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Repurposing disulfiram for treatment of *Staphylococcus aureus* infections

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ABSTRACT

Background: Antimicrobial resistance is an urgent threat affecting healthcare systems worldwide. Identification of novel molecules capable of escaping current resistance mechanisms and exhibiting potent activity against highly drug-resistant strains is the unmet need of the hour.
Methods: Whole cell growth inhibition assays were used to screen and identify novel inhibitors. The hit compounds were tested against Vero cells to determine the selectivity index, followed by time-kill kinetics against *Staphylococcus aureus*. The ability of disulfiram to synergize with several approved drugs utilized for the treatment of *S. aureus* was determined using fractional inhibitory concentration indexes, followed by its ability to decimate staphyloccocal infections ex vivo. Finally, the in-vivo potential of disulfiram was determined in a neutropenic murine model of *S. aureus* infection.
Results: The screening showed that disulfiram has equipotent antibacterial activity against *S. aureus*, including clinical drug-resistant strains (minimum inhibitory concentration 8–16 mg/L). Disulfiram exhibited concentration-dependent bactericidal activity (~7 $\log_{10}$ colony-forming units/mL reduction), synergized with linezolid and gentamycin against *S. aureus,* eradicated staphylococcal biofilms (64-fold better than vancomycin), decimated intracellular *S. aureus* better than vancomycin, exhibited longer post antibiotic effect than vancomycin, and reduced bacterial counts in murine thigh as well as vancomycin at 50 mg/kg.
Conclusion: Taken together, disulfiram exhibits all the characteristics required for repurposing as an antibacterial targeting staphylococcal infections.

1. Introduction

The World Health Organization (WHO) has recognized antimicrobial resistance (AMR) to be a significant threat to advances in medical health care worldwide [1]. In fact, the dwindling antibiotic pipeline, coupled with increasing cases of highly drug-resistant infections caused due to nosocomial and community-acquired pathogens, has prompted the 10x'20 declaration by Infectious Diseases Society of America to target ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens [2]. To spur novel drug discovery, WHO released a list of pathogens for which new antibiotics are urgently needed [3]. In the critical category, carbapenem-resistant *A. baumannii*, an endemic nosocomial pathogen which causes frequent infection outbreaks with high morbidity and mortality, is followed by meticillin- and vancomycin-resistant *S. aureus*, one of the most frequent causes of community and hospital-acquired infections. In the USA alone, 75 000 deaths from 722 000 reported cases of nosocomial infections have been reported, while 23 000 deaths occur annually in the USA from drug-resistant infections [4]. Thus, new drugs with novel mechanisms of action are urgently required to combat such infections.

Development of a new antimicrobial is a very slow process and is frequently beset with numerous pitfalls. Repurposing of approved drugs is a promising alternative strategy that reduces the time and cost of antibiotic development as it takes advantage of existing toxicology and pharmacokinetic data from preclinical and clinical trials [5–7].

In an effort to identify non-antibiotics that exhibit antibiotic activity, the authors found that disulfiram exhibited potent antimicrobial activity against *S. aureus*. Chemically, disulfiram is an organo-sulphur pro-drug used orally since the 1950s to treat chronic alcoholism (Antabuse) with a well-established safety profile [8]. Disulfiram irreversibly binds and inhibits acetaldehyde de-

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hydrogenase, resulting in accumulation of toxic acetaldehyde leading to an amplified hangover effect after consumption of alcohol.

The antimicrobial activity of disulfiram is in agreement with published reports where it was found to exhibit potent activity against *E. faecium* (16 μg/mL), *S. aureus* (8 μg/mL) and *Staphylococcus epidermidis* (1 μg/mL), but limited activity against Gram-negative pathogens *A. baumannii* and *K. pneumoniae* (64 μg/mL) [9,10]. Although identified previously for its antimicrobial activity, there is limited biological analysis available for repurposing disulfiram [10,11]. Thus, this study presents detailed bioanalysis of disulfiram as an antimicrobial, including in-vivo efficacy against *S. aureus* in a murine neutropenic thigh infection model.

