($p < 0.0001$) (Figure 6). Tukey's multiple comparisons showed no significant difference between DSF and DDTC ($p > 0.05$). However, a significant difference was observed between CCCP, DSF ($p < 0.0001$), which shows that the decrease in the membrane potential was more significant with positive control CCCP when compared with the treatments.

Figure 6

Membrane Potential in S. aureus JE2



Note. S. aureus JE2 treated with DSF, DDTC at 6.25μM and CCCP (1 μM) statistical significance depicted with asterisks (****$p < 0.001$).

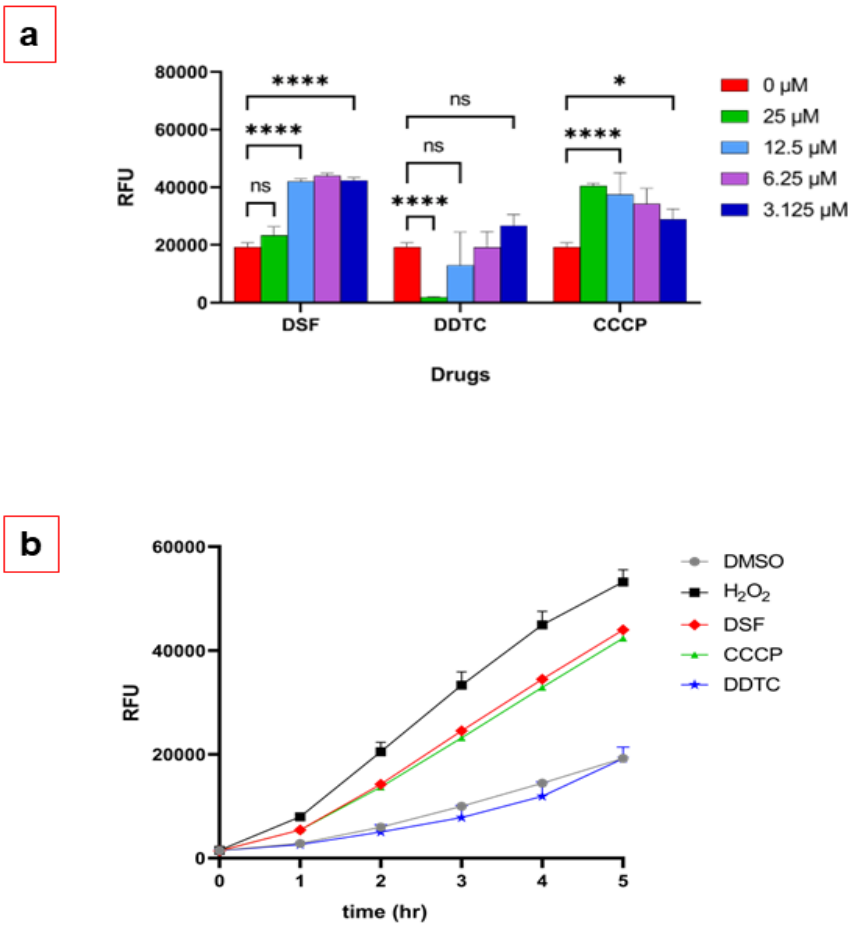
Reactive oxygen species (ROS) Assay

Figure 7.a depicts the ROS assay results after 5 hrs. for MRSA JE2 labeled with the oxidant-reactive dye H_2DCFDA and treated with 0 – 25 μM DSF, DDTC, and CCCP. The results showed increased ROS production when the cells were treated with DSF and CCCP up to 25 μM. Furthermore, Tukey's multiple comparisons showed a significant increase in the RFUs for DSF treatments of 12.5 μM, 6.25 μM, and 3.125 μM compared to untreated; however, surprisingly 25 μM DSF gave the lowest RFUs across all concentrations. The study also found the treatment with DDTC at 25 μM was the only concentration that showed a significant decrease in ROS levels. In contrast, no statistically significant variability was observed with other concentrations of DDTC when compared with untreated ($p > 0.05$).

We also examined the change in ROS level over time in MRSA JE2 treated with 6.25 μM of DSF, DDTC, and CCCP. H_2O_2 (100 μM) and CCCP were used as a positive control Figure 7.b shows that DSF, H_2O_2 and CCCP were the only treatments that exhibited increased ROS production compared to untreated control; however, cells treated with DDTC had similar ROS levels to the untreated control.

Figure 7

Reactive Oxygen Species Levels in S. aureus Strain JE2



Note. **(a)** Arbitrary fluorescence (RFU) for 5 hr. treatments of DSF, DDTC, and CCCP at different concentrations and statistical significance depicted with asterisks (ns = $p > 0.05$, *$p \leq 0.05$, ****$p < 0.000001$) **(b)** ROS assessed against time with DSF, DDTC, and CCCP at 6.25 µM concentration compared to 100 µM H_2O_2

Discussion

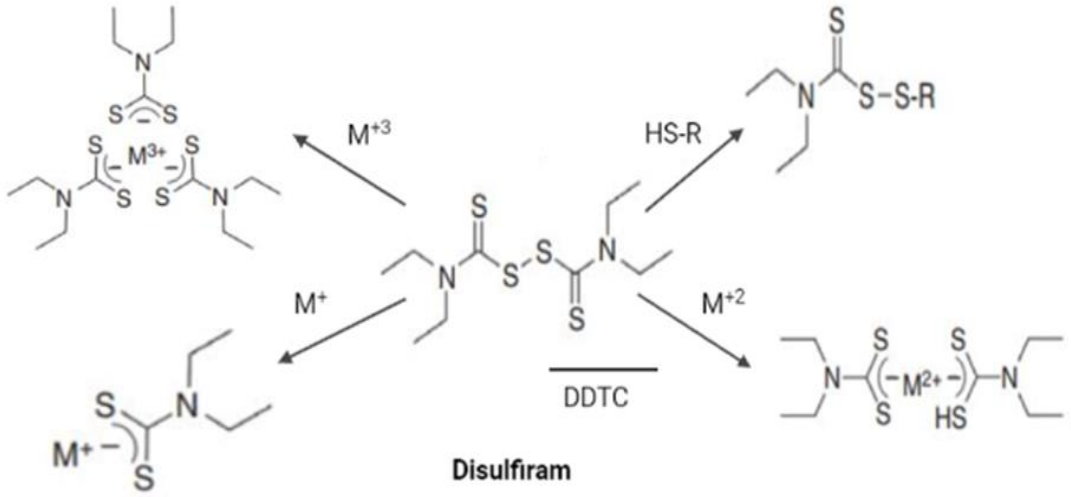
During cellular metabolism, host-pathogen interaction and antibiotic treatment, *S. aureus* is exposed to various redox active species, such as reactive oxygen, electrophile, nitrogen, chlorine, and sulfur species. These reactive species cause several post-translational thiol modifications in proteins, which could activate or deactivate specific transcription factors, further disturbing the redox balance of the cell and damaging cellular components, such as macromolecules, lipids, proteins, etc. (Loi, Rossius, & Antelmann, 2015; Linzner, Loi, Fritsch, & Antelmann, 2021). As a defense mechanism *S. aureus* employs low-molecular-weight thiol reductants such as CoASH and BSH to detoxify these reactive species and often contribute to resistance and virulence of the pathogen. CoASH is an important cofactor and a substrate for the biosynthesis and oxidation of pyruvate and fatty acids. These functions depend on the ability of CoASH to form thioester bonds as in CoAS-Ac. Previous studies showed that DSF is a potent oxidizer of protein sulfhydryl's (RSH) and oxidize thiols like glutathione (GSH) through mixed disulfide reaction (Stromme, 1963; Eneanya, Bianchine, Duran, & Andresen, 1981; Kitson, 1981) and this reaction was also observed with the cysteine residues (Cys) to inhibit aldehyde dehydrogenase (ALDH) (Shen et al., 2000; Johansson, 1992).

We think that DSF is readily cleaved by thiophilic CoASH through thiol-disulfide exchange reactions (Figure 8), resulting in the simultaneous addition and release of the chelating DDTC. This was indirectly attributed to the results from the ATP assay, which showed significantly decreased viability in all the *S. aureus* strains (JE,25923, RN1) evaluated with DSF, DDTC, and CCCP. DSF could alter the metabolism of *S. aureus* by decreasing the production of ATP by interacting with the CoASH, an essential cofactor required for the synthesis of ATP, as the bacterial cells require an acylated form of CoASH to enter into the

Krebs cycle/tricarboxylic acid TCA cycle which is the part of aerobic respiration producing nicotinamide adenine dinucleotide NADH (Long, 2017; Boylan et al., 2006; Potula et al., 2020). In the electron transport chain, NADH is involved in the synthesis of ATP through oxidative phosphorylation (Figure 9). It is proposed that DSF inhibition of CoASH causes decreased NADH affecting the electron transport chain and finally affecting the ATP production. Data from the membrane potential assay also revealed that CoASH could be a target for DSF, and a decrease in the membrane potential could be caused by reduced NADH synthesis.