2. Materials and methods

2.1. Growth media and reagents

All bacterial media and supplements including Mueller–Hinton cation supplemented broth II (MHBII), Mueller–Hinton agar (MHA) and tryptic soy broth (TSB) were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). All other chemicals and antibiotics were procured from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute Medium (RPMI) and fetal bovine serum (FBS) were purchased from Lonza (Greenwood, SC, USA). All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Bacterial strains

Disulfiram was screened against a bacterial panel consisting of ESKAPE pathogens, namely *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), *K. pneumoniae* (BAA-1705), *A. baumannii* (BAA-1605), *P. aeruginosa* (ATCC 27853) and *Enterococcus* spp. The panel was further expanded to include drug-resistant clinical *S. aureus* and enterococci strains, including those resistant to vancomycin and other clinically utilized antibiotics. These strains were procured from Biodefense and Emerging Infections Research Resources Repository/Network on Antimicrobial Resistance in *Staphylococcus aureus*/American Type Culture Collection (BEI/NARSA/ATCC, USA), and routinely cultivated on MHA and MHBII. Before starting the experiment, a single colony was picked from an MHA plate, inoculated in MHBII and incubated overnight at 37°C with shaking for 18–24 h to obtain the starter culture.

2.3. Antibiotic susceptibility testing

Antibiotic susceptibility testing of disulfiram was conducted according to the Clinical Laboratory Standards Institute guidelines using the broth microdilution assay [12]. Stock solutions, 10 mg/mL, of test compounds were prepared in DMSO. Bacterial cultures were inoculated in MHBII, and optical density (OD) was measured at 600 nm, followed by dilution to achieve $\sim 10^6$ colony-forming units (CFU)/mL. The compounds were tested from 64 to 0.5 mg/L in two-fold serial diluted fashion, with 2.5 μL of each concentration added to the wells of a 96-well round bottomed microtitre plate. Later, 97.5 μL of bacterial suspension was added to each well containing either test compound or an appropriate control. The plates were incubated at 37°C for 18–24 h, and then the minimum inhibitory concentration (MIC) was determined. The MIC is defined as the lowest concentration of the compound at which there is absence of visible growth. For each test compound, MIC determinations were carried out independently three times using duplicate samples.

2.4. Cell cytotoxicity of disulfiram

Cell toxicity was performed against Vero cells using the MTT assay [13]. Approximately 10^3 cells/well were seeded in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere. After 24 h, the compound was added, ranging from 100 to 12.5 μg/mL concentration, and incubated for 72 h. After incubation, MTT was added to each well, incubated at 37°C for a further 4 h, residual medium was discarded, 0.1 mL of DMSO was added to solubilize the formazan crystals, and OD was taken at 540 nm for the calculation of CC_{50}. CC_{50} is defined as the lowest concentration of compound which leads to a 50% reduction in cell viability. Doxorubicin was used as the positive control and each experiment was repeated in triplicate.

2.5. Bacterial time-kill kinetics with disulfiram

The presence or absence of bactericidal activity was assessed by the time-kill method as described previously [14]. Briefly, *S. aureus* ATCC 29213 was diluted $\sim 10^6$ CFU/mL in MHBII, treated with 1X and 10X MIC of disulfiram and vancomycin, and incubated at 37°C with shaking for 24 h. One-hundred microlitre samples were collected at 0, 1, 6 and 24 h, serially diluted in PBS, and plated on MHA followed by incubation at 37°C for 18–20 h. The kill curves were constructed by counting the colonies from plates and plotting the CFU/mL of surviving bacteria at each time point in the presence and absence of compound. Each experiment was repeated three times in duplicate and the mean data were plotted.

2.6. Drug interaction of disulfiram with FDA-approved drugs

Interaction of disulfiram with drugs approved by the Food and Drug Administration (FDA), namely vancomycin, levofloxacin, ceftazidime, linezolid and gentamycin, was tested by the checkerboard method. Serial two-fold dilutions of each drug were freshly prepared prior to testing. Disulfiram was diluted two-fold along the abscissa ranging from 8 to 0.0008 mg/L (12 dilutions), while the antibiotics were serially diluted along the ordinate (eight dilutions) ranging from 2 to 0.03 mg/L for vancomycin and gentamycin, 4–0.06 mg/L for linezolid, 1–0.015 mg/L for levofloxacin and 32–0.25 mg/L for ceftazidime in 96-well microtitre plates. Ninety-five microlitres of $\sim 10^5$ CFU/mL was added to each well and plates were incubated at 37°C for 24 h. After incubation, the ΣFICs (fractional inhibitory concentrations) were calculated as follows: $\Sigma FIC = FIC\ A + FIC\ B$, where FIC A is the MIC of Drug A in the combination/MIC of Drug A alone and FIC B is the MIC of Drug B in the combination/MIC of Drug B alone. The combination is considered synergistic when ΣFIC is ≤ 0.5, indifferent when ΣFIC is >0.5 to 4, and antagonistic when ΣFIC is >4 [15].

2.7. Determination of activity of disulfiram against S. aureus biofilm

Determination of the antibiofilm activity of disulfiram was performed as described previously [16]. Briefly, *S. aureus* ATCC 29213 were grown overnight in 1% TSB with shaking (180 RPM) at 37°C. The overnight culture was diluted in fresh TSB broth (1:100), and 0.2 mL of freshly diluted culture was transferred into a 96-well polystyrene flat bottomed plate, covered with an adhesive foil lid for maintaining low oxygen, and incubated in static conditions for 48 h at 37°C. After incubation, media were decanted and the plate was rinsed gently three times with 1X PBS (pH 7.4) to remove the planktonic bacteria. Plates were refilled with TSB with different drug concentrations and incubated for 24 h at 37°C. After drug treatment, the media was decanted, washed three times with 1X PBS (pH 7.4), and biofilm was fixed by incubating the plate at 60°C for 1 h. After fixing, the biofilm was stained by 0.06% crystal violet for 10 min, rinsed with PBS and dried at room temperature. For quantification of biofilm, the bound crystal violet was eluted by 30% acetic acid (0.2 mL). Absorbance was measured on a microtitre plate reader at 600 nm for biofilm quantification.

2.8. Determination of activity of disulfiram against intracellular S. aureus

The J774.A1 mouse macrophage cell line was seeded at 50 000 cells/well in a 12-well tissue culture plate and was infected with *S. aureus* ATCC 29213 for 1 h at a multiplicity of infection of 1:100 [17]. After infection, cells were washed with 1X PBS (pH 7.4) to remove extracellular bacteria, and wells were replaced by RPMI medium containing different concentrations of drugs containing gentamycin (0.5 mg/L) to prevent extracellular bacterial growth. The plates were incubated for 24 h at 37°C in 5% CO_2. Following incubation, the cells were washed three times with 1X PBS (pH 7.4) and lysed with RIPA buffer (#89901 Thermo). The cell lysates were serially diluted, plated on TSA, and incubated for 24 h at 37°C for enumeration of CFU.

2.9. Determination of post antibiotic effect of disulfiram

To determine the post antibiotic effect (PAE) of disulfiram, overnight culture of *S. aureus* ATCC 29213 was diluted in MHBII ~10^5 CFU/mL, exposed to 1X and 5X MIC of vancomycin, levofloxacin and disulfiram, and incubated at 37°C for 1 h. Following incubation, culture was centrifuged and washed twice with prewarmed MHBII to remove any traces of antibiotics. Finally, cells were resuspended in drug-free MHBII and incubated further at 37°C. Samples were taken every 1 h, serially diluted and plated on TSA for enumeration of CFU. PAE was calculated as PAE = T – C; where T is the difference in time required for 1 $\log_{10}$ increase in CFU vs. CFU observed immediately after the removal of drug, and C in a similarly treated drug-free control [18].