Figure 8

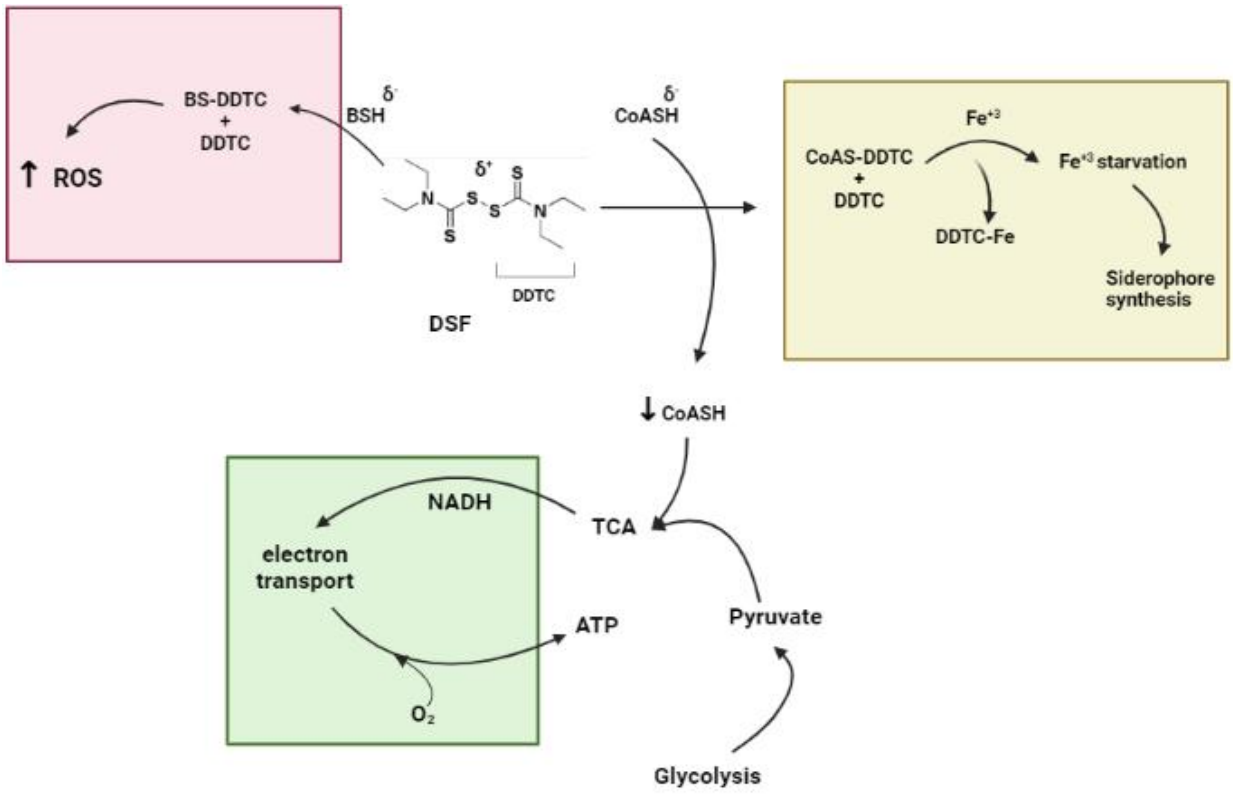
Thiol-Disulfide Exchange Reactions of DSF



Note. Figure depicts thiol-disulfide exchange reactions of DSF resulting in the formation of DDTC that have the ability to chelate metal ions (Frazier et al., 2019).

Figure 9

A Proposed Mechanism of DSF and DDTC Targeting CoASH and BSH



Note. Image created using *BioRender.com.*

S. aureus does not utilize the glutathione thiol/disulfide redox system employed by eukaryotes and many bacteria. Instead, this organism produces BSH as its major low molecular weight antioxidant thiol to help maintain the reducing environment of the cytoplasm and maintain redox homeostasis (Loi et al., 2015). Since its discovery in 2009, BSH has been a focus of attention because it contributes to resistance under oxidative stress and detoxification of electrophiles, such as the antibiotic fosfomycin (FOS), moreover, could be a novel drug target for therapeutic development in *S. aureus* (Perera, Newton, & Pogliano, 2015). A study

has shown decreased survival of *S. aureus* in blood lacking BSH (Posada et al., 2014) and increased sensitivity to FOS (Roberts et al., 2013) reinforcing that BSH might be a potential target against *S. aureus*. Previous studies revealed that DSF lowered MIC of FOS in an MRSA strain JE2 (Frazier et al., 2019) and increased bactericidal effects with the combination was observed, suggesting BSH could likely be a target. HPLC analyses from this study revealed a significant decrease in the cytosolic thiol levels of BSH and CYS with DSF, showing that it reacts with BSH, altering its levels intracellularly (Lewis et al., 2022). We hypothesized that DSF reacts with BSH (an antioxidant) leading the bacterial cells to oxidative stress. The results from the ROS assay demonstrated that DSF assessed at different concentrations evoked the production of ROS (Figure 7a) when tested at 6.25, 12.5, 3.125 µM; however, no statistical significance in ROS levels were observed at 25µM DSF (i.e., ~0.5 x MIC) compared to the vehicle which was attributed to complete cessation of metabolic activity in the cell. This was confirmed by a kinetic study that demonstrated increased ROS production over time tested at 6.25 µM DSF (i.e., ~0.125 x MIC) (Figure 7b) further validating our hypothesis.

Although disrupting the BSH and CoASH systems could play a vital role in the *S. aureus* growth inhibition, the distinct structure activity relationship of the DSF, and its analogs suggest the involvement of other mechanisms. One of them could be the sequestering of metal cofactors (e.g., Fe^{3+}) by its metabolite, DDTC, leading to decreased iron availability for the cell (Figure 9). DSF has a high affinity for metals and could form metal-DDTC complexes. Iron is an essential nutrient for all bacteria and has a prominent role in bacterial pathogenesis. In response to low iron levels *in vivo*, bacteria produce siderophores, low molecular weight iron chelators with a high affinity for iron uptake (Courcol, Trivier, Bissinger, Martin, & Brown,

1997). Previous studies revealed that DSF could chelate metals, forming stable complexes (e.g., copper, iron, zinc) (Viola-Rhenals et al., 2018).

To conclude, this *in vitro* study determined that DSF and DDTC significantly impact energy production and exert oxidative stress on *S. aureus,* attributing to its antibacterial activity. As part of future directions, we will test the hypothesis that DSF and/or its metabolite reacts with Fe^{+3} leading to decreased iron, stressing the bacterial cells to increase the production of siderophores (Figure 9). Overall, though this research established probable target pathways of DSF that contribute to the mechanism of antibacterial action in *S. aureus* and further studies are needed to corroborate these findings.

Chapter 3

In Vitro Antimicrobial Pharmacodynamics of Vancomycin and Disulfiram in *Staphylococcus aureus*

Chapter overview

This chapter focuses on the use of disulfiram (DSF) to enhance the killing effect of vancomycin (VAN) against methicillin-resistant *Staphylococcus aureus* (MRSA) infections. As a prospective intervention, this study examined the *in vitro* antimicrobial pharmacodynamic interaction between DSF and VAN. The standard treatment for systemic infections caused by MRSA is intravenous VAN. For isolates with an intermediate level of VAN resistance, it is more challenging to accomplish the pharmacokinetic/pharmacodynamic target indices for VAN therapy. Overall, this investigation showed that DSF either additively or synergistically decreased the minimum inhibitory and bactericidal concentrations of VAN. The VAN intermediate-resistant strain (VISA) Mu50 was successfully treated with a 4/8 μg/ml VAN/DSF combination and provided the best bactericidal effects and growth inhibition, but not with either antibiotic alone. The enhanced killing effect at the cellular level was confirmed with flow cytometry, moreover, revealing that DSF may increase susceptibility to the bactericidal effects of VAN by counteracting the muropeptide fortification mechanism in VISA isolates.

Introduction

Methicillin resistance is defined as the strains of *S. aureus* that are nonsusceptible to penicillins such as methicillin, oxacillin and flucloxacillin and are cross-resistant to most other β-lactam classes. The methicillin resistance is caused by the *mecA* or *mecC* gene located on the staphylococcal cassette that encode penicillin-binding protein 2a (PBP2a), a mutated transpeptidase that is able to form peptidoglycan crosslinks of the bacterial cell wall even in the

presence of penicillins (Loomba, Taneja, & Mishra, 2010). Until today, VAN has been the drug of choice to treat serious infections caused by MRSA, penicillin-resistant pneumococci, and other Gram-positive bacteria (Bruniera et al., 2015). Recently, infections caused by MRSA with reduced susceptibility to VAN have emerged and these isolates are classified into three groups by the Clinical and Laboratory Standards Institute (CLSI): VAN-susceptible *S. aureus* (VSSA) with MIC ≤ 2 μg/ml; VAN-intermediate *S. aureus* (VISA) with MIC of 4–8 μg/ml; and VAN-resistant *S. aureus* (VRSA) with MIC ≥ 16 μg/ml) (Cong et al., 2020).

The VISA variant is believed to be initiated from heterogeneous VAN-intermediate *S. aureus* (hVISA), which is defined as an *S. aureus* strain with a VAN MIC within the susceptible range (≤2 μg/ml). The molecular mechanisms underlying VISA development are incompletely defined; however, it is generally accepted that VISA is a result of the gradual accumulation of the VISA-associated mutated genes. Of particular importance are the genes encoding two-component regulatory systems, such as *WalKR* (Peng et al., 2017; J. Hu, Zhang, Liu, Chen, & Sun, 2015), *GraSR* (Yoo et al., 2013), and *VraSR* (Q. Hu, Peng, & Rao, 2016). Even though their genetic lineages varied, VISA isolates generally display common phenotypes such as thickened cell wall, less crosslinked peptidoglycan, reduced autolytic activity, and decreased virulence. VRSA strains also have been found to have thicker cell walls than the VAN-sensitive strains (Daum, Gupta, Sabbagh, & Milewski, 1992), however with VISA strains, there is also increased peptidoglycan synthesis. Exchange of genetic material is yet another mechanism postulated for VRSA and is thought to be mediated by the *VanA* operon carried by the transposon Tn1546 acquired from the VAN-resistant *Enterococcus faecalis* (VRE) (Loomba et al., 2010; Cong et al., 2020).

The discovery, routine administration, and universalization of antibiotic use to treat infections have transformed modern medicine and reshaped the therapeutic model. Unfortunately, the overuse of antibiotics has resulted in the escalation of antibiotic resistant infections that the Center for Diseases Control and Prevention (CDC) attributes to an estimated 23,000 fatalities each year in the United States (Munita & Arias, 2016). To screen for resistance, antibacterial test results are important to confirm the susceptibility of the clinical isolates to avoid treatment failure (Jorgensen & Ferraro, 2009). In general, susceptibility testing uses standardized breakpoints to classify isolates as susceptible, intermediate resistant, or resistant. Minimum inhibitory concentrations (MICs) are fundamental units for setting breakpoints; however, zone diameter values are sometimes used when breakpoint MICs have not been established (Turnidge & Paterson, 2007). Clinicians depend on pharmacokinetic (PK) and pharmacodynamic (PD) data to administer intravenous antibiotics for systemic infections (e.g., bacteremia) in pharmacotherapy. Such infections might need to be treated with two antibiotics that, when combined, can achieve the PK/PD target indices (e.g., AUC/MIC) recommended by clinical practice guidelines (Levison & Levison, 2009).