2.10. Activity of disulfiram in murine neutropenic S. aureus thigh infection model

The in-vivo potential of disulfiram to decimate *S. aureus* infection was determined by utilizing the murine neutropenic *S. aureus* infection model as described [14]. Briefly, BALB/c mice weighing ~22–24 g were used throughout the study and were rendered neutropenic by a series of cyclophosphamide injections given intraperitoneally (IP) 4 days and 1 day before infection. This was followed by injection of *S. aureus* ATCC 29213 in the right thigh of mice to establish infection. After 3 h of infection, disulfiram and vancomycin at 50 mg/kg and 50 mg/kg of body weight, respectively, were injected IP into mice twice, with 3 h between injections. The control animals were administered saline in the same volume and frequency as those receiving treatment. After 24 h, the mice were sacrificed, and thigh tissue was collected, weighed and homogenized in 5 mL of saline before serial dilution and plating on MHA plates for CFU determination. After incubation for 18–24 h at 37°C, CFU were enumerated. Each experiment was repeated three times in duplicate and the mean data are plotted.

2.11. Statistical analyses

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). Comparison between three or more groups was analysed using one-way analysis of varaince, with post-hoc Tukey's multiple comparisons test. *P*-values <0.05 were considered to indicate significance.

3. Results and discussion

3.1. Disulfiram exhibits broad-spectrum activity

Disulfiram was identified in a screen for non-antibiotics expressing antibiotic activity against the ESKAPE pathogens. As can be seen in Table 1, it is potent against Gram-positive pathogens such as *S. aureus* (MIC=8 mg/L) but is less potent against *Enterococcus* spp. (MIC=16–32 mg/L), and is inactive against Gram-negative pathogens (MIC=64–>64 mg/L) with the exception of *A. baumannii* (MIC=16 mg/L). Intriguingly, when tested against an expanded panel of clinical, highly drug-resistant strains of *A. baumannii*, the MIC ranged from 8 to 64 mg/L (data not shown). These results correlate well with published data, with the exception of *A. baumannii* [10].

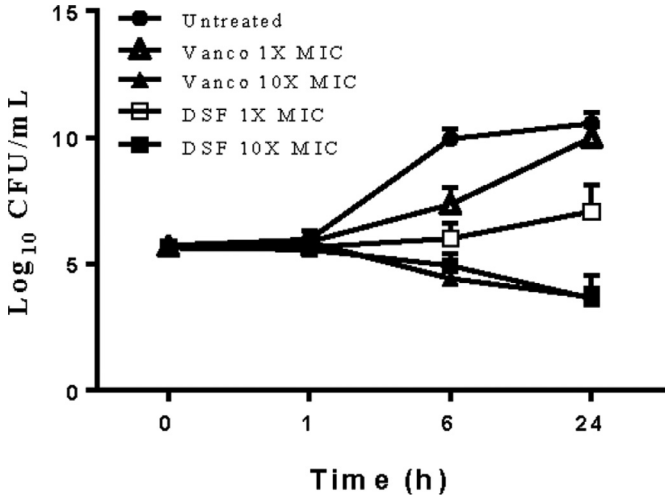

Fig. 1. Bacterial killing kinetics with disulfiram (DSF) and vancomycin (vanco) against *Staphylococcus aureus* ATCC 29213.

The antimicrobial activity of disulfiram was determined against an extended panel of MRSA and vancomycin-resistant *S. aureus* (VRSA) strains, consisting of clinical strains with well-defined and characterized resistance patterns. As shown in Table 1, disulfiram was equipotent against drug-sensitive and drug-resistant strains, and there was no significant difference in MIC with respect to virulence factor, such as Panton–Valentine Leucocidin, as well as different types of *mec* cassettes, suggesting a new mechanism of action and lack of cross-resistance with existing drugs.