The Clinical guidelines recommend an AUC/MIC ≥ 400 as the targeted PK/PD parameter for patients with severe MRSA infections. This AUC/MIC ratio is associated with serum trough concentrations of > 15 μg/ml and attainable with VAN doses of 15-20 mg/kg administered every 8 to 12 hrs. as an intravenous (IV) infusion for MRSA infections that are VAN susceptible (assuming a VAN MIC of ≤ 1 μg/ml) to achieve clinical efficacy and improve patient safety (Baddour et al., 2015; Rybak et al., 2020). The probability of achieving the recommended AUC/MIC target is low when the MIC for VAN exceeds 1 μg/ml, therefore, alternative treatment strategies are necessary to achieve these individual targets (Rybak et al.,

2020). Synergistic combinations are utilized in therapy because they have benefits over monotherapeutic regimens, including broadening the spectrum of antibiotics and reducing the emergence of resistance (Zhu, Tse, Weller, Chen, & Blainey, 2021). A recent drug library screening revealed DSF as a possible antibiotic adjuvant in the pursuit for agents that can lower the MIC of VAN in multidrug-resistant *Staphylococcus* (Moore et al., 2020). Additionally, preliminary isobologram analyses using *S. aureus* revealed cumulative fractional inhibitory concentrations (FICs) for VAN/DSF co-treatments of 0.5 to 1, indicating additive to synergistic interactions and were strain dependent (Moore et al., 2020; Long, 2017). Recent research has also revealed that VAN and diethyldithiocarbamate (DDTC), the major metabolite of DSF, had significant synergistic effects against different MRSA strains (Frazier et al., 2019). Similar results were observed with DDTC and VAN in the presence of Cu^{2+} against MRSA biofilms (Kaul et al., 2022) and other infectious agents (Kaul, Suss, Zannettino, & Richter, 2021). Moreover, DSF and VAN, individually or in combination did not exhibit cytotoxicity against a eukaryotic cell line (Serafin et al., 2022). Considering these findings, a more thorough analysis of the antimicrobial PD interactions of VAN/DSF in *S. aureus* was required. Here, the *in vitro* studies accompanying this chapter focus on strains with intermediate levels of VAN resistance and illustrate how VAN/DSF affects MRSA growth and survival over time.

Methods

Synergism studies

Synergistic interaction between DSF and VAN was evaluated using the microdilution checkerboard assay **(**Moody J.A. (1992)**.** A 0.5 McFarland standard prepared in saline from an overnight agar culture was diluted to 10^5 cfu/ml in cation-adjusted Mueller-Hinton broth (CAMHB). A transparent 96-well microtiter plate was prepared with two-fold serial dilutions of

DSF (range 1 – 16 µg/ml) and VAN (range 0.125 – 32 µg/ml). Afterwards, an equal volume of culture was added, and the plate was incubated in a water-jacketed incubator at 37 °C. After 48 hrs. MICs were recorded for the treatments that had complete visual growth inhibition. To determine the minimum bactericidal concentrations (MBCs), 4 µl samples from each well were applied to Mueller-Hinton agar (MHA) and the plates were incubated for 48 hrs. to identify the concentrations that conferred complete growth inhibition. The ratio of fractional inhibitory and fractional bactericidal concentrations (FIC/FBC) was calculated by dividing the MIC or MBC of VAN/DSF treatment by the MIC or MBC of individual treatments. The lowest summative calculated from the FIC/FBC values were interpreted according to indices: synergism ≤ 0.5 (++); additive 0.5< to 1 (+); indifferent 1< to 4 (±); antagonism 4 < (–) (Doern, 2014)**.**

Growth Studies

Time-kill experiments were performed by measuring the colony forming units (cfu) after 24 hrs. incubation. These experiments were conducted in 1 ml of CAMHB, in Gibco Dulbecco's Modified Eagle Medium (DMEM) or/and 5% fetal bovine serum (FBS) supplement or Hank's balanced salt solution (HBSS) supplemented with 1% glucose. An overnight culture of *S. aureus* was used to prepare an early log-phase inoculum by diluting it with 9 ml fresh broth and growing it to an OD_{600} of 0.6-0.8. From that, a 0.5 McFarland standard (approximately 10^8 cfu/ml) was prepared in saline and later diluted in fresh broth or media. Afterwards 1 ml of cultures treated with an antibiotic(s), or an equal volume of vehicle (DMSO) were incubated with shaking (150 rpm) at 37 °C. At each time point, ten-fold serial dilutions of samples were spread on MHA. The plates were incubated at 37 °C for 24 hrs. and the number of cfu/ml was determined. Treatments were characterized as bactericidal if there was ≥ 3 $\log_{10}$ cfu/ml reduction (i.e., ≥99.9%) and bacteriostatic if the decrease was 0 to <3 $\log_{10}$ cfu/ml compared to

the initial inoculum ((Basri & Khairon, 2012)). Synergism was further defined as a decrease of ≥ 2 $\log_{10}$ cfu/ml for VAN/DSF when compared to most active agent alone (Pankuch & Appelbaum, 2006). Additive and indifferent effects were similarly defined as reductions of 1 to 2 $\log_{10}$ cfu/ml and 0 to < 1 $\log_{10}$ cfu/ml, respectively.

S. aureus strain Mu50 growth over time was evaluated based on turbidity measurements. An overnight Mu50 culture was prepared in 1 ml of broth. From that, a 0.5 McFarland standard (approximately 10^8 cfu/ml) was prepared in saline and later diluted in fresh broth to prepare an inoculum size of approximately 5.5 x 10^5 cfu/ml. In a clear flat bottom, 96-well microtiter plate, 100 µl of drugs [DSF 8 µg/ml, VAN 4 µg/ml and VAN/DSF (4/8)] or vehicle (DMSO) cultures were dispensed and incubated at 37 °C for 48 hrs. Optical density (OD) readings were recorded on a Molecular Devices SpectraMax® 384 plate reader at 600 nm following 5-sec agitation.

The growth over time was also assessed based on the cfu/ml counts. The same cultures from the turbidity measurement were used to monitor colony growth. 100 µl of samples at time points 0, 2, 4, 8, 24, and 48 hrs. were taken, and 20 µl was serially diluted in a microtiter plate containing 180 µl sterile saline. Afterwards, 10 µl was added from the wells in microtiter plate to the MHA agar plate starting from the highest dilution to the lowest dilution. With a sterile loop, the samples were spread on the agar from highest to lowest dilution, and colonies were counted after 48 hrs. incubation. Growth curves were plotted against time (hr.) from the mean OD_{600} and cfu values using Prism 9.0.2 software (GraphPad Software, Inc.).

Flow Cytometry

An overnight Mu50 culture was prepared in 1 ml of broth. From that, a 0.5 McFarland standard (approximately 10^8 cfu/ml) was prepared in saline and diluted in fresh CAMHB to prepare an inoculum size of approximately 5.5 x 10^5 cfu/ml. The CAMHB cultures were then

treated with either DSF (8 or 16 µg/ml), VAN (2 or 4 µg/ml), DSF+VAN (8+4 or 16+2 µg/ml), or DMSO (null). Following incubation (37 °C, 22 hr., 150 rpm), samples of 100 µl were combined with 100 µl PBS and stained with 1 µl of 1.5 mM propidium iodide (PI, Thermo Scientific™) and 0.5 mM SYTOX Green (Invitrogen™). For positive controls of >99.9% permeated cells, 5 µl of 10 mM cetyltrimethylammonium bromide (CTAB) was added to a Mu50 null sample. The bacteria were incubated in the dark for 15 mins and analyzed on an ACEA Novocyte 2000R flow cytometer equipped with a 488 nm excitation laser and 530/30 (green) and 675/30 (red) emission filters for detection of SYTOX Green and PI, respectively. Forward scatter (FSC), and side scatter (SSC) plot comparison of unstained cells was used to calibrate the acquisition gate. For each sample, ca. 20,000 events were collected and plotted using NovoExpress 1.3.0 software (Agilent Technologies, Inc.).

The same method was applied for detecting VAN binding sites using 0.1 µg/ml VAN BODIPY™ FL Conjugate (Invitrogen™) which is a green-fluorescent analog of VAN in place of SYTOX green. The Mu50 cultures were incubated for 22 and 44 hrs., with vehicle (DMSO), treatments (DSF at 1 x MIC and/or VAN at 0.5 x MIC) and stained with (VAN-BDP FL). The mean fluorescence intensity (MFI) was measured via fluorescein isothiocyanate (FITC) channel and graphs with statistical analysis were conducted by two-way analysis of variance (ANOVA), followed by a Tukey's multiple-comparison test using Prism 9.0.2 software.

Post-Antibiotic Effect

The post-antibiotic effect (PAE) was determined by cfu counts by the drop plate method on MHA (Herigstad, Hamilton, & Heersink, 2001). An overnight culture of *S. aureus* was prepared in MHB. 1 ml of this overnight culture was diluted with 9 ml (1:10) of CAMHB and incubated till the OD_{600} reading reached 0.7. A 5 ml of 0.5 McFarland standard was prepared

and serially diluted in 45 ml broth to prepare approximately 10^6 cfu/ml early log-phase inoculum, which was treated with different concentrations of antibiotic(s) and incubated with shaking (150 rpm at 37 °C) (Pankuch & Appelbaum, 2006). After 2 hrs., the cultures were diluted 1:1,000 and incubated at 37 °C. Using the drop plate method by applying 5 µL on MHA in duplicate, bacterial viability was then assessed over time from the initial time point (T_0). Following 24 hrs. incubation, the time required to increase the cfu counts by 1 $\log_{10}$ from the initial time point (T_0) was determined. The PAE was then calculated as *T-C,* where *T* is the difference in time for a 1 $\log_{10}$ increase in cfu from T_0 and *C* is the corresponding time for the untreated control.

Results

Synergistic Studies

The MIC and MBC values for optimal VAN/DSF combinations based on the lowest ΣFIC and ΣFBC calculated from isobologram (checkerboard) analyses (Moody, 2004) are presented in Table 4. The results from the VAN/DSF synergistic studies demonstrated a 50% reduction of VAN MICs in VSSA strains JE2 and COL with an additive effect (ΣFIC range of 0.53-0.75). Similarly, the other six VISA strains (MIC VAN 8 µg/ml) demonstrated synergistic (VISA AR-217,218,220) and additive effect (VISA Mu50, VISA AR-216, AR-219) with ΣFICs of 0.5 to 0.75. Furthermore, the addition of 2-4 µg/ml of DSF lowered the MIC of VAN in 50 percent of the VISA strains to an MIC level observed in VSSA strains. Moreover, the VAN/DSF combination effect was prominent in the VISA Mu50 as well as VAN resistant *S. aureus* (VRSA-MI) isolate (MIC for VAN; 1,024 µg/ml) (Finks et al., 2009) where the addition of 4 µg/ml DSF reduced the MIC of VAN to the VSSA breakpoint value of 2 µg/ml, which was a significant finding in this study.

Table 4

Comparison of MIC and MBC Values for VAN and/or DSF Treatments

MRSA strain	MIC (µg/ml)			MBC (µg/ml)		
	VAN	DSF	VAN/DSF[a, b]	VAN	DSF	VAN/DSF[a, b]
VSSA JE2	1	16	0.5/4 (+)	1	>16	0.5/4 (+)
VSSA COL	2	16	1/0.5 (+)	2	16	1/0.5 (+)
hVISA Mu3	2	16	1/0.5 (+)	2	16	1/0.5 (+)
VISA Mu50	8	8	2/4 (+)	8	16	8/8 (±)
VISA AR-216	8	8	2/4 (+)	8	16	4/1 (+)
VISA AR-217	8	8	4/2 (++)	16	16	4/2 (++)
VISA AR-218	8	8	2/2 (++)	8	>16	0.5/4 (+)
VISA AR-219	8	8	4/2 (+)	8	>16	2/8 (++)
VISA AR-220	8	8	4/2 (++)	16	16	4/2 (++)
VRSA-MI	>32	16	2/4 (++)	>32	>16	1/16 (++)

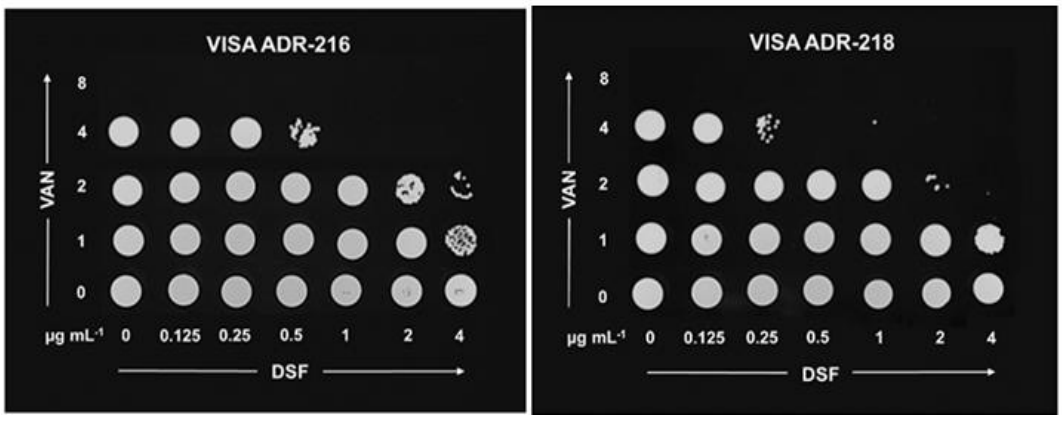
Note. [a] the lowest concentrations in combination

[b] synergism (++); additive (+); indifferent (±); antagonism (–)

The inclusion of DSF also resulted in a decrease in the MBC of VAN in five of the six VISA strains, including VRSA-MI, reference strains JE2 (CA-MRSA), COL (HA-MRSA) as well as the heterogeneous VISA Mu3 (hVISA). Table 4 demonstrates that DSF did not suppress the bactericidal effects of VAN. Moreover, Figure 10 shows the increased bactericidal activity with VAN/DSF combination in VISA AR-216,218 with ΣFBC's 0.5-0.75

Figure 10

DSF Lowers the MBC of VAN in VISA



Growth Studies

Table 5 represents the influence of treatments DSF, VAN, VAN/DSF on cfu counts after 24 hr. on an MRSA panel that included four VISA strains with a VAN MIC of 8 µg/ml. The VISA Mu50 was used a reference strain to evaluate various treatment combinations, inoculum sizes, and culture media. In CAMHB, VAN/DSF doses of 4/8 (Figure 11) and 2/16 µg/ml produced bactericidal effects (i.e., 99.9% kill) with synergy. The treatments with 4/4 (99.1%), 2/8 (89.7%) µg/ml also decreased Mu50 cfu counts with a bacteriostatic effect in an additive manner. A similar effect [bacteriostatic (59.1%)] was observed when the inoculum size was increased from 10^5 to 10^7 cfu/ml with VAN/DSF 4/8 µg/ml combination.

Table 5

Effects of VAN, DSF, and Combined Treatments on cfu/ml Counts After 24 hrs.

MRSA strain	medium	inoculum[a]	µg/ml		cfu/ml (24 hr)[a]			interaction[b]
			VAN	DSF	VAN	DSF	VAN/DSF	
VISA Mu50	CAMHB	$2.5 \pm 2.1 \times 10^5$	1	8	$>10^9$	$>10^9$	$1.5 \pm 0.7 \times 10^6$	—
	CAMHB	$4.6 \pm 1.9 \times 10^5$	2	8	$>10^9$	$>10^9$	$4.7 \pm 6 \times 10^4$	bacteriostatic (+)
	CAMHB	$4.5 \pm 0.4 \times 10^5$	2	16	$>10^9$	2×10^5	3.0×10^2	bactericidal (++)
	CAMHB	$3.0 \pm 1.4 \times 10^5$	4	4	2.0×10^5	$>10^9$	2.5×10^3	bacteriostatic (++)
	CAMHB	$5.5 \pm 2.1 \times 10^5$	4	8	$2.5 \pm 2.1 \times 10^8$	$>10^9$	$6.5 \pm 0.7 \times 10^2$	bactericidal (++)
	CAMHB	$1.5 \pm 0.7 \times 10^6$	4	8	$>10^9$	$>10^9$	$6.5 \pm 4.9 \times 10^5$	bacteriostatic (±)
	CAMHB	$1.1 \pm 0.21 \times 10^7$	4	8	$>10^9$	$>10^9$	$4.5 \pm 3.5 \times 10^6$	bacteriostatic (±)
	CAMHB+FBS	$5.5 \pm 6.3 \times 10^5$	4	8	$>10^9$	$>10^9$	$3.0 \pm 1.4 \times 10^2$	bactericidal (++)
	HBSS	$2.1 \pm 1.2 \times 10^5$	4	8	$2.0 \pm 1.4 \times 10^4$	$1.5 \pm 0.7 \times 10^3$	4.0×10^2	bactericidal (++)
VISA ADR-217	CAMHB	$2.5 \pm 0.7 \times 10^5$	4	8	$>10^9$	$>10^9$	$6.5 \pm 2.1 \times 10^5$	—
VISA ADR-219	CAMHB	$2.0 \pm 1.3 \times 10^5$	4	8	$1.3 \pm 0.9 \times 10^8$	$6.0 \pm 4.2 \times 10^7$	2.0×10^4	bacteriostatic (+)
VISA ADR-220	CAMHB	$3.0 \pm 1.4 \times 10^5$	4	8	$9.0 \pm 5.6 \times 10^7$	$2.5 \pm 0.7 \times 10^6$	1.0×10^3	bacteriostatic (+)
VRSA-MI	CAMHB	$1.8 \pm 2.1 \times 10^5$	2	8	$>10^9$	$>10^9$	$3.5 \pm 0.7 \times 10^5$	—
	CAMHB	$1.8 \pm 2.1 \times 10^5$	4	4	$>10^9$	$>10^9$	$>10^9$	—
	CAMHB	$4.5 \pm 0.7 \times 10^5$	4	8	$>10^9$	$>10^9$	$1.5 \pm 2 \times 10^5$	bacteriostatic (±)
hVISA Mu3	CAMHB	$2.5 \pm 0.7 \times 10^5$	1	8	$>10^9$	$>10^9$	$2.5 \pm 2.1 \times 10^4$	bacteriostatic (+)
VSSA COL	CAMHB	$3.0 \pm 1.4 \times 10^5$	1	8	$>10^9$	$>10^9$	7.0×10^8	—
	CAMHB	$2.5 \pm 0.7 \times 10^5$	1	16	$>10^9$	$>10^9$	1.0×10^2	bactericidal (++)