3.2. Evaluation of cell cytotoxicity of disulfiram against eukaryotic cells

The CC_{50} of disulfiram was determined against Vero cells to be 250 mg/L. As such, the selectivity index (SI=CC_{50}/MIC) was calculated to be 31.25, which is sufficiently promising to continue with repurposing.

3.3. Disulfiram exhibits concentration-dependent bactericidal killing

The bacterial killing kinetics of disulfiram were assessed at 1X and 10X MIC with vancomycin as control. As depicted in Fig. 1, disulfiram exhibits modest killing kinetics at 1X MIC, where it caused a ~3 $\log_{10}$ CFU/mL reduction. However, at 10X MIC, disulfiram caused a ~7 $\log_{10}$ CFU/mL reduction in 24 h compared with the drug-free control, which is comparable to vancomycin at 10X MIC. Thus, disulfiram exhibits concentration-dependent bactericidal activity comparable to vancomycin. This compares well with a previous report [10], although this previous study also found that disulfiram exhibits time-dependent activity.

3.4. Disulfiram potentiates antimicrobial efficacy of clinically utilized drugs

As combination therapy is needed urgently to combat rising AMR, the ability of disulfiram to synergize with FDA-approved

Table 1
Minimum inhibitory concentration (MIC) of disulfiram against ESKAPE and expanded pathogen panel.

Strains		Antibiotic resistant to	Molecular details of strains	MIC of disulfiram (mg/L)
MSSA	*S. aureus* ATCC 29213	None		8
MRSA	NR 100	Meticillin, ceftriaxone, meropenem, tetracycline	• Positive for mec (subtype I) • Large variety of virulence factors	8
	NR 119	Meticillin, ceftriaxone, meropenem, gentamycin and linezolid	• Positive for mec (subtype IV) • G2576T mutation in domain V in one or more 23S rRNA genes	8
	NR 10129	Meticillin, ceftriaxone, meropenem	• Also known as TCH60	16
	NR 10186	Meticillin, ceftriaxone, meropenem	• Community-acquired MRSA • PFGE typed as USA300 • PVLvirulence factor positive • Contains mec type IV cassette	8
	NR 10191	Meticillin, ceftriaxone, meropenem	• Community-acquired MRSA • PFGE typed as USA600 • PVL factor negative • Contains mec type II cassette	16
	NR 10192	Meticillin, ceftriaxone, meropenem	• Community-acquired MRSA • PFGE typed not as USA100-1100 • PVL factor negative • Contains mec type II cassette	8
	NR 10193	Meticillin, ceftriaxone, meropenem	• Community-acquired MRSA • PVL factor negative • Contains mec type II cassette	16
	NR 10194	Meticillin, ceftriaxone	• Community-acquired MRSA • PVL factor positive • Contains mec type V cassette	16
	NR 10198	Meticillin, ceftriaxone, meropenem	• Community-acquired MRSA • PFGE typed as USA100 • PVL factor negative • Contains mec type II cassette	8
VRSA	VRS 1	Meticillin, ceftriaxone, meropenem, gentamycin, vancomycin, teicoplanin	• Contains mec subtype II cassette and vanA • Negative for vanB, vanC1, vanC2, vanD, vanE, PVL and ACME • PFGE typed as USA100	32
	VRS 4	Meticillin, ceftriaxone, meropenem, vancomycinmycin teicoplanin	• Contains mec subtype II cassette and vanA • Negative for vanB, vanC1, vanC2, vanD, vanE, PVL and ACME • PFGE typed as USA100	16
	VRS 12	Meticillin, ceftriaxone, meropenem, vancomycin teicoplanin	• Data not available	16
Enterococcus spp.	NR 31903	Ampicillin	Complete genome is sequenced (GenBank: AJDX00000000)	32
	NR 31884	Gentamicin	Haemolytic, cytolytic isolate Complete genome is sequenced (GenBank: AIRF00000000)	32
	NR 31885	Gentamicin	Cytolytic isolate Complete genome is sequenced (GenBank: AIRH00000000)	32
	NR 31912	Vancomycin	Complete genome is sequenced (GenBank: AJDX00000000)	16
Gram-negative strains	*Escherichia coli* ATCC 25922	None	Type strain	>64
	Klebsiella pneumoniae BAA-1705	Imipenem, ertapenem	Type strain	64
	Acinetobacter baumannii BAA-1605	Ceftazidime, gentamicin, ticarcillin, piperacillin, aztreonam, cefepime, ciprofloxacin, imipenem, meropemem	Type strain	16
	Pseudomonas aeruginosa ATCC 25923	None	Type strain	>64