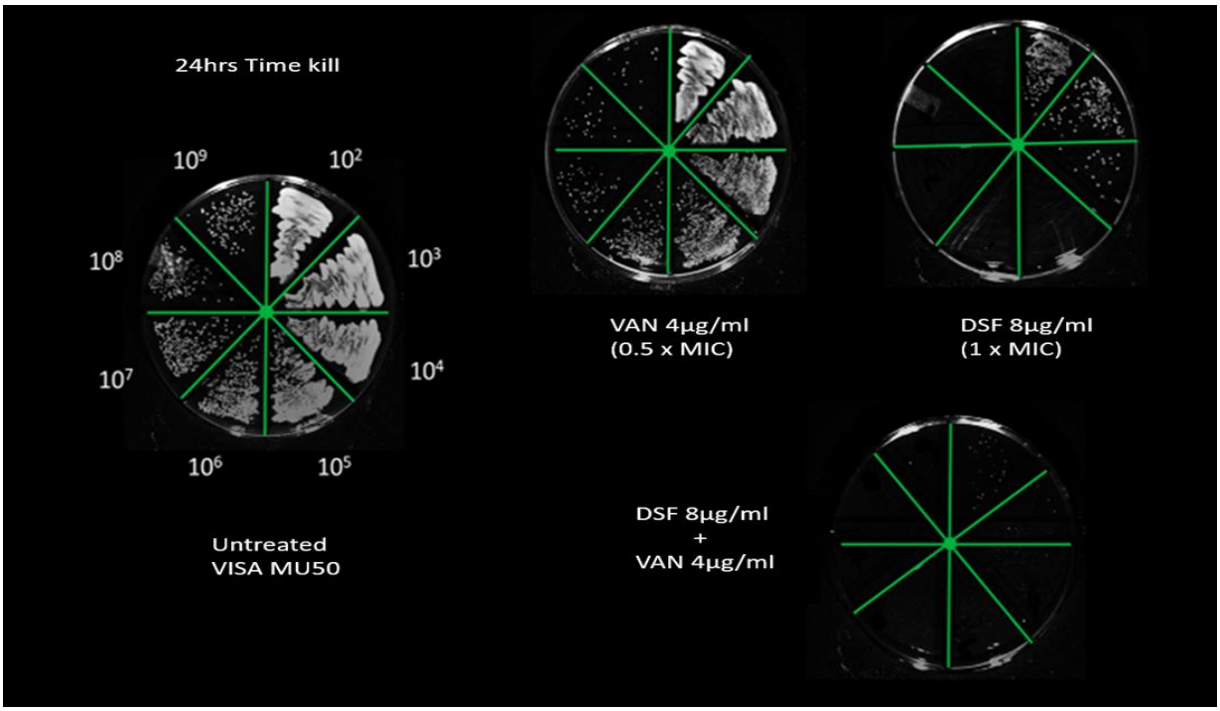
Note. [a] mean of two replicates from separate experiments

[b] synergism (++), additive (+), and indifferent (±) (Moody, 1992)

Significant bactericidal activity (99.8%) was noted against non-dividing Mu50 cells in HBSS buffer medium similar to the 4/8 µg/ml performance in CAMHB. Furthermore, adding a 5% FBS supplement to CAMHB conferred no antagonistic effects. The VAN/DSF (4/8 µg/ml) combination was effective against VRSA-MI and other VISA mostly in a bacteriostatic manner (Table 5). Likewise, the hVISA Mu3 strain also demonstrated bacteriostatic inhibition but with the 1/8 µg/ml combination.

Figure 11

Increased Bactericidal Activity With DSF and VAN Combination



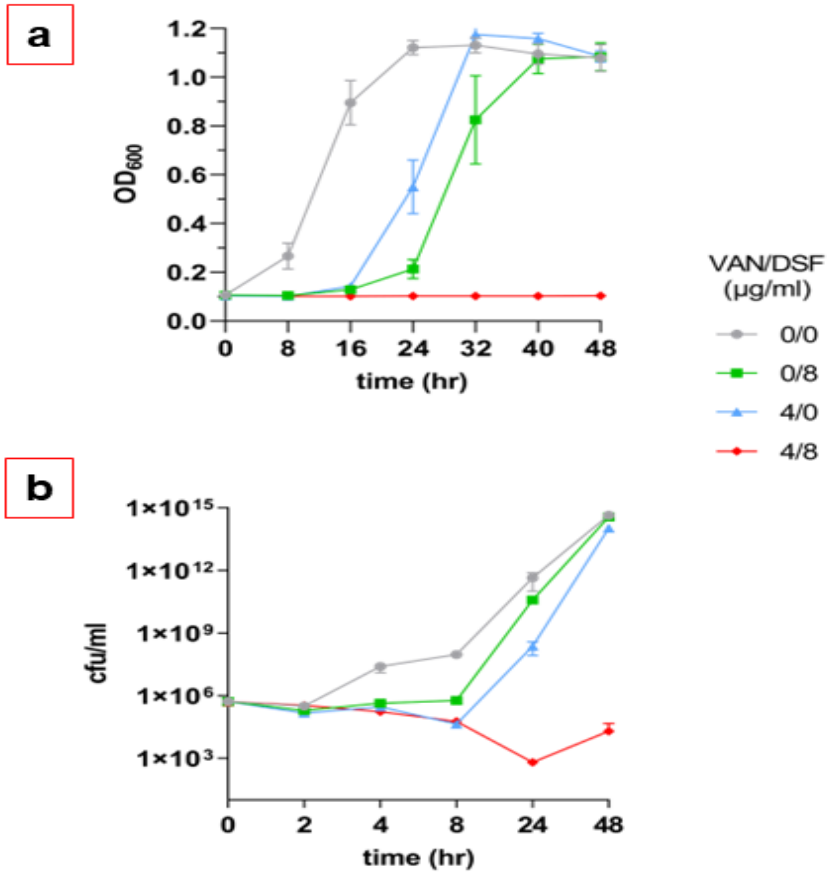
Note. Figure depicts enhanced bactericidal activity with DSF (1 x MIC) and VAN (0.5 x MIC) compared to individual treatments DSF, VAN, and DMSO.

The results from Mu50 growth vs time studies, as seen in Figure 12a, demonstrated that DSF decreased the MIC of VAN and continued to inhibit optical growth for up to 48 hrs. These results were confirmed with the time-kill curve (Figure 12b), which indicated that the

VAN/DSF combination exhibited increased bactericidal effects within 4 hrs. of treatment and continued over the course of 24 hrs. when compared to individual treatments alone. Figure 13 reaffirmed the enhanced bactericidal activity with 1 x MIC of DSF and 0.5 x MIC of VAN treatment on agar following 3-4 hr. exposure. Additionally, Figure 13 also indicated that DSF manifested a concentration-dependent killing effect on the Mu50 strain as evident by the decreased abundance of growth from 1 x to 8 x MIC at the 10-hr. time point.

Figure 12

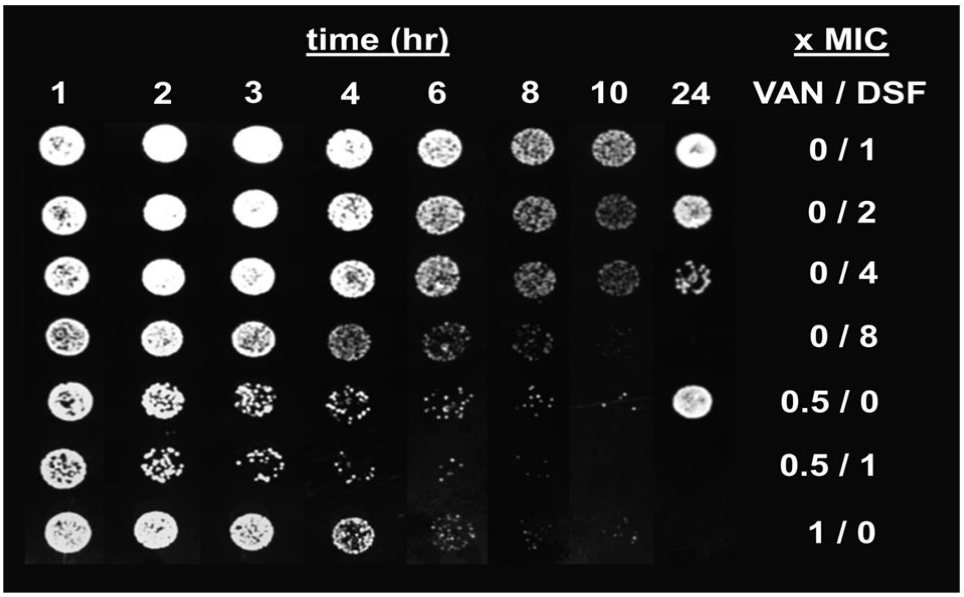
Effects of VAN and DSF on VISA Mu50 Growth and Viability Over Time



Note. Mu50 growth and viability over time measured by **(a)** optical density and **(b)** colony forming unit (cfu) measurements.

Figure 13

DSF Showing Concentration Dependent Antibacterial Activity and Increased Killing Effects With the Addition of DSF and 0.5 x MIC VAN



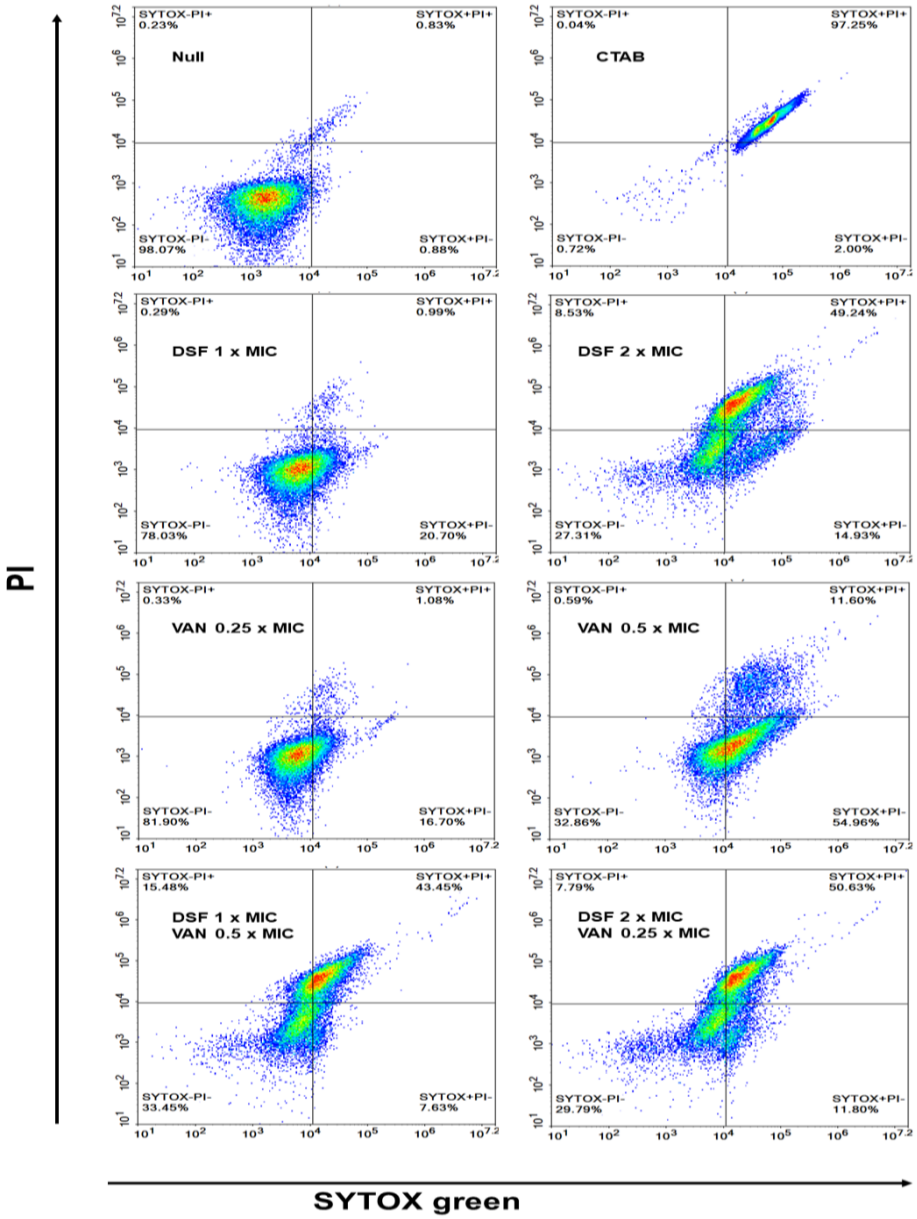
Flow cytometry studies

Flow cytometry with a double stain [SYTOX (green) and PI (red)] procedure was used to evaluate the antimicrobial PD of the VAN and DSF treatments on the VISA Mu50 at a cellular level. This technique (Roth, Poot, Yue, & Millard, 1997) involves two nucleic acid dyes, which stain the nonviable bacteria based on the extent they penetrate the cell membrane. The DMSO and CTAB treated were used as reference plots to distinguish the viable untreated (null) and non-viable SYTOX+/PI+ (Figure 14 top left, right), respectively. Concentrations of DSF at 1 x MIC and VAN at 0.25,0.5 x MIC showed a moderate increase in the SYTOX – labeled cells; however, exposure to 2 x MIC DSF demonstrated increased density of non-

viable cells. Figures 14, 15 also showed the increased double stained (SYTOX+/PI+) cells, reassuring the enhanced bactericidal activity and the potency of the combination.

Figure 14

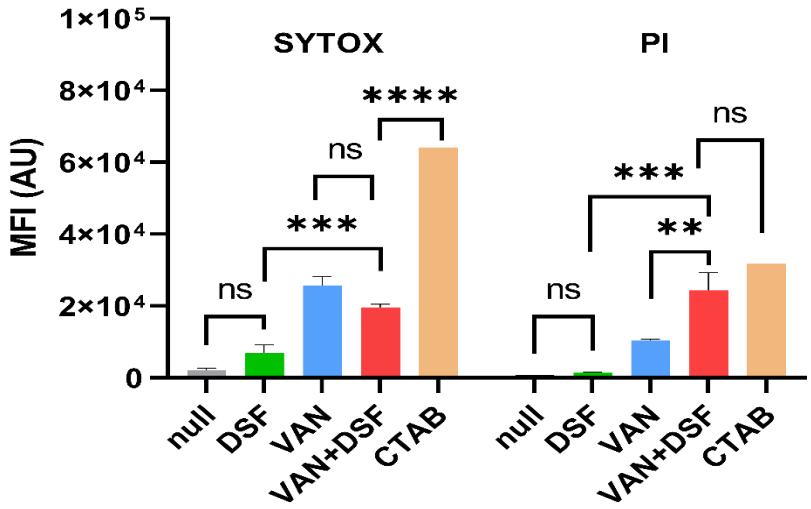
Analysis of VISA Mu50 Viability by Flow Cytometry



Note. Flow cytometry analysis of VISA Mu50 after overnight treatment with DSF, VAN, and VAN+DSF. The CTAB and DMSO treated cells permeable to SYTOX Green, and PI were used to reference nonviable bacteria (top right) and viable cells (top left), respectively.

Figure 15

Mean Fluorescence Intensity (MFI) of VISA Mu50 by Flow Cytometry



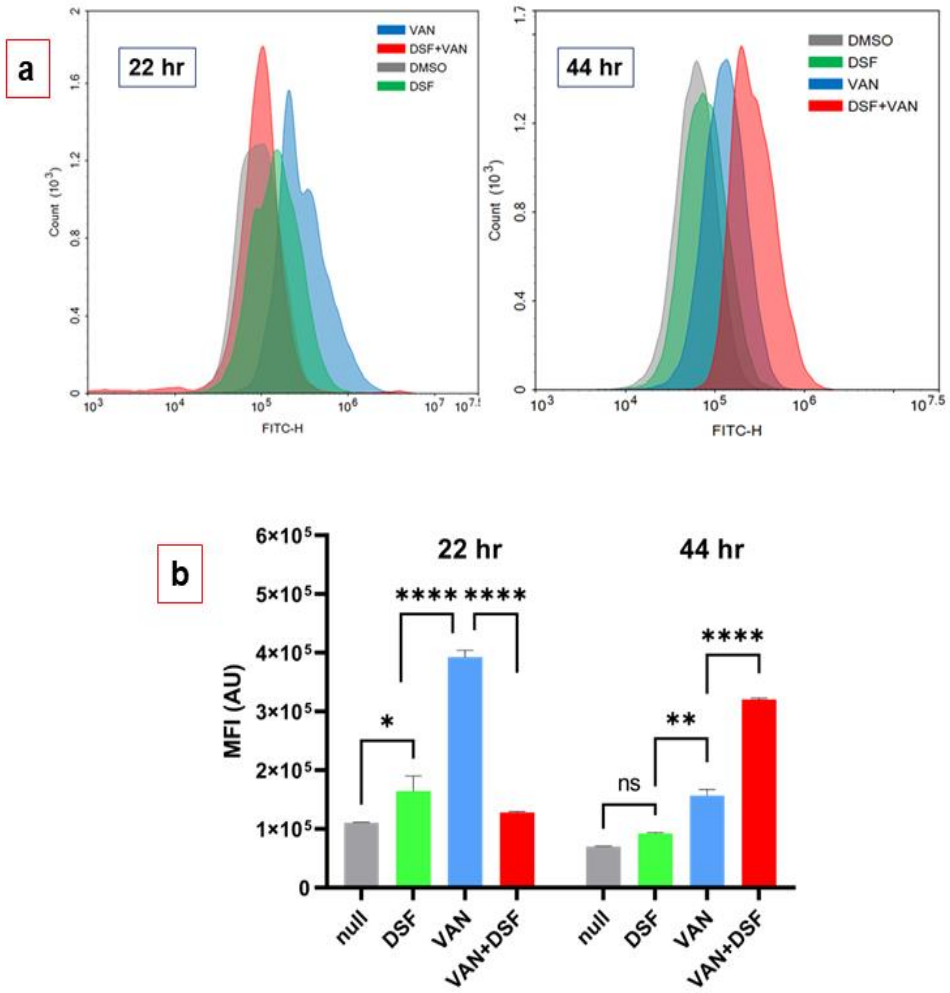
Note. Bar graphs depict the mean fluorescence intensity (MFI) of VISA Mu50 by flow Cytometry and statistical significance depicted with asterisks (ns = $p > 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$) (number of experiments and replicates- 1,2 respectively)

In addition, flow cytometry was also used for the differential investigation of VAN binding to cell wall muropeptides using 4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (abbreviated to BODIPY) preparation of VAN (VAN-BDP FL) dye (Figure 16) revealed that the cultures treated with DSF showed similar VAN-BDP FL mean fluorescence intensities (MFIs) as non-treated (null) cells after 22 and 44 hrs., whereas cultures treated with VAN demonstrated significantly high VAN-BDP FL binding at both time points. Surprisingly, the cultures treated with the VAN/DSF combination showed similar MFIs as DSF, suggesting that DSF may inhibit the fortification mechanism (Figure 17) by decreasing the "false" muropeptides formation, which acts as binding targets for VAN in Mu50 (Cui, Murakami,

Kuwahara-Arai, Hanaki, & Hiramatsu, 2000; Hanaki, Kuwahara-Arai, et al., 1998). However, at the 44-hr. mark, VAN/DSF cultures demonstrated similar MFIs as VAN samples at 22 hrs., indicating the subsiding effects of DSF and increased muropeptide binding of VAN-BDP FL.

Figure 16

Flow Cytometry With VAN-BDP FL



Note. **a.** Comparison of flow cytometry histograms of Mu50 labeled with following 22 and 44 hr. treatment with 1 x MIC DSF and/or 0.5 x MIC VAN **b.** Data represent the mean fluorescence intensity (MFI) and statistical significance depicted with asterisks (ns = $p > 0.05$; *$p \leq 0.05$;**$p \leq 0.01$; ****$p < 0.001$). (number of experiments and replicates- 1,2 respectively)

Post-antibiotic effect (PAE) studies

The impact of DSF on the PAE of VAN was the final PD parameter evaluated in this study. We tested the hypothesis that co treatment with DSF could increase the PAE of VAN. Table 6 showed that the PAEs were strain-, concentration-, and time-dependent for both VAN and DSF. The PAEs for VAN against VISA were similar to or greater than the PAEs against VAN susceptible strains and interestingly the VAN PAE for the VISA strain Mu50 doubled upon increasing the concentration and time of incubation, which could be related to the changes in the cell wall by "entrapping" VAN molecules with free muropeptides (Sieradzki & Tomasz, 1997). Overall, treatment with 1 x MIC of VAN for 1 hr. demonstrated PAE of 1.25 hr. and increased duration for 2 hr. incubation which was a persistent finding compared to previous studies (Aeschlimann, Hershberger, & Rybak, 1999; Suller & Lloyd, 2002). However, DSF exhibited shorter PAEs after 1 and 2 hr. incubation which was an expected finding considering the rapid and extensive metabolism of the drug (Custodio et al., 2022). Similarly, the addition of DSF showed shorter PAEs compared to VAN alone for all combinations at the 1 and 2 hr. intervals in Mu50 and JE2, but not COL.

Table 6

Comparison of PAEs Following VAN and/or DSF Treatments for 1 and 2 hr.