MSSA, meticillin-susceptible *Staphylococcus aureus*; MRSA, meticillin-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*; PFGE, pulsed-field gel electrophoresis; PVL, Panton–Valentine leukocidin; ACME, arginine catabolic mobile element.

drugs utilized for the treatment of *S. aureus* was tested by the chequerboard assay. As can be seen in Table 2, disulfiram synergizes with linezolid and gentamycin against *S. aureus* ATCC 29213. This synergy with gentamycin and vancomycin has been reported previously [10,19], although there was no interaction between vancomycin and disulfiram.

In addition, disulfiram was tested for its ability to sensitize VRSA to vancomycin. As can be seen in Table 3, addition of

Table 2
Synergy of disulfiram with other drugs approved by the Food and Drug Administration against *Staphylococcus aureus* ATCC 29213

Drug	MIC of drug alone	MIC of drug in presence of disulfiram	MIC of disulfiram alone	MIC of disulfiram in the presence of drug	FIC index	Indication
Vancomycin	1	0.5	16	8	1	No interaction
Levofloxacin	0.25	0.25	16	1	1	No interaction
Ceftazidime	8	8	16	8	1.5	No interaction
Linezolid	2	0.5	16	1	0.312	Synergy
Gentamycin	0.25	0.03	16	0.5	0.15	Synergy

MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration.

Table 3
Checkerboard assay results for disulfiram synergy with vancomycin in vancomycin-resistant *Staphylococcus aureus* clinical isolates

Bacteria	MIC of vancomycin alone	MIC of vancomycin in presence of disulfiram	MIC of disulfiram alone	MIC of disulfiram in presence of vancomycin	FIC index	Indication
VRS 1	1024	64	16	4	0.312	Synergy
VRS 4	1024	32	16	4	0.281	Synergy
VRS 12	1024	512	16	4	1.25	No interaction

MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration.

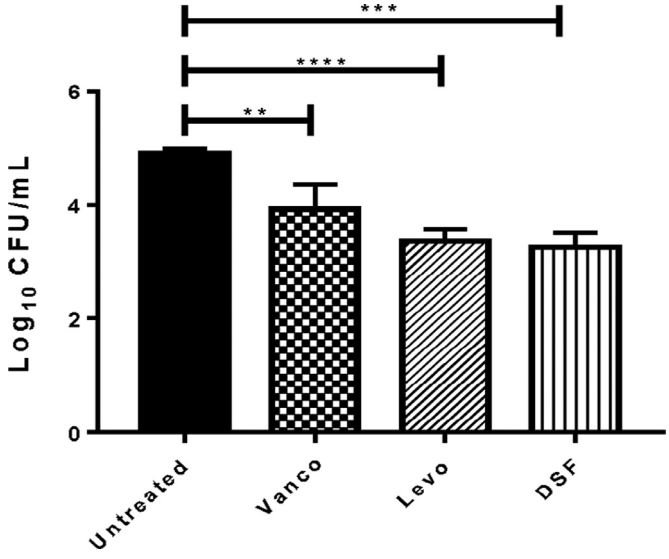

Fig. 2. Evaluation of disulfiram (DSF), vancomycin (vanco) and levofloxacin (Levo) (10X minimum inhibitory concentration) in eradicating the intracellular infection of *Staphylococcus aureus* ATCC 29213 in J774.A mouse macrophage cell line. CFU, colony-forming units.