MRSA strain	µg/ml		time (hrs.)	PAE (hr.)		
	VAN	DSF		VAN	DSF	VAN/DSF
VISA Mu50	4	8	1	0.75	0.25	0.50
	8	8	1	1.25	0.50	0.75
	8	8	2	3.25	0.75	1.25
VSSA COL	2	16	1	0.50	0.50	1.50
	2	16	2	1.25	1.50	1.50
VSSA JE2	1	16	1	1.25	0.75	1.00
	1	16	2	2.25	1.25	1.25

Discussion

The first-line medication for the treatment of MRSA bacteremia is VAN and has been profusely used in the past few years. Reports showed that *S. aureus* clinical isolates with reduced susceptibility to VAN have emerged (Centers for Disease & Prevention, 2000; Hiramatsu et al., 1997; Rotun et al., 1999). PD parameters are among the factors that influence how clinical responses to anti-MRSA antibiotics respond for VAN (Kollef, 2007). Moreover, MRSA systemic infections need immediate treatment to reduce the onset of complications and prevent the spread of the disease. Failure rates of VAN treatment were correlated with the rise in MICs and a decline in the rate of bactericidal activity (Rhee, Gardiner, & Charles, 2005). In addition, a study demonstrated a significant decrease in VAN treatment efficacy for MRSA isolates with VAN MIC values ≥ 1 µg/ml (9.5 % clinical success rate) compared to the isolates, which exhibited VAN MIC of ≤ 0.5 µg/ml (55.6 % clinical success rate) (Sakoulas et al., 2004). According to findings of this study (Figure 10, Table 4), adding DSF expedited VAN's lethal ability against *S. aureus* and significantly reduced its MIC/MBC values. Moreover, DSF demonstrated concentration-dependent antibacterial activity against Mu50 whereas VAN exhibited time-dependent and concentration-independent bactericidal activity (Figure 13).

Multidrug therapy can potentially increase the range of pathogens that can be treated, prevent the development of antibiotic resistance, and enhance therapeutic effectiveness. In clinical practice, concentration-dependent and time-dependent antibiotics are sometimes combined for their synergistic potential. For instance, a study reported the use of aminoglycoside with penicillin or/and VAN for treating infectious endocarditis (IE) caused by *S. aureus* (Baddour et al., 2015). Likewise, VAN demonstrated an additive effect with an aminoglycoside (arbekacin) against MRSA in an *in vitro* IE model (Lee et al., 2003). Recent

studies used β-lactam antibiotics in combination with VAN against MRSA bacteremia for their significant synergistic antibacterial effect (Wilsey, Burgess, & Burgess, 2020; Tran & Rybak, 2018). The results of this study (Figure 10, 11, 13) revealed that VAN/DSF exhibited a synergistic killing effect against multiple MRSA strains. Likewise, from a previous report, DSF also demonstrated synergistic effects with FOS, which acts by inhibiting cell wall biosynthesis by covalently modifying a cysteine residue in UDP-*N*-acetylglucosamine enol pyruvyl transferase (MurA) (Skarzynski et al., 1996; Du et al., 2000), which could share a common target with DSF. Moreover, DSF with FOS displayed synergy (ΣFIC 0.25), as well as enhanced bactericidal activity against FOS-resistant JE2 (*fos*B^{+}), which could be attributed to the drug-mediated reduction of fosB-dependent antioxidant BSH (i.e., through a thiol-disulfide exchange reaction) (Figure 8) (Frazier et al., 2019; Lewis et al., 2022). However, other cell wall-related factors and mechanisms will need to be considered for the enhanced killing effect shown with the Mu50 strain since these mechanisms are unlikely to be applicable to the VAN/DSF interaction.

VAN is a peptidoglycan biosynthesis inhibitor, which acts by binding to the C-terminal D-Ala-D-Ala residue of the peptidoglycan precursor (UDP-linked MurNAc) at the division septum (main location for cell wall synthesis), forming a stable VAN complex, which results in the intracellular accumulation of UDP-linked MurNAc-pentapeptide precursors, which are required for the cell wall synthesis (Pootoolal, Neu, & Wright, 2002; Reynolds, 1961; Reynolds, 1989; Pereira, Filipe, Tomasz, & Pinho, 2007). The *S. aureus* cell wall is composed of highly cross-linked peptidoglycan (composed of amino acid-bound sugars called murein), teichoic acids and surface proteins. In VISA strains, thickened cell walls and decreased murein cross-linking are thought to be the major contributing factors for VAN resistance. The thick wall

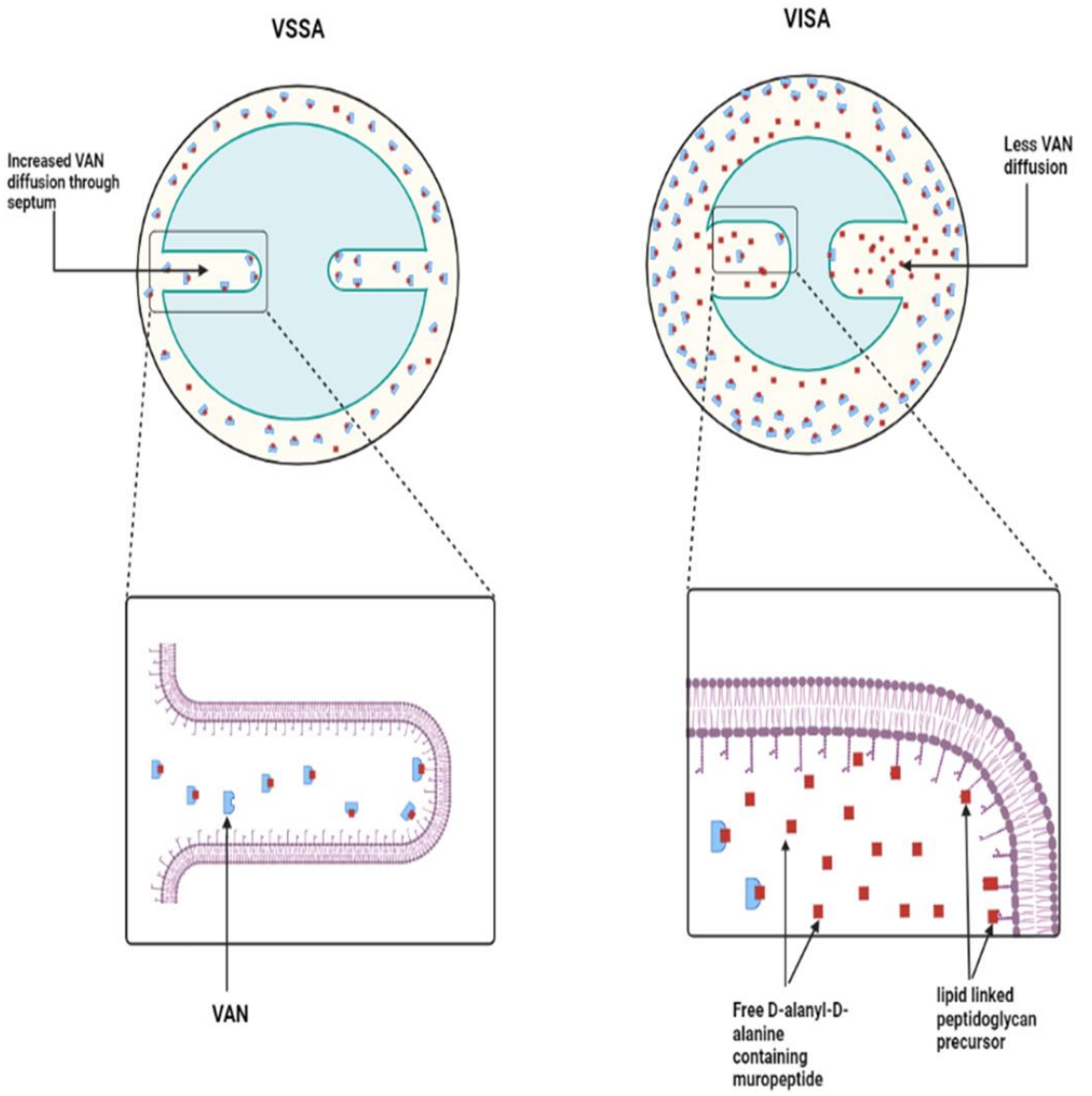
usually has less cross-linked peptidoglycan, which increases the free D-Ala-D-Ala residues/uncross-linked muropeptides that act as "pseudo" or "false" targets to entrap the VAN to protect the cell from the antibiotic (Figure 17) (Q. Hu et al., 2016; Howden, Davies, Johnson, Stinear, & Grayson, 2010). Previous research on Mu50 and a VRSA isolate lacking either the *vanA* or *vanB* genes to produce D-lactate terminating peptidoglycan precursors has shown greater VAN tolerance due to these false binding sites, decreasing the potency of the drug near the division septum of the cell wall (Cui et al., 2000; Sieradzki & Tomasz, 1997).

Moreover, it was established that the VISA/VRSA strains tested have higher VAN MICs compared to the VSSA isolates, which is attributed to the elevated muropeptide targets (Table 4). The flow cytometry results from this study support this mechanism. Figure 16 show a higher level of VAN-BDP FL bound with VAN at 22 hrs. when compared to the Mu50 samples treated with vehicle, DSF, or VAN/DSF. The VAN/DSF combination MFI levels were similar for untreated Mu50 indicating that the combination may have inhibited the muropeptide fortification from consuming VAN. The increased lethal impact of VAN within the first 24 hrs. is thought to be partially explained by the unaltered muropeptide composition with DSF co-treatment. By the 44-hr. time point, the MFI data indicates that the cellular effects of DSF had diminished, and that VAN had caused the remaining live bacteria to produce more muropeptide, allowing VAN-BDP FL to bind and increase fluorescence.

When choosing antibiotic dose regimens, the post-antibiotic effect (PAE), a PD parameter, may be investigated. Moreover, it is described as the period of time after a brief antibiotic exposure when bacterial growth is reduced (Pankuch & Appelbaum, 2006). The final objective of this study was to describe the impact of DSF on the PAE of VAN.

Figure 17

Model Comparing the Changes Seen in the Cell Wall Associated With VSSA and VISA Phenotypes



Note. Figure depicts the cell wall changes in VSSA and VISA in addition to the VAN site of action (i.e., at the division septum). In VISA, the effective concentration of VAN reaching the precursor drug target is decreased because of its lower rate of diffusion to the septum due to increased "false" binding sites to entrap the VAN molecules. Adapted from (Howden et al., 2010). Image created using *BioRender.com.*

With the addition of DSF, shorter Mu50 durations were observed, indicating an antagonistic interaction with VAN. Although the cause of the PAE variations between VAN and VAN/DSF is not yet clear, the decreased ATP production raises the possibility that metabolism factors may be involved. Furthermore, the results from the ATP assay (Figure 5) established that the decreased ATP synthesis by the VAN/DSF combination may have resulted in the production of fewer muropeptides for the VAN to bind up and further continued to inhibit growth even after removal from the media. Due to the reduced PAE of VAN when co-treated with DSF, this finding may have translational implications that need a modified interval dosage regimen for VAN.

Though this investigation into the *in vitro* PD parameters revealed the potent antibacterial effect of the VAN/DSF combination against VISA strains, further clinical studies would be needed to evaluate the potential side effects of the combination. The substantial metabolism of DSF may produce metabolites that could increase parenteral VAN-associated toxicities (such as ototoxicity, and nephrotoxicity) (Jeffres, 2017) and/or change excretion rate, despite its usage as a maintenance medication and the minimal occurrence of negative effects in the absence of alcohol (Rybak et al., 2020). The PK parameters of the combination will consequently need to be defined in translational studies. Additionally, DSF was FDA-approved in 1951 (Johansson, 1992), so its clinical PK needs to be better defined by contemporary standards. Further research will determine the best way to employ DSF as an adjunctive therapy for anti-infective medications.

To conclude, this research established that DSF might be effective as an antibiotic adjuvant in VAN treatment for MRSA infections, and our flow cytometry data further validated the increased lethal effects of the VAN/DSF combination. Moreover, it also showed that DSF

might work against the muropeptide fortification mechanism in VISA strains that prevent VAN as an cell wall biosynthesis inhibitor.

Chapter 4

Summary and Future Directions

This research established that disulfiram (DSF) reduces the minimum inhibitory concentration (MIC) of vancomycin (VAN) against several VAN intermediate susceptible *S. aureus* (VISA) strains and partially explains the mechanism of action of DSF against *S. aureus*. The important findings of this study include that DSF disrupts the metabolism in bacteria, affects the redox homeostasis, and antagonizes the muropeptide mechanism in VISA strain Mu50, which could be reasons for enhanced bactericidal activity with the VAN/DSF combination treatment. The results obtained in this study were completely done *in vitro* and further *in vivo* investigation to determine the potential effects of the DSF ± VAN is required. Future study focuses on evaluating the *in vivo* efficacy of DSF with and without VAN in methicillin resistant *S. aureus* (MRSA) infection model and we will test the hypothesis that DSF with VAN could significantly decrease or inhibit *S. aureus* burden in a mouse model.

Future Aim:

Aim: To Determine the therapeutic efficacy of DSF with and without VAN in a VISA peritonitis infection model.

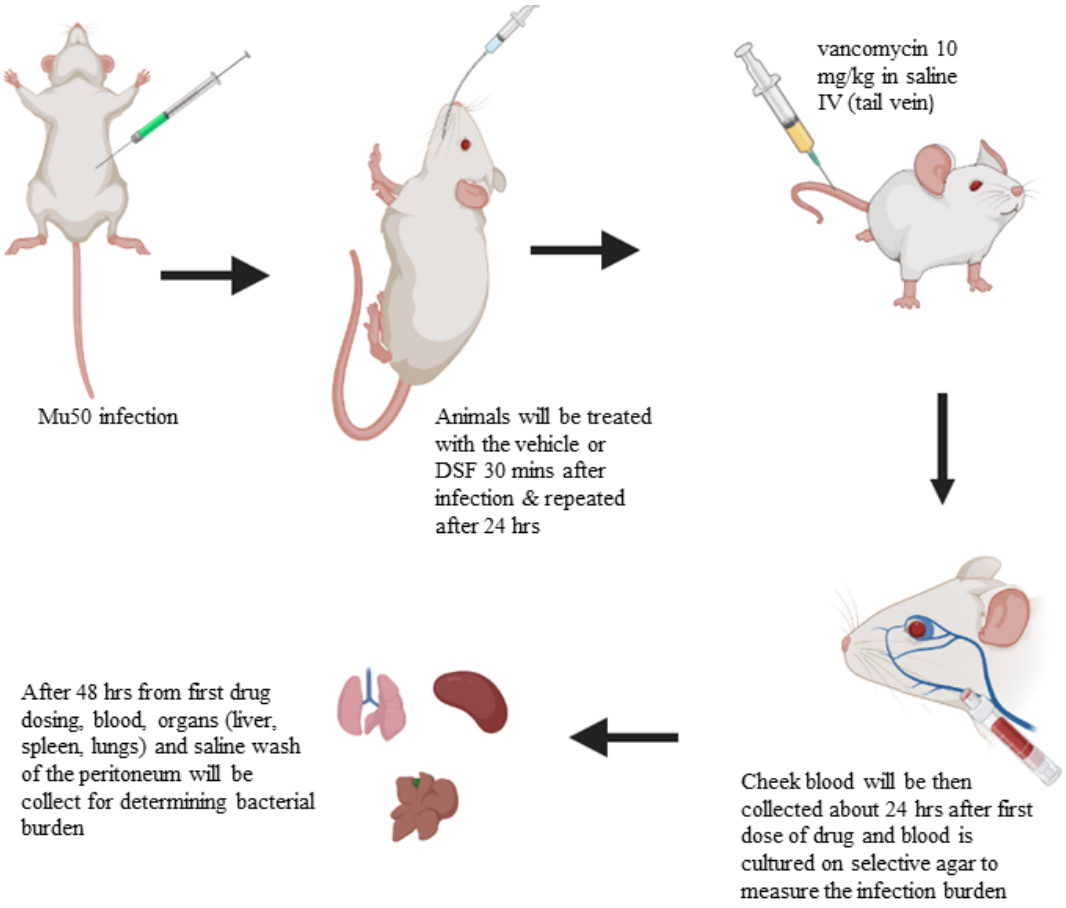
Experimental Design:

- We will use 32 C57BL/6 mice (8 weeks old, 20-30 g) in groups of 8 infected with VISA strain Mu50 for a subtotal of 64 mice plus a 10% excess of 6 animals.
- After a 12 h fasting period, mice will be infected by intraperitoneal (i.e.) injection with 500 µl of 10^7 cfu/mouse of bacteria in sterile saline containing 5% (wt./vol) mucin.

- Then, the animals will be treated with the vehicle or antibiotic(s) orally or I.V for about 30 minutes after infecting the mice. (Our preliminary studies showed that the mice did not show any rejection of DSF when it was suspended in 50% sweetened milk).
- Simultaneously, mice will be evaluated for moribund euthanasia criteria as mentioned in the protocol. (In prior studies, moribund conditions (e.g., fever, >15% loss in body weight) were not observed over a one-week period).
- About 24 hrs. after infection, the animals will be treated with an antibiotic(s) or vehicle again
- About 30 minutes after infection, the right or left cheek will be sterilized with a 70% alcohol wipe, and blood will be collected through the right or left mandibular vein either with a lancet or 20–25-gauge needle.
- The collected blood will later be cultured on a selective agar medium to grow VISA Mu50 bacteria to measure the infection burden, and animals will be returned to the cage simultaneously.
- About 48 hrs. post-infection, the animals will be euthanized by carbon dioxide inhalation followed by cervical dislocation.
- Blood, tissues (liver, kidney, spleen, lungs), and saline wash of the peritoneum will be collected with sterile instruments.
- The samples will later be homogenized with PBS or sterile saline and spread on agar media to determine bacterial burden.

Figure 18

Schematic Diagram to Determine the Therapeutic Efficacy of DSF ± VAN in a Mu50 Infection Model



Note. Image created using *BioRender.com.*

Other aims:

1. Evaluate the siderophore synthesis with DSF.

Previous studies revealed that DSF could chelate metals (e.g., copper, iron, zinc), forming stable complexes (i.e., $M[DDTC]_2$) (Viola-Rhenals et al., 2018). We will test the hypothesis that DSF and/or its metabolite reacts with Fe^{+3} leading to decreased iron, stressing

the bacterial cells to increase the production of siderophores. This potential antibacterial mechanism will be investigated by measuring siderophores production via a modified chrome azurol S (CAS) agar plate assay (Milagres, Machuca, & Napoleao, 1999).

2. Quantify the muropeptide levels when treated with DSF and VAN in the Mu50 strain compared to a VSSA strain.

Our results from the *in vitro* pharmacodynamics (PD) studies established that DSF could affect the fortification mechanism by muropeptides, and other cell wall associated factors in the VISA Mu50 strain. In depth analysis of the VAN/DSF combination effect on the bacterial cell wall is needed for a better understanding of the mechanism of action and the possible reasons for the increased susceptibility for VAN when co-treated with DSF. The procedure includes primary separation of peptidoglycan, digesting and reducing it, followed by fractioning of muropeptides by using high performance liquid chromatography (HPLC) (Hanaki, Labischinski, et al., 1998).

In conclusion, future objectives of this research further include defining how DSF affects *S. aureus* metabolism utilizing transcriptome data from previous unpublished research and performing PK/PD experiments using a VISA infection model. The "*in vivo* experiments" further aim to establish the PK parameters of VAN/DSF treatments and determine whether orally administered DSF accelerates parenteral VAN's ability to clear an MRSA infection. In addition, the use of RNA-Seq differential expression analysis for the identification of controlled metabolic pathways that DSF may alter will make it easier to understand the mechanism(s) through which the susceptibility of MRSA to VAN increases when cotreated with DSF.

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Appendix: IRB Approval Letter

MARSHALL UNIVERSITY
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Office of Research Integrity

August 11, 2022

Yogesh Meka
1675 6th Ave, Apt 2
Huntington, WV 25703

Dear Yogesh:

This letter is in response to the submitted thesis abstract entitled *"Pharmacology of Disulfiram as an Antimicrobial Agent."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #738. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract, you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director