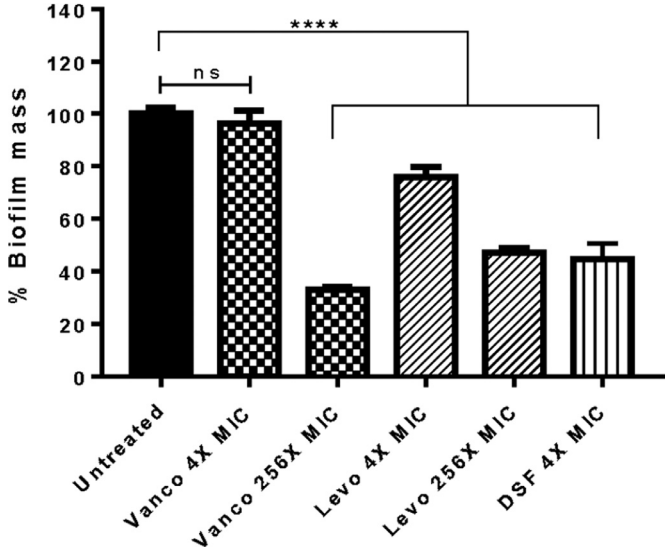

Fig. 3. Anti-biofilm activity of disulfiram (DSF) against the *Staphylococcus aureus* ATCC 29213 biofilm. Vanco, vancomycin; levo, levofloxacin; MIC, minimum inhibitory concentration.

disulfiram lowers the MIC of vancomycin from 1024 to 64 mg/L against two of three VRSA clinical isolates, which agrees with published data [10]. This augurs well for the ability of disulfiram to be utilized as combination therapy for the treatment of drug-resistant *S. aureus* infections.

3.5. Disulfiram eradicates S. aureus biofilm

It has been demonstrated that bacteria under different stresses resort to making biofilm in order to protect themselves, leading to therapeutic failure. Most approved drugs have very limited activity against pathogens in biofilms, so it is imperative to determine the antibiofilm activity of molecules in development. In this context, disulfiram at 4X MIC exhibited potent antibiofilm activity as it reduced biofilm by 56% compared with untreated (Fig. 3). On the other hand, a similar reduction in biofilm was achieved at 256X MIC of vancomycin and levofloxacin, respectively. Thus, disulfiram is 64-fold more potent in reducing biofilm compared with vancomycin and levofloxacin, which augurs well for its repurposing potential.

3.6. Disulfiram demonstrates efficient intracellular killing of S. aureus

Intracellular infections due to *S. aureus,* a facultative intracellular pathogen, are increasingly being recognized as a major reason for their re-occurence since the intracellular infection acts as a reservoir of repeated infections, as seen in staphylococcal endocarditis [20]. Thus, it is important to determine if a molecule under investigation possesses the ability to decimate intracellular infections. As can be seen in Fig. 2, at 24 h, disulfiram caused a ~1.6 $\log_{10}$ reduction in bacterial count at 10X MIC, compared with untreated. In comparison, at 24 h, vancomycin and levofloxacin caused ~0.98 $\log_{10}$ and ~1.4 $\log_{10}$ reductions in bacterial count at 10X MIC. Taken together, these results highlight the ability of disulfiram to eradicate intracellular *S. aureus* more effectively than vancomycin but comparably with levofloxacin (Fig. 2).

3.7. Disulfiram exhibits prolonged post antibiotic effect against S. aureus ATCC 29213

Possessing a long PAE is an asset for any molecule as it helps to minimize the dosages required for therapeutic clearance. In this context, disulfiram was found to exhibit a prolonged PAE of 5 h

Table 4
Post antibiotic effect of disulfiram

Compound	Time (h) for 1 $\log_{10}$ increase	Post antibiotic effect (h)
Untreated *Staphylococcus aureus* ATCC 29213	2	0
Disulfiram 1X MIC	3	1
Disulfiram 5X MIC	7	5
Vancomycin 1X MIC	3	1
Vancomycin 5X MIC	4	2
Levofloxacin 1X MIC	3	1
Levofloxacin 5X MIC	6	4

MIC, minimum inhibitory concentration.

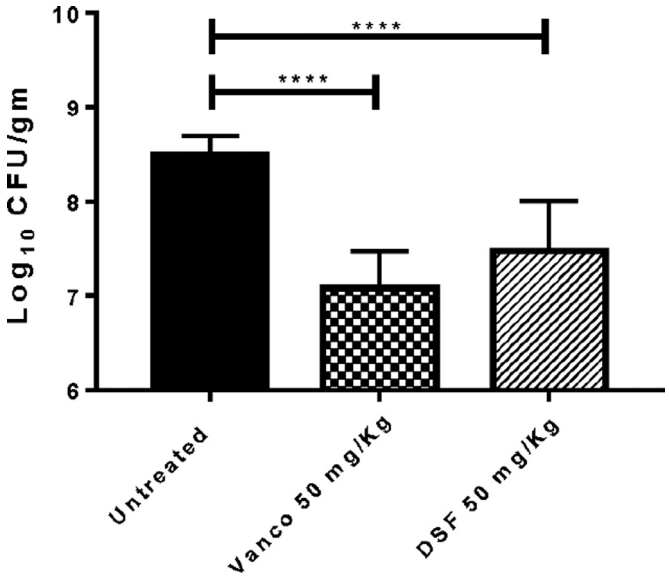

Fig. 4. In-vivo efficacy of disulfiram (DSF, 50 mg/kg) and vancomycin (vanco, 50 mg/kg) in a neutropenic murine thigh infection model. The mean $\log_{10}$ colony-forming units (CFU) is plotted for mouse thigh after treatment with DSF and vanco.

at 5X MIC, which is better than that of vancomycin (2 h at 5X MIC) but comparable to levofloxacin (4 h at 5X MIC) (Table 4). Thus, disulfiram exhibits concentration-dependent bactericidal activity with prolonged PAE.

3.8. In-vivo activity of disulfiram in murine neutropenic thigh infection model

As disulfiram exhibited potent bactericidal activity, its efficacy was tested in a murine neutropenic thigh infection model. The maximum tolerated dose of disulfiram was determined to be >200 mg/kg (data not shown). Briefly, the thigh of neutropenic mice was infected with *S. aureus*, followed by two IP injections of disulfiram and vancomycin at 50 mg/kg. Saline- and vancomycin-treated mice groups were considered as negative and positive controls, respectively. As seen in Fig. 4, treatment with disulfiram at 50 mg/kg significantly reduced mean bacterial counts (~1.1 $\log_{10}$) in thigh compared with the control group ($P<0.05$), which is comparable to vancomycin at 50 mg/kg (~1.4 $\log_{10}$). These results revealed that disulfiram is as effective as vancomycin in reducing the bacterial load in infected mice.

The dwindling drug discovery effort in past decades and rapid emergence of drug-resistant pathogens have left us with very limited options to combat the increasing menace of AMR. Thus, the discovery of novel molecules with potentially new mechanisms of action is urgently needed. This study showed that disulfiram possesses potent bactericidal activity against clinical drug-resistant isolates of *S. aureus* and *A. baumannii*, has a high safety index, is able to clear intracellular *S. aureus* infection better than vancomycin, eradicates *S. aureus* biofilm at 64-fold lower concentration than vancomycin, synergizes with linezolid and gentamicin, potentiates vancomycin against VRSA, and causes a significant reduction in bacterial load in murine models of *S. aureus* infection. In addition, it possesses an excellent clinical record for a 500-mg daily dosage with a half-life of 7.3 h [21]. Taken together, disulfiram exhibits all the properties required for repurposing as an antibacterial targeting *S. aureus*.

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Competing interests

None declared.

Ethical approval

The use of mice for infectious studies (IAEC/2014/139, 03.12.2014) was approved by Institutional Animal Ethics Committee at CSIR-CDRI, Lucknow.

